

Reverse Genetics of the Negative-Sense Influenza A Virus: Aspects and Applications

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Received: Mar 12, 2015; Accepted: May 03, 2015; Published: June 01, 2015

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Abstract

Viruses with completely or predominantly negative-sense RNA genomes include a number of human and animal pathogens such as influenza, ebola and rabies viruses. The study of these viruses has greatly benefited from the use of advances in reverse genetics systems developed to manipulate viral genomes. Reverse genetics technology provided a platform to study different aspects of viral replication, pathogenesis, interaction with vectors, and to develop genetically engineered vaccines. This review summarizes major technical breakthroughs in the development of reverse genetics technologies for negative-sense RNA viruses using Influenza A Virus (IAV) as a model system. The varied applications of the technology as well as current trends, such as 2A 'self-cleaving' peptides for co-expression of foreign genes, are also outlined.

Introduction

Reverse Genetics Systems for Negative-Sense RNA Viruses

Reverse genetics, a technique used to engineer specific mutations into viral genomes, was first performed for DNA viruses, either by transfecting cells with plasmids encoding the viral genome or by heterologous recombination of plasmids bearing viral sequences with the virus genome [1,2]. They were followed by manipulations of positive-sense RNA genomes. Transfection of plasmids, or RNA transcribed from plasmids, containing the poliovirus genome, into susceptible cells led to recovery of infectious poliovirus [3,4]. However, the genomes of negative-sense RNA viruses were less amenable to artificial manipulations in comparison with the DNA and positive-sense RNA viruses. In contrast to positive-sense RNA viruses, the genome of which is also a functional messenger RNA (mRNA), the naked genomic RNA of a negative-sense RNA virus is not able to initiate infection when expressed in or transfected into a permissive cell line. Their genomes are the complement of mRNA and therefore cannot be directly translated to give viral proteins without first being copied into complementary mRNA (Figure 1). The minimal infectious particle is the transcriptionally active Ribonucleoprotein (RNP) complex which is composed of the genomic viral RNA (vRNA) complexed with the viral Nucleoprotein (NP) and the RNA-dependent RNA polymerase

protein. The viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome RNA template due to the fact that animal cells do not possess such an enzyme. This function must be supplied preformed in the input virion as genomic RNA on its own cannot initiate infection. Moreover, the mRNA is different to the positive complementary RNA intermediate which is used for replication in that it is 3' truncated. As a result, the mRNA does not contain all the viral specific information required for production of new genomes and cannot serve as template for transcription.

Viruses with completely or predominantly negative-sense RNA genomes span seven viral families: the non-segmented *Bornaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, and *Filoviridae*, and the segmented *Arenaviridae* (2 segments), *Bunyaviridae* (3 segments), and *Orthomyxoviridae* (6-8 segments). These virus families include a number of human and animal pathogens such as influenza, measles, mumps, rabies, Respiratory Syncytial Virus (RSV), Vesicular Stomatitis Virus (VSV), Lassa, Ebola, Marburg,

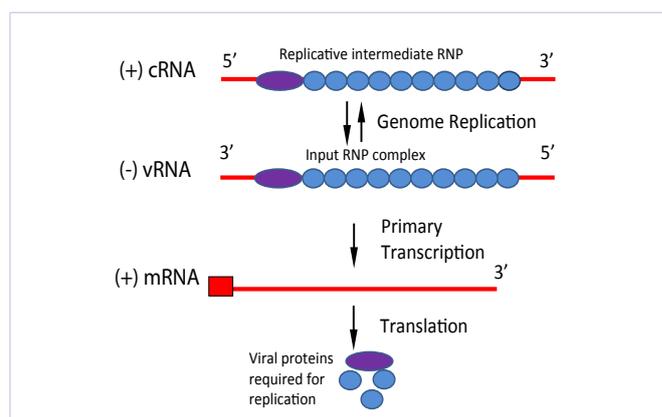


Figure 1: Replication of Negative-Sense RNA Viruses: The input genome is a ribonucleoprotein (RNP) complex which cannot be translated. RNA-dependent RNA polymerase is associated with the input genome and transcribes mRNA molecules as the first step of replication. Translation of primary transcripts produces proteins which together with full-length (+) cRNA form RNP replicative intermediate. This then serves as template for new genome synthesis. Both vRNA and cRNA are exactly complementary and encapsidated by nucleocapsid (N) protein, while mRNA is 3' truncated.

canine distemper, human parainfluenza and hantavirus. The recovery of infectious negative-sense RNA viruses from cloned complementary DNAs (cDNAs) in the 1990s was among the most important breakthroughs in RNA virology; it became the basis for directed engineering of the viral genomes and facilitated a detailed understanding of the function of the viral genes and their products. Reverse genetics technology opened the possibility of deleting genes for studying their function, introducing mutations to determine genetic factors of virulence and developing heterologous viral expression vectors for vaccine and gene therapy.

In the present review, we use IAV as a model system to summarize the achievements in the development of this technology for negative-sense RNA viruses. Each year, IAV infections hospitalize 3-5 million individuals resulting in substantial morbidity, mortality and economic burdens. Several applications of IAV recombinant viruses, as well as other viral systems, are outlined.

Reconstitution of Biologically Active RNPs *in vitro*

The reconstitution of RNPs using synthetic or purified vRNAs became the basis for the genetic engineering of influenza virus. In the isolated RNPs, the polymerase complex and NP are associated with the vRNA. In order to generate genetically engineered viruses, synthetic RNA needs to be assembled into replication competent RNPs. *In vitro* reconstitution of RNPs resulted in the transcription of a synthetic RNA template and, more important, of full-length vRNAs purified from virions [5-7]. Honda et al. [8] isolated RNPs and separated the RNA polymerase-RNA

fraction from NP by differential centrifugation of isolated RNP cores through a discontinuous CsCl-glycerol gradient. The RNA polymerase-RNA complex could initiate RNA synthesis, but it was unable to synthesize full-length RNA unless NP was added to the reaction, indicating that the polymerase complex is sufficient to initiate RNA synthesis, whereas NP is needed for elongation of the RNA transcript. Incubation of influenza virus naked RNA, either transcribed from plasmids *in vitro* or purified by phenol extraction, with free NP/polymerase fractions, resulted in the formation of transcriptionally active complexes [8].

