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Segmented, Negative-Sense RNA Viruses of Humans: Genetic Systems and Experimental Uses of Reporter Strains

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Abstract

Negative-stranded RNA viruses are a large group of viruses that encode their genomes in RNA across multiple segments in an orientation antisense to messenger RNA. Their members infect broad ranges of hosts, and there are a number of notable human pathogens. Here, we examine the development of reverse genetic systems as applied to these virus families, emphasizing conserved approaches illustrated by some of the prominent members that cause significant human disease. We also describe the utility of their genetic systems in the development of reporter strains of the viruses and some biological insights made possible by their use. To conclude the review, we highlight some possible future uses of reporter viruses that not only will increase our basic understanding of how these viruses replicate and cause disease but also could inform the development of new approaches to therapeutically intervene.

INTRODUCTION

Negative-stranded RNA viruses (of the phylum Negarnaviricota) are a large group of enveloped viruses that infect a broad range of hosts. While their physical characteristics and genomic size and structure can vary dramatically, this group is thought to have originated from a common ancestor (1). Current phylogenetic analyses subdivide this group into two major subphyla, the Haploviricotina, which encompass nonsegmented genomes (such as Rhabdoviridae, Filoviridae, and Paramyxoviridae), and the Polyploviricotina, which are defined by segmented genomes (such as Orthomyxoviridae, Arenaviridae, and Phenuiviridae) (2) (**Figure 1**).

Like all obligate intracellular pathogens, these viruses must enter the cell and co-opt host factors to facilitate productive replication. Additionally, there are several broadly conserved defining features of all negative-sense RNA viruses. Perhaps most obvious is that the incoming viral RNA (vRNA) is not directly translatable but requires a viral RNA-dependent RNA polymerase (RdRp) to generate positive-sense messenger RNA (mRNA) before viral protein can be made. The vRNA is also thought to be exclusively found bound to a virally encoded RNA binding protein, a structure known as the ribonucleoprotein (RNP) complex. Despite these similarities, the hosts and host range, routes of transmission, disease presentations (or lack thereof), and mechanisms of viral replication can be highly variable.

As a group, negative-sense, segmented RNA viruses have received a large amount of attention as they include a number of human pathogens. Orthomyxoviruses such as influenza A virus (IAV) and influenza B virus (IBV) alone, for example, can infect up to an estimated 20% of the global population every year, leading to as many as 650,000 deaths (3). In addition to the cost in human life, the economic burden imposed by these viruses is estimated to be in the billions of dollars annually (4). IAVs also have previously caused a number of global pandemics, and they retain the potential to lead to new ones (5). There are also neglected and emerging zoonotic negative-sense, segmented RNA viruses such as lymphocytic choriomeningitis virus (LCMV), which can cause fetal developmental defects (6), and Rift Valley fever virus (RVFV), which poses threats to both humans and livestock biosecurity (7). Thus, understanding the biology that underlies viral replication and pathogenesis remains an important area of scientific investigation.

Viral reverse genetics, which is the process of introducing a precise genetic change into a viral genome so that the resulting virus can be studied or subsequently used in experimentation, is a powerful approach that has revolutionized our understanding of negative-sense, segmented

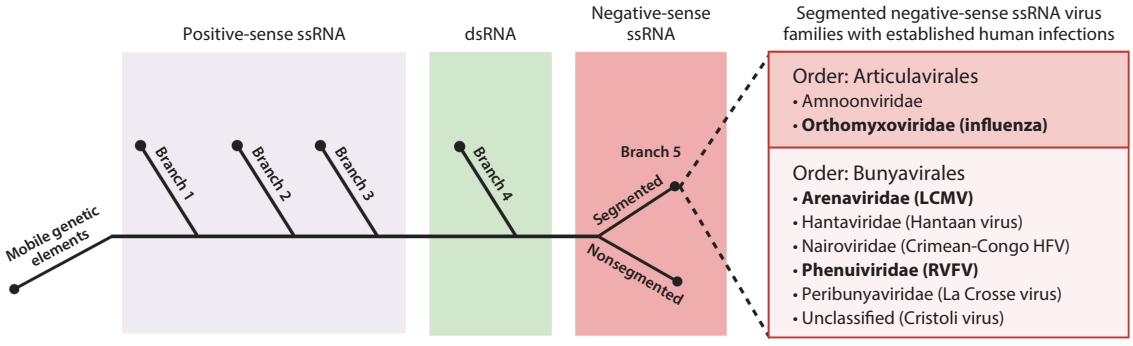

Figure 1

Representation of the RNA virus phylogeny with an emphasis on the segmented, negative-sense ssRNA families that have known human infections. Bolded families indicate viruses covered in this review. Abbreviations: dsRNA, double-stranded RNA; HFV, hemorrhagic fever virus; LCMV, lymphocytic choriomeningitis virus; RVFV, Rift Valley fever virus; ssRNA, single-stranded RNA.