Luytjes et al. [9] devised the first system to modify influenza viruses by constructing a plasmid that contained the coding region for chloramphenicol acetyltransferase (CAT) in place of the NS gene in the antisense orientation flanked by the 3' and 5' untranslated regions (UTRs) of the vRNA of the influenza virus segment eight (Figure 2). This cassette was flanked by a T7 RNA polymerase promoter and a restriction enzyme site that allowed the production of *in vitro* transcripts containing an exact virus-like 3' end. *In vitro* transcription from the T7 promoter resulted in a RNA molecule containing the terminal non-coding sequences, matching those found in influenza virus segment eight, flanking an antisense copy of the CAT gene. This RNA could not be translated to give active CAT protein unless it was itself first used as a template to make positive-sense mRNA. Since eukaryotic cells do not possess either RNA-dependent RNA polymerase or CAT activities, the new approach was a sensitive reporter system. RNA transcripts were mixed with purified NP and polymerase proteins to allow the formation of RNP complexes. Prior to or after their transfection with RNPs, the cells were infected with helper

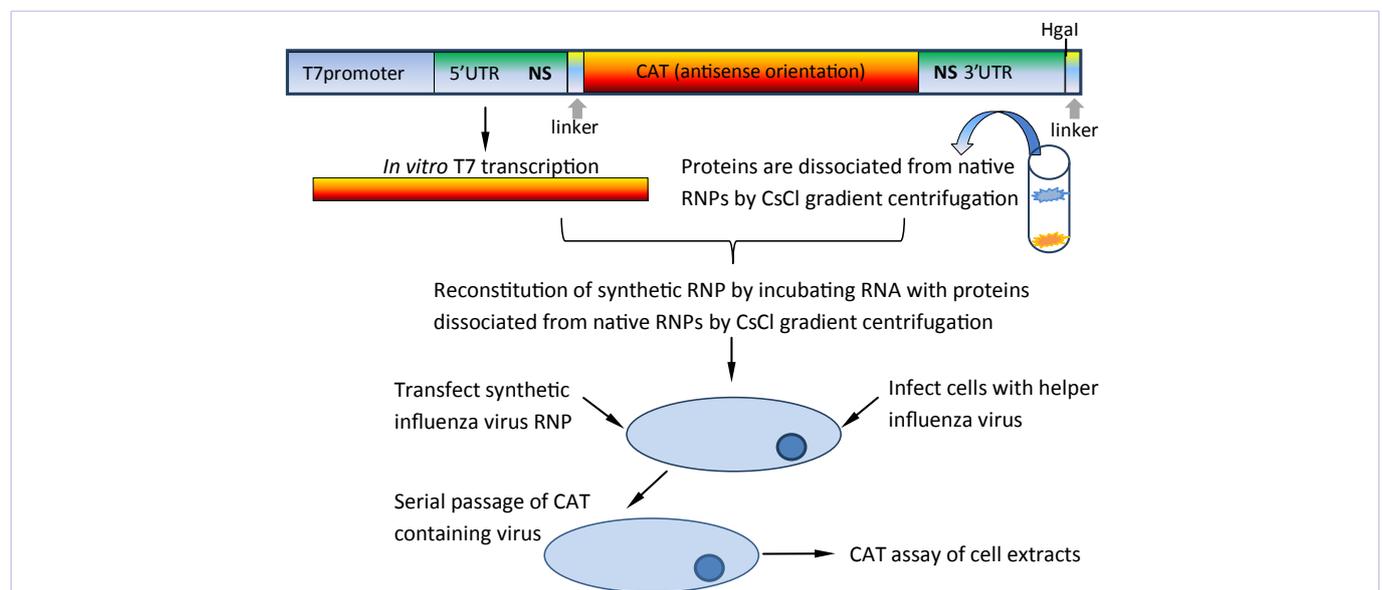


Figure 2: RNP Transfection Method for the Rescue of Transfectant Influenza Virus: The coding sequence for CAT (in the antisense orientation) is flanked by short linker sequences (introduced for cloning purposes), the 5' and 3' untranslated regions (UTRs) of vRNA, and a restriction site to generate vRNA-like 3' ends. *In vitro* transcription by T7 polymerase results in a synthetic vRNA that can be packaged into infectious influenza virus. Purified NP and polymerase proteins are assembled with *in vitro* synthesized vRNA to form RNP complexes. Following RNP transfection and helper virus infection, the transfectant virus, containing RNA derived from cloned cDNA is selected. The recombinant CAT RNA is not only transcribed and replicated, but also packaged into progeny virus particles as demonstrated by the ability of the media from transfection experiments to induce CAT activity in cells after serial passage (adapted from [9]).

influenza virus to provide the viral proteins required for RNA amplification. CAT activity in lysates derived from transfected and infected cells indicated the transcription of vRNA-like CAT transcripts to give mRNAs. The recombinant CAT RNA was not only transcribed and replicated, but also packaged into progeny virus particles as demonstrated by the ability of the media from transfection experiments to induce CAT activity in cells after serial passage even after RNase A treatment. These experiments demonstrated that the UTRs of influenza virus RNAs contain all the signals required for transcription, replication, and packaging of CAT-vRNA.

This technique was subsequently refined to create influenza virus containing Neuraminidase (NA) proteins derived from plasmid cDNAs [10]. It relied on reconstitution of viral RNPs from *in vitro*-transcribed RNA and purified NPs. The protein-RNA complex was transfected into cells, followed by infection with a helper influenza virus with a strong counter-selectable phenotype which was well characterized. Influenza virus A/WSN-HK is a reassortant containing seven segments from influenza A/WSN/33 and the NA gene segment from influenza virus A/HK/8/86. This reassortant virus can only form plaques in Madin-Darby Bovine Kidney (MDBK) cells when the cell culture media is supplemented with a protease, whereas the parent WSN/33 strain replicates and forms large plaques without the requirement for exogenous protease. WSN/NA segment responsible for protease-independence was supplied in the form of a synthetic RNP. Selection of the virus containing the synthetic WSN/NA segment from the helper virus could be achieved by excluding protease from the cell culture media. Constructs were made to verify the plasmid origin of the WSN segment by introducing five silent point mutations in the NA coding sequence. Incorporation of the synthetic RNP into influenza virus was proved by the presence of mutations in the viruses recovered in the absence of protease. This system allowed, for the first time, site-directed mutagenesis of an IAV gene, however, it depended on helper-virus infection and strong selection necessary to distinguish recombinant virus from the wild-type helper virus. Since its first report, the RNP transfection method has been improved or modified in several ways: coupling *in vitro* transcription with RNP reconstitution [11]; using electroporation instead of DEAE transfection [12]; preparing the NP and polymerase proteins from infected cells rather than purified virus [13], and by adding native RNP cores instead of using helper influenza virus [14].

Recombinant Vaccinia Virus / Stable Cell Lines as a Source of T7 Polymerase

Concurrent with efforts to perform reverse genetics with influenza virus, techniques to manipulate the genomes of non-segmented negative-sense RNA viruses were being developed and were often based on transcription of vRNA by the T7 RNA polymerase. A major achievement was made by Pattnaik et al. [15] who employed a method that enables rescue, without VSV helper virus, of virus like particles that originate entirely from cDNA. The cDNA corresponding to a Defective Interfering (DI) genome was placed under control of bacteriophage T7 promoter such that transcription would initiate on the first DI-specific

nucleotide. The 3' end of the transcript was cleaved at the last DI nucleotide by placing Hepatitis Delta Virus (HDV) antigenomic ribozyme downstream of the DI RNA. The ribozyme was positioned such that autocatalytic cleavage released DI genome RNA with the exact termini required. Plasmid derived RNA was successfully produced *in vivo* by transfecting this construct into cells previously infected with a recombinant vaccinia virus vTF7-3 as a source of T7 polymerase [16]. Co-transfection of further T7 constructs containing VSV genes enabled efficient encapsidation and replication of the DI RNA. In the presence of all VSV genes the DI RNA could be packaged into VSV DI particles which budded from the cells [15].

A similar approach was later used by Schnell et al. [17] who successfully recovered recombinant rabies virus. These authors co-transfected vaccinia virus-infected cells with plasmids, encoding the viral nucleocapsid protein (N) and the polymerase proteins (L and P) under the control of T7 promoters, together with a plasmid encoding a full-length antigenomic RNA under the control of T7 promoter at the 5' end and a self-cleaving ribozyme at the 3' end. After transcription of RNAs from the T7 promoters and translation of the encoded proteins, nucleocapsid proteins assemble around the antigenomic RNAs, and polymerase proteins then replicate these RNPs to form RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and translation, infectious virus is assembled. This reverse genetics technique was adapted by laboratories studying other non-segmented negative-sense RNA viruses, resulting in the rescue of VSV [18,19], measles virus [20], RSV [21], Sendai virus [22,23], human parainfluenza virus 3 [24,25], and simian virus 5 [26]. It was thought that the synthesis of a positive-sense anti genomic RNA from cDNA was the key to successful virus rescue. This RNA, in contrast to negative-sense genomic RNA, cannot hybridize to positive-sense mRNA encoding viral proteins and thus does not interfere with virus generation. Also, premature abortion of T7 RNA polymerase transcription can be caused by stretches of uridine residues followed by hairpin structures present in some negative-sense RNA viruses [19].

Some modifications to the original technique have been made, such as the use of stably transfected cell lines expressing the T7 RNA polymerase protein, or one or more of the viral proteins required for genome replication [20]. For example, Kato et al. [23] provided the second report of recovery of Sendai virus from cDNAs. In this report, virus was recovered from both negative and positive sense RNAs transcribed *in vivo* in the vaccinia T7 system, or transfected into the system after being transcribed *in vitro*. The efficiency of recoveries from antigenomic RNA constructs was much higher than reported by Garcin et al. [22]. These results were achieved by: i) truncation of the T7 promoter (by removing the guanosine triplet) thus providing a precise 5' end to the viral RNA transcripts; ii) optimization of the NP, P, and L plasmid ratios, and iii) inhibition of vaccinia cytopathic effect by incubating in the presence of both AraC and rifampicin [23]. In most of the methods, the positive-sense antigenomic RNA was used as opposed to the negative-sense genome RNA. This is critical because of an antisense problem. If the negative-sense genome is used instead, mRNAs encoding viral proteins can hybridize to

the naked genomic RNA and prevent the critical assembly of the genome into the RNP [27]. As previously mentioned, the negative-sense viruses always keep their genome in RNP form, probably in part to avoid this antisense problem. Once in RNP form, the positive strand can then be replicated to form full-length minus sense RNPs that are wrapped into NPs as nascent RNA chains and thus immune to interference from mRNAs.

Bunyavirus promoter elements and the viral proteins that are required for transcription and replication were studied using a reverse genetics approach. Dunn et al. [28] cloned the CAT gene in the negative-sense orientation between the 5' and 3' UTRs of the Bunyamwera bunyavirus S RNA segment. As with influenza virus, the terminal sequences of bunyavirus RNAs are complementary, highly conserved and crucial for promoter activity. Cells were transfected with constructs expressing the proteins encoded by the L and S segments followed by transfection with *in vitro* transcribed RNA, which resulted in CAT activity. The bunyavirus S segment encodes two proteins, N and NSs, in overlapping reading frames. To determine whether both of these proteins are required for transcription and replication, constructs expressing only N or NSs were tested. N protein expression, together with L protein expression, resulted in CAT activity from the reporter RNA, whereas no CAT activity was detected with the L and NSs expression constructs. Thus, it was concluded that the L and N proteins are sufficient for transcription and replication of a bunyavirus-like RNA. In addition, it was shown that, like the influenza virus polymerase complex, the bunyavirus polymerase protein can apparently start transcription and/or replication internally [28].

A year later, the first recovery of the *Bunyamwera virus* entirely from cDNAs was reported by Bridgen and Elliott [29]. In contrast to the influenza system, which required helper virus, a helper-free system was employed. Each antigenomic RNA construct was expressed from a T7 promoter and had the self-cleaving hepatitis delta virus ribozyme at the 3' end. Each antigenome transcript contained two extra nonviral guanosine residues at the 5' end. Three plasmids were transfected expressing the three antigenomic viral segments (L, M, and S) along with three T7 plasmids expressing the viral mRNAs encoding all the viral proteins (N, NSs, G 1, G2, NSm, and L) into HeLa cells infected with the recombinant vaccinia virus expressing T7 polymerase. To increase the number of bunyavirus particles relative to the number of vaccinia virus particles, the authors took advantage of the ability of *Bunyamwera virus* to replicate in mosquito cells and introduced a passage step through *Aedes albopictus* C6/36 cells. Therefore, extracts of the cells harvested after transfection were used to infect C6/36 cells, and after 1 week, supernatants from these cells were assayed for the presence of *Bunyamwera virus* by plaque formation on BHK cells. The rescue efficiency was about 10-100 plaques per 10⁷ cells in the original transfection, and the transfectant viruses grew with the same kinetics and to the same titre as authentic *Bunyamwera virus* [29].