RNA viruses. While the specific details of reverse genetic systems typically vary between individual viruses, the basic principles are the same. First, the full-length RNA viral genome is reverse transcribed and cloned into DNA plasmids so that they can be manipulated in vitro. The genome encoding plasmids (usually along with helper protein expression plasmids) are then introduced into cells, and the recombinant virus is produced. While this technology can be used in a number of different applications, reverse genetic systems to generate viruses that harbor reporter proteins have been particularly well utilized. By generating viruses that, for example, make an infected cell fluorescent, one can better understand viral replication rates or viral tropisms in animal models of infection. Alternatively, bioluminescent reporters can allow precise quantification of viral replication and represent a quick and cost-effective way to evaluate the efficacy of experimental antiviral therapeutics and vaccines.

In this review, we explore the history of experimental genetic systems as applied to negative-sense, segmented RNA viruses while emphasizing conserved approaches and applications illustrated by some of the prominent members such as IAV/IBV, LCMV, and RVFV. We also describe some of the reporter strains that have been generated, as well as the insights provided by their experimental use. Finally, we highlight some areas of potential future investigation with the use of reporter virus strains that will help complete our understanding of viral biology, pathogenesis, and intervention strategies.

ORTHOMYXOVIRUSES

Viruses belonging to the family *Orthomyxoviridae* can infect and cause disease in insects, birds, fish, livestock, and humans among other hosts. Perhaps the most notable human pathogens are IAV and IBV, which cause annual epidemics and occasionally devastating pandemics (8). Although IBV infection is typically restricted to humans, IAV infects a wide variety of hosts, including mammals and birds, with waterfowl considered a natural reservoir (9). Additionally, IAVs can swap genomic segments, leading to reassortment and the generation of novel viruses, which pose a significant threat to animal and human health (9).

The Genomic Organization of Influenza Viruses

IAVs and IBVs contain eight negative-sense, single-stranded RNA segments, each expressing at least one protein, and share general genomic organization. The coding sequence for each segment is flanked by short untranslated regions (UTRs), which contain the sequences and secondary structures required for transcription and replication (10, 11). A small region comprising the UTRs and part of the coding sequence serve as packaging signals and are required for segment assembly into newly formed virions (12).

The three largest segments of IAV encode the proteins PB2, PB1, and PA, which together form the heterotrimeric RdRp responsible for transcribing and replicating the genomic segments. Segments four and six encode the two major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which facilitate viral entry and release, respectively. Nucleoprotein (NP), which coats the genomic segments and together with the RdRp forms viral RNP (vRNP) complexes, is encoded in segment five. Segment seven encodes the structural protein matrix 1 (M1), which forms the base layer of the viral lipid envelope, and matrix 2 (M2) is required for nascent virion assembly. Finally, segment eight encodes the nonstructural proteins NS1 and nuclear export protein (NEP), which are responsible for innate immune system antagonism and nuclear export of viral mRNAs, respectively. Although IBV generally shares these proteins, there is very little sequence homology, and each virus (or even strains within viruses) employs different strategies to express these and additional accessory proteins, such as NB by IBV (13–18).

Early Influenza Virus Reverse Genetic Systems

While there was initial success with generating reverse genetic systems for positive-sense RNA viruses, negative-sense RNA viruses posed a distinct challenge in that their nucleic acid genomes are noninfectious on their own. Adding to this challenge is the unique structure of the influenza virus genome, which consists of eight individual genomic segments. Therefore, any approach required the replication and transcription of all eight individual segments, rather than just one.