A similar approach to the one used by Dunn et al. [28] was subsequently utilized for Rift Valley fever (RVF) *phlebovirus* which has an ambisense S RNA segment: the N and NSs proteins

are encoded in distinct ORFs, with the NSs ORF being in the vRNA sense. Both proteins are translated from specific subgenomic mRNAs. In the reverse genetics system developed for RVF virus [30,31], the antisense CAT reporter cDNA was also expressed using the T7-vaccinia virus system, while the L and N proteins were supplied from vaccinia virus recombinants.

Although the T7 RNA polymerase approach was used for transcription of single IAV genes [32,33], rescue of recombinant IAV using T7 RNA polymerase approach was only described in 2007 [34]. In this study, T7 RNA polymerase was expressed from plasmid DNA and recombinant virus was produced in avian, canine and human cell lines. Rescue of virus using this approach was more efficient than with the RNA polymerase I (pol I) unidirectional system, however, production of recombinant influenza virus A/PR/8/34 was not as efficient as with the bidirectional RNA pol I system (discussed below). The authors suggested that developing a bidirectional T7 RNA polymerase system may enhance rescue efficiency of the T7 RNA polymerase approach [34].

Polymerase I as an Alternative Approach to *In vitro* Reconstitution

Development of a new approach for reverse genetics of influenza virus was reported by Neumann et al. [35-37] who pioneered the use of an RNA pol I-based system. This approach eliminated the need for protein purification, *in vitro* transcription, and *in vitro* RNP reconstitution by taking advantage of the characteristics of RNA polymerase I transcription. Unlike the mRNA transcripts produced by polymerase II (pol II), RNA polymerase I catalyzes the synthesis of ribosomal RNA (rRNA), which lacks 5' cap and 3' poly (A) structures. Therefore, artificial influenza virus RNA segments with precise 5' and 3' ends could be produced using pol-I driven transcription [35]. RNA polymerase I localizes to the nucleus, where influenza virus transcription and replication occur. Hence RNA polymerase I transcription systems can be used to generate vRNA-like transcripts intracellularly [36,37]. An RNA polymerase I-based system for reverse genetics of influenza viruses was established by cloning a cassette containing the coding region for CAT (in antisense orientation) flanked by the UTRs of HA between the mouse RNA polymerase I promoter and terminator sequences [35]. Helper influenza virus infection, followed by transfection of the RNA polymerase I-HA-CAT construct, resulted in CAT activity, demonstrating that the recombinant HA-CAT template was intracellularly transcribed by RNA polymerase (Figure 3A)[36]. Moreover, the recombinant HA-CAT vRNA was packaged into progeny virions. Pleschka et al. [38] used this technique to replace the vRNA segment encoding the NA glycoprotein with a plasmid-based construct, showing that the technique could substitute for RNP reconstitution in replacing single vRNA segments. Expression of the RNA segment of interest was under the control of a truncated pol I promoter at the 5' end and pol I terminator at the 3' end. The RNP protein components (PB1, PB2, PA and NP), shown previously to be the minimal proteins required to reconstitute influenza virus polymerase activity [39], were encoded on plasmids under the control of pol I promoters. These plasmids were transfected into

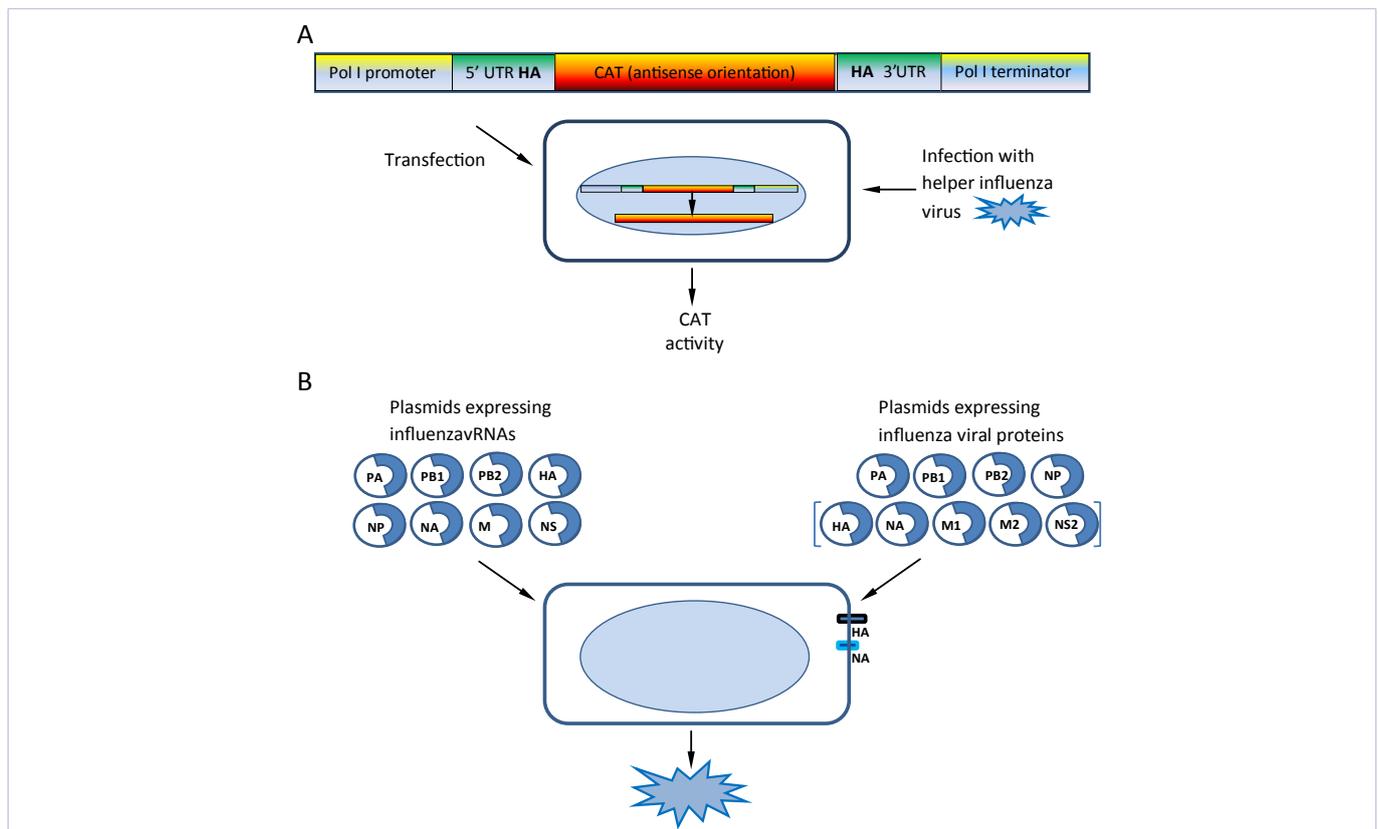


Figure 3: RNA Polymerase I Reverse Genetics System: (A) A plasmid containing the coding region for CAT (in antisense orientation) flanked by the UTRs of HA between mouse RNA polymerase I promoter and terminator sequences is transfected into eukaryotic cells. The cells are also infected with helper influenza virus. Intracellular transcription by RNA polymerase I results in expression of the CAT gene and CAT activity which can be measured (adapted from [36]). (B) Recovery of influenza virus from transfectants. Plasmids containing the RNA polymerase I promoter, a cDNA for each of the eight vRNAs, and the RNA polymerase I terminator are transfected into cells together with protein expression plasmids. Although infectious viruses can be generated with plasmids expressing PA, PB1, PB2, and NP, expression of all remaining structural proteins (shown in brackets) increases the efficiency of virus production (adapted from [40]).

cells and the RNPs assembled intracellularly.

The system described by Neumann et al. [40] represents the conclusion of this work and resulted in the ability to manipulate every gene in the influenza virus genome. They developed a system that utilizes the host cell for making the equivalent of newly released RNPs by cotransfecting eight plasmids encoding each of the influenza virus genomic RNA segments under control of the RNA pol I promoter and transcription terminator along with four plasmids encoding the polymerase complex proteins and NP cDNAs under control of an RNA pol II promoter (Figure 3B). The concept of co-transfecting multiple plasmids to reconstitute a biochemical activity was first used for studying herpes virus DNA replication [41]. The scale of transfection used by Neumann et al. [40] for influenza virus, utilizing 12 plasmids, was very impressive. The lack of a helper influenza virus allows the virus from the initial transfection to be characterized immediately, thus limiting the chance of viruses containing reversion or second-site mutations from becoming significant contaminants.

After this, the primary objective was to generate influenza virus from the least number of plasmid constructs that can be transfected into cultured cells to provide high viral yields.

Hoffmann et al. [42] reduced the number of plasmids down to eight to develop a more efficient reverse genetics system: the RNA polymerase I/II system. They inserted viral cDNAs between a human pol I promoter and a mouse pol I terminator to produce negative-sense vRNAs, while viral proteins were synthesized by inserting the pol I transcription cassette between a human CMV pol II promoter and a bovine polyadenylation signal. This unique approach allowed for production of both negative-sense vRNAs, synthesized in the forward direction, and positive-sense mRNAs, synthesized in the reverse direction. Reducing the number of plasmids used for IAV rescue increased the transfection efficiency of mammalian cells, enabled the use of cell lines with low transfectability and resulted in higher virus yields [42].

While the twelve-plasmid and eight-plasmid reverse genetics systems for the generation of IAV from cDNAs are the most widely used, three-plasmid and one-plasmid systems were also developed to further improve transfection efficiency and virus yield. Neumann et al. [43] developed a three-plasmid IAV rescue system in which cDNAs were placed between the human pol I promoter and the mouse pol I terminator. These transcription cassettes (eight in total) were then cloned into one plasmid.

The viral proteins PB2, PB1 and PA or NP were expressed from two plasmids with the cDNA flanked by a pol II promoter and a polyadenylation sequence.

A one-plasmid reverse genetics system was developed by Zhang et al. [44]. cDNAs encoding PB2, PB1, PA and NP were first inserted between a pol I promoter terminator and a pol II promoter-terminator. The pol II promoter was omitted from the cassette containing HA, NA, M or NS. All eight cassettes were then ligated to form a single plasmid. Virus yields generated using the 23.6 kb eight-unit plasmid system were comparable with the eight-plasmid system in chicken embryonic fibroblasts, while their co-culture with Madin-Darby Canine Kidney (MDCK) cells further improved the yields. Since RNA Pol I is species specific, the choice of cell line used to generate influenza virus using the one-plasmid system depends on the type of Pol I promoter used. The interesting factors that may have reduced intraplasmid recombination events and ensured plasmid stability are a low-copy-number vector (p15A *ori*, approximately 15 copies per bacterial cell), minimized number of CMV promoters and decreased length of homologous sequences by alternate placement of a single promoter and a dual-promoter cassette.

While pol I-based reverse genetics systems were developed for influenza virus, Flick and Pettersson [45] turned to the RNA pol I expression system to use it as an alternative approach for developing a reverse genetics system for *Bunyaviridae*. As described earlier for influenza virus, in the pol I system, cDNAs coding for viral RNA segments, or reporter genes flanked by viral sequences, are cloned between the RNA pol I promoter and terminator to generate transcripts that have correct 5' and 3' ends without modifications such as a cap structure and a poly(A) tail [35, 46]. Flick and Pettersson [45] used the pol I system to express reporter genes flanked by the 5' and 3' untranslated sequences of the mRNA segment of Uukuniemi (UUK) virus, a member of the *Phlebovirus* genus. They showed that the pol I system could be used to synthesize chimeric RNA templates, which, despite lacking a cap structure and poly(A) tail, are transported to the cytoplasm, where they are amplified and transcribed by the UUK virus replicase components supplied either by superinfection with UUK virus or by expression of viral proteins from separate plasmids. The L and N proteins were found to be necessary and sufficient for transcription and replication. One important question is whether the pol I transcript is amplified by replication. As was shown by Flick and Pettersson for UUK virus, although not directly quantifying RNA synthesis, they observed high expression level of CAT and Green Fluorescent Protein (GFP) that could not have been achieved unless replication had occurred. Based on their previous experience, the overall level of CAT activity was much higher than that obtained in the influenza virus up-regulation mutant. Finally, the fact that extracellular medium from transfected and UUK virus-superinfected cells could be used to serially passage CAT activity strongly suggests that the pol I transcript must have been amplified and packaged.

Vaccinia virus (VV) has been used either to direct the synthesis of the T7 RNA polymerase [16], which then drives the expression of the reporter construct and the viral helper proteins

[18,21,24,26,47,48] or to express the viral helper proteins directly [28,30,31]. However, the pol I system offers several advantages over the VV-based reverse genetics systems. For example, VV introduces into the cell a number of unwanted enzymatic activities, which are avoided by using the pol I system. In addition, there is no need to remove the VV, by physical or biochemical means [17-19], by passaging the virus through cells not permissive to VV or by using a variant VV (MVA-T7) which does not replicate in mammalian cells [49]. However, these problems can be avoided by using cell lines that stably express bacteriophage T7 polymerase or by expressing it from plasmids. The pol I system also has the advantage of generating the exact 5' and 3' ends of the RNA transcripts, thus avoiding the need for expressing runoff transcripts from restriction enzyme-cleaved plasmids or the use of a ribozyme to produce the correct 3' end. However, it should be borne in mind that: i) unlike influenza virus, transcription of RNA by most negative sense viruses occurs in the cytoplasm whereas polymerase I produces RNA in the nucleus, and ii) RNA pol I transcription has stringent species specificity which prevents the utilization of this system in every cell type.

Applications of the Reverse Genetics Systems

Understanding the Biology of these Viruses

RNP reconstitution *in vivo* and *in vitro* has allowed detailed analysis of the viral promoter and other *cis*-acting signals important for the regulation of transcription and replication (e.g., polyadenylation signals, *cis*-acting signals within the untranslated regions). Reverse genetics has also made it possible to study the functional importance of viral proteins during infection, structure-function relationships of viral gene products and molecular aspects of viral pathogenicity. Transfectant viruses generated by this technique can also be used to address issues regarding virus-host cell interactions in transport and assembly processes of viral components.

The development of reverse genetics systems for the *rhabdoviruses*, *paramyxoviruses* and *orthomyxoviruses* has provided tools to investigate in more detail the roles of the *cis*-acting RNA elements involved in replication and transcription. Applying this approach, Hoffman and Banerjee [24] demonstrated that nucleotides 1-12 (from the terminus) of the leader promoter formed a domain critical for human parainfluenza virus type 3 replication. In addition, they showed that no mutations in these regions caused defects in transcription, although mutations in the intergenic sequence and the gene start found at the leader-gene junction did disrupt transcription.

The functional analysis of the influenza viral RNA promoter *via* reverse genetics led to the proposed 'corkscrew' model for the 5' and 3' vRNA terminal sections in their coordinate binding to viral RNA polymerase [46,50]. Originally it was believed that the double-sense panhandle formed by the termini of all influenza virus gene segments might be involved in polymerase recognition, however, application of this approach led to the conclusion that 3' terminal sequences alone could optimally function as a promoter [6]. Moreover, it was shown that various base-pair exchanges not only restored promoter function, but

resulted in increased levels of promoter activity, especially when positions 3 and 8 in the 3' branch or 3 and 8 in the 5' branch of the vRNA promoter structure were involved in such complementary double exchange.

Construction of cRNA promoter variants through RNA polymerase I reverse genetics allowed determination of the RNA polymerase-associated, base-paired conformation in a reporter gene read-out system. It turned out to adhere to the 'corkscrew' model, similar, but slightly different in its binding interactions from the corresponding vRNA conformation. The observation of two transcription reactions, initiated in either direction from influenza vRNA and cRNA template molecules, allowed construction of bicistronic, ambisense RNA molecules for simultaneous expression of two proteins from a single segment of vRNA [51].

Two other studies used the RNA polymerase I system to determine the function of influenza virus proteins. Neumann et al. [52] generated virus-like particles that entirely lacked or possessed mutations in the NS2 gene and examined the effect of these modifications on vRNP nuclear export. This study confirmed a previous finding by O'Neill et al. [53] that NS2 is critical for vRNP nuclear export, mediated by a nuclear export signal in the N-terminal region of NS2. Watanabe et al. [54] studied the role of the M2 ion-channel protein. Viruses were generated that lacked or contained mutations in the M2 transmembrane domain, indicating that influenza A viruses can undergo multiple cycles of replication without M2 ion-channel activity in cell culture. However, viruses defective in M2 ion-channel activity did not efficiently replicate in mice, demonstrating that this activity is critical for the viral life cycle. Similar approaches can be employed to determine the functions of other influenza virus proteins or cellular events involving specific viral proteins [54].

Viral attenuation as a result of reverse genetics through specific mutations has practical significance in vaccine development. Such attenuating mutations include those eliminating gene products that are nonessential for replication in tissue culture, those rearranging gene order, and those deleting the cytoplasmic tails of viral glycoproteins [55-59]. Deletion mutants generally cannot revert, thus permanent attenuation should be possible in such recombinants.

Using a reverse genetics approach it is possible, for example, to produce a master strain of influenza virus with multiple attenuating mutations in the genes encoding internal proteins. This can be used to produce a high-yield reassortant virus that possesses the HA and NA from a currently circulating strain, exploited in the production of inactivated vaccines and as a potentially useful vector for gene transfer into mammalian cells. Studies with helper virus-dependent reverse genetics systems have demonstrated that influenza virus can accommodate additional genetic material. For several short polypeptides, including the V3 loop of HIV-1 gp 120 protein [60], a highly conserved epitope from the ectodomain of HIV-1 gp41 [61], and a B-cell epitope from the outer membrane protein F of *Pseudomonas aeruginosa* [62], insertion in the antigenic sites of HA resulted in immune responses against the foreign epitope (reviewed in [63]).

Applications of the reverse genetics system can also be demonstrated using VSV as an example. It was shown that genes encoding foreign membrane glycoproteins can either be incorporated as extra genes in VSV or can be incorporated in place of the VSV G gene [64-66]. Therefore, it is possible to obtain viruses containing the foreign proteins in their envelopes by swapping the endogenous VSV G gene for genes encoding foreign glycoproteins. These viruses lack the normally broad tropism conferred by VSV G and can be targeted to specific cells. For example, VSV recombinants expressing the HIV receptor and a co-receptor in place of G incorporate both foreign proteins and are targeted specifically to cells infected with HIV-1 which display the HIV-1 envelope proteins on their surface [66].

Recombinant VSVs expressing foreign antigens have potential in vaccine application and have been shown to elicit protective immunity in experimental animals. Vaccination of mice with a single dose of recombinant VSV expressing the influenza hemagglutinin provided complete protection from influenza challenge [27]. Other examples cited include: expression and incorporation of the IAV neuraminidase (NA) proteins by Kretzschmar et al. [67], the HIV-1 envelope protein (gp 160) with a VSV-G cytoplasmic tail by Johnson et al. [68], the MV fusion (F) and hemagglutinin (HA) proteins by Schnell et al. [64], the RSV Glycoprotein (G) and Fusion (F) protein and the cellular proteins CD4, CXCR4, and CCR5 by Schnell et al [66].

To summarize, reverse genetics approaches have now been described for representatives of most groups of negative-sense RNA viruses and gave an opportunity to study different aspects of viral replication, pathogenesis, interaction with vectors, and to develop genetically engineered vaccines.

Expression of 2A-Linked Recombinant Polyproteins within Influenza Virus Segments

Several bicistronic strategies have been used for the expression of foreign genes from the genome of IAV. These employ internal promoters [69], mini-genes [70], overlapping (e.g. -UAAUG-) or highly proximal stop/start sequences [71], and Internal Ribosome Entry Site (IRES) elements [72]. Each of these is associated with a number of limitations [73,74] but one particular disadvantage is uneven/unreliable protein expression levels. While the IRES allows cap-independent translation of a second protein from a single transcript the level of the second protein is often significantly reduced [75]. An alternative strategy is the use of self-processing viral 2A peptides which gives approximately equal expression of multiple genes under the control of a single promoter [76,77]. Analysis of recombinant foot-and-mouth disease virus (FMDV) polyproteins and artificial polyprotein systems in which 2A was inserted between two reporter proteins showed that just 2A, plus the N-terminal proline of the downstream protein (-LLNFDLLKLAGDVESNPG¹P-) was sufficient to mediate a highly efficient co-translational "cleavage" [76,78,79]. Briefly, the 2A region of the polyproteins manipulates the ribosome to "skip" the synthesis of the glycyl-prolyl peptide bond at its own carboxyl terminus leading to the release of the nascent protein followed by translation of the remaining downstream sequence. In this

manner multiple, discrete, translation products are derived from a single open reading frame. This technology has been crucial for human gene therapies targeting cancer, production of induced human pluripotent stem cells for regenerative medicine, creation of transgenic animals and plants with improved nutritional properties and the production of high-value proteins for the pharmaceutical industry [80,81]. When using the 2A system it should be borne in mind that the 2A oligopeptide remains as a C-terminal extension of the upstream fusion partner. In the case of proteins translocated to the Endoplasmic Reticulum (ER) a strategy was adopted to include furin cleavage sequences ($^{-1}$ RRRR-, $^{-1}$ RKRR-, $^{-1}$ RRKR-) between the upstream protein and 2A to remove the “unwanted” tag [82]. In plants the first nine amino acids (SN⁴AADEVAT) of the LP4 peptide of *Impatiens balsamina* was connected to the 20aa 2A to generate a similar hybrid linker peptide [83,84].

Using reverse genetics techniques, several 2A-based strategies have been developed to generate recombinant influenza viruses expressing additional heterologous proteins. The genome of IAV consists of eight segments of negative-sense RNA. Among them, the smallest (NS, segment 8), encoding two proteins (NS1 and NEP) through an alternate splicing mechanism, is a suitable target for genetic manipulation. NS1 has been shown to tolerate

relatively long insertions and foreign protein is produced in large quantities in infected cells. For instance, the segment was modified to express NS1-GFP and NEP as a single polyprotein with a self-cleaving 19aa porcine teschovirus-1 (PTV-1) 2A site between them to release the upstream fusion protein from NEP during translation (Figure 4A). Although the strategy depends on mutation of the splice acceptor signal to prevent splicing of this segment the GFP reporter virus replicated efficiently in different cell populations and caused significant pathogenicity in mice. In addition, the constructed GFP expressing viral vector was used as a tool for the tracking of influenza virus infection in animals treated with two antiviral agents, amantadine and oseltamivir, which block virus uncoating and virus spreading, respectively [85]. A novel approach for the sensitive quantification of viral infection and spread by analysis of enzymatic activity in cell culture supernatant was used by Eckert et al. [86] who inserted the *Gaussia* Luciferase (GLuc) reporter into segment 8 flanked by complete NS1 and 2A-NEP genes (Figure 4A). Independently, Heaton et al. [87] developed a luciferase-expressing IAV and identified the polymerase PB2 segment as a second site in the genome to tolerate a reporter gene insertion. In this virus, GLuc was connected to PB2 *via* a foot-and-mouth 2A sequence and had a KDEL, ER retention signal for *in vitro* characterization (Figure 4B). The luciferase virus, PR8-GLuc, was subsequently used for *in*

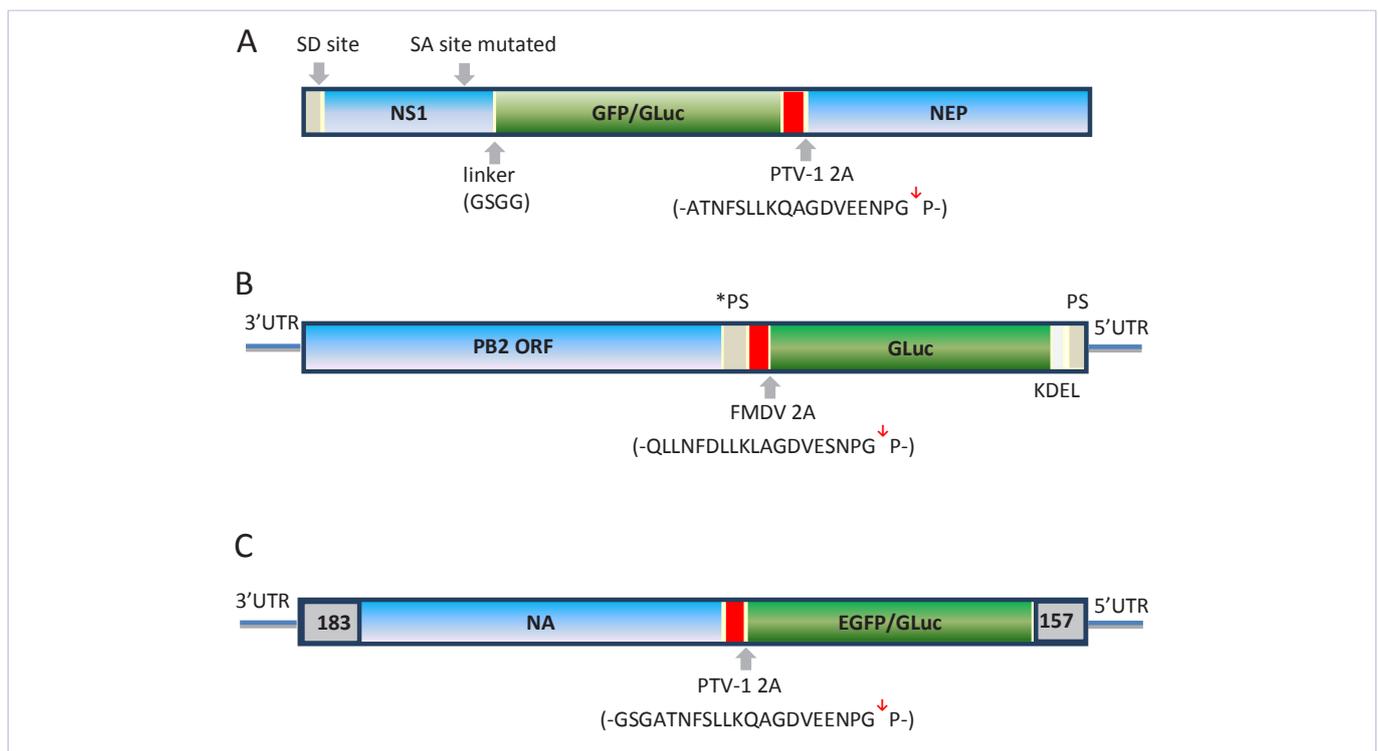


Figure 4: Construction of Reporter Influenza A Viruses: (A) Diagram of the recombinant NS1 protein expressed by influenza A vectors where complete NS1 and NEP/NS2 genes flank a reporter gene, either GFP or secreted GLuc. Additional features such as Gly-Ser-Gly-Gly linker between NS1 and reporter gene and the Porcine Teschovirus-1 (PTV-1) 2A “StopGo” sequence are indicated, as are the Splice Donor (SD) and mutated Splice Acceptor (SA) site. (B) Schematic representation of the PB2 segment of PR8-GLuc encoding the GLuc transgene with a FMDV 2A peptide-coding sequence and KDEL ER retention signal. *PS represents silent mutations of the original packaging signal; PS represents the duplicated original packaging sequence. (C) Schematic diagram of chimera NA + EGFP or NA + GLuc. The 3'- and 5'- end UTRs, and 183nt and 157nt in the NA-coding regions were responsible for the efficient incorporation of vRNA into virus particles. The reporter gene was inserted into the C-terminal region of NA linked by a Gly-Ser-Gly spacer ahead of the PTV-1 2A cleavage sequence.

vivo experiments to study the efficacy of monoclonal antibodies that bind to the conserved IAV hemagglutinin stalk and protect mice in challenge studies.

It has been suggested that immunization with influenza virus NS vectors expressing the 6 kDa early secretory antigenic target protein (ESAT-6) derived from *Mycobacterium tuberculosis* might be a potent vaccination strategy against tuberculosis (TB) [88]. ESAT-6 was expressed as a fusion protein with the 125 N-terminal amino acid residues from the influenza virus NS1 protein *via* a FMDV 2A linker. In this work, the NS vectors could induce an *M. tuberculosis*-specific CD4⁺ T-cell response after intranasal immunization in mice. Moreover, vaccination of mice and guinea pigs provided protection against tuberculosis equivalent to that provided by the widely used Bacillus Calmette-Guérin (BCG) vaccine. The coding capacity of this selected vector to maintain longer inserts allowed cassettes expressing two mycobacterium antigens, ESAT-6 and Ag85A (ESAT6-2A-Ag85A), to induce a broader TB-specific immune response [89]. In contrast to Ag85A, ESAT-6 protein is absent in BCG, raising the possibility of performing a BCG-prime influenza vector-boost immunization regimen.

Efforts have also been made to insert foreign sequences into the open reading frame of hemagglutinin and neuraminidase segments 4 and 6 respectively. The essential NA and HA proteins project through the viral envelope and are also expressed on the surface of influenza virus-infected cells. There have been several attempts to generate replication-competent recombinant influenza viruses carrying a reporter gene in the NA segment [69,90-92]. Indeed, it was reported that the insertion of the bacterial CAT gene with the 2A sequence into NA vRNA allowed CAT and NA to be stably expressed in infected cells [90]. Interestingly, the disruption of NA vRNA packaging signals at the 3' end in this study may have resulted in attenuation of the virus. Several studies suggest that segment-specific packaging sequences found in both 3'- and 5'- UTRs and coding sequences flanking each open reading frame have critical roles in packaging segments into the virion [93-95]. An improved strategy for generating replication-competent recombinant IAV carrying EGFP in NA vRNA, as either EGFP + NA or NA + EGFP, retained the 3'- UTR and adjacent 183 nucleotides (nt) of the coding region and the 5'- UTR and adjacent 157nt of the coding region (Figure 4C)[92]. A follow-up report for this application described the generation of IAV expressing a NA-2A-GLuc fusion protein for monitoring the course of infection in real time in living animals [96].

Conclusions and Remarks

Procedures developed during the last 25 years to genetically manipulate the genomes of negative-sense RNA viruses, commonly referred to as reverse genetics, have enhanced the investigation of viral gene expression and the dissection of *cis*-acting regulatory sequences that are important for replication and transcription. These new methods allow us to study viruses that are present only in low titers in infected cells or whose isolation is problematic. Information has also been garnered on natural or induced RNA recombination and mechanisms generating DI RNAs.

A common feature of these various systems is that the template RNA is derived from a cDNA clone containing authentic viral terminal sequences. However, there are certain variations in the developed approaches: the delivery of the template, the sequences present in the template, the source of viral proteins and the type of promoter to drive the transcription and expression. The template can be transcribed within the cell or delivered into the cell either as a transfected naked RNA or RNP complex. The sequences present in the template may also differ and contain either authentic viral transcripts or defective interfering RNA sequences or reporter genes or mutated RNAs. Transcription of the template RNA requires viral proteins that can be supplied either by recombinant sources or by helper virus infection. To direct transcription of the template and expression of viral proteins, various types of promoters have been utilized. These included the bacteriophage T7 promoter, RNA polymerase type I promoters and RNA polymerase type II promoters.

Chimeric polyproteins incorporating 2A have been widely used as tools for co-expression of two (or more) proteins in biomedicine and biotechnology. An advantage of using 2As is the ability to consistently express a number of proteins at an equal level. Using an IRES to co-express proteins usually results in the upstream protein being expressed 10 times more than the downstream one [75]. After "fine-tuning" of the foot-and-mouth 2A sequence, we suggest that researchers opt for 2A₃₀ (+11aa derived from the capsid protein 1D). This 2A proved to be the most favorable in terms of both length and cleavage efficiency and was unaffected by the sequence of the upstream gene [97,98]. In the case of shorter 2As, cleavage efficiency has been improved by insertion of various spacer sequences such as Gly-Ser-Gly or Ser-Gly-Ser-Gly ahead of the 2A sequence [99,100]. Although the FMDV 2A sequence has been the most widely used, biotechnologists should also be aware that other 2A-like sequences have been utilized successfully, including *Equine rhinitis A virus* (ERAV), PTV-1 and *Thosea asigna virus* (TaV) 2As [99,101,102].

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