The first established reverse genetic systems for IAV leveraged information gathered from seminal studies performed in the 1980s and 1990s, which demonstrated the three polymerase proteins (PB2, PB1, and PA), along with NP, were sufficient to transcribe and replicate vRNAs (11, 19–22). In a proof-of-concept study, Luytjes et al. (23) applied these findings to modify the influenza virus genome to express a foreign gene. Here, a viral-like RNA encoding a reporter protein was generated from complementary DNA (cDNA) under a T7 promoter in vitro and then incubated with polymerase and NP proteins, purified from influenza viruses, to form vRNPs. These vRNPs were transfected into human cells and, upon infection with a helper influenza virus, reporter activity could be detected. Based on reporter activity in cells infected with passaged virus, the authors concluded the reporter RNA was packaged into new virions. Building on this approach, Enami et al. (24) introduced several site-specific mutations to the influenza virus structural protein NA that were incorporated into progeny virions.

Over the next several years, modest improvements were made to this system. First, the efficiency of the vRNA transfectants system was improved by coupling RNA transcription and RNP complex formation, rather than approaching these two activities as separate steps (25). Second, and perhaps most importantly, the T7 promoter was replaced with the eukaryotic Pol I promoter (26). In this system, viral-like RNAs, without a 5′ cap or 3′ poly-A tail, were generated in vitro from a cDNA template flanked by Pol I promoter and terminator sequences. These viral-like RNAs, upon infection with influenza virus, could be recognized by the viral polymerase for transcription, replication, and packaging into nascent virions (26, 27).

However, while this system demonstrated influenza viruses could be genetically manipulated for the first time, there were several drawbacks. First, the use of helper viruses required the desired virus progeny to be selected from the output of infection, using either temperature-adapted (25), protease-dependent (24), or antibody-based selection systems (25). Second, it required isolating purified polymerase and NP proteins from virions, which was time-consuming. Third, viral yields were relatively low compared to wild-type stocks grown in embryonated chicken eggs.

Improved and Current Influenza Virus Reverse Genetic Systems

A major step forward in the development of influenza virus reverse genetic systems was made when two independent groups (at the University of Wisconsin and the Mount Sinai School of Medicine) successfully rescued IAVs without the use of a helper virus (28, 29). In both approaches, all eight IAV genes were cloned in separate cDNA plasmids under a Pol I promoter, which drove the expression of negative-sense vRNAs. These eight cDNAs were then transfected into cells along with plasmids that expressed the polymerase and NP proteins under a Pol II promoter. Here, the newly synthesized replicase proteins recognized the vRNAs generated by Pol I and transcribed both mRNA and complementary RNA, leading to viral gene expression, replication, and production of nascent virions. Both groups introduced mutations to viral genes through these approaches, which were incorporated in rescued viruses.

This approach opened many new doors including those for the development of influenza virus vaccines that had previously relied exclusively on recombination following the coinfection of cells with a contemporary, circulating influenza virus and the high-growth laboratory-adapted strain

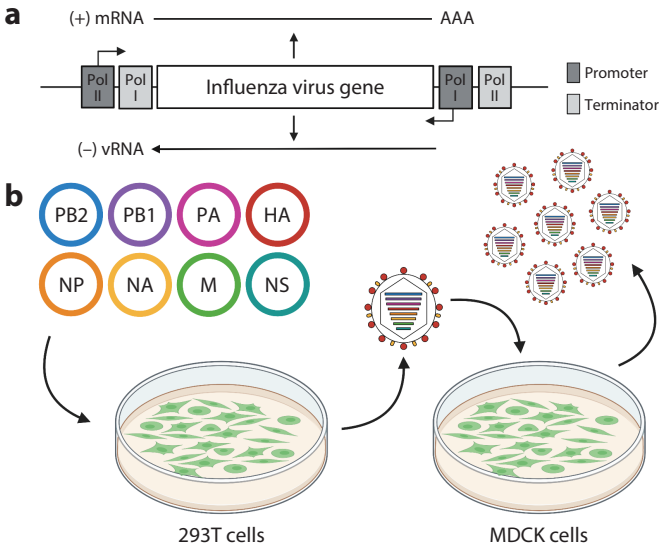

Figure 2

Current reverse genetic systems for influenza virus rescue. (*a*) Schematic of bidirectional approach to generate vRNA and mRNA from the same cDNA template. (*b*) Current protocol for generating influenza viruses from cDNA plasmids. Plasmids encoding the eight genomic segments are transfected into 293T cells. Supernatant-containing rescued viruses are then amplified on MDCK cells to generate high titer stocks. Abbreviations: cDNA, complementary DNA; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; mRNA, messenger RNA; NA, neuraminidase; NP, nucleoprotein; vRNA, viral RNA.

A/Puerto Rico/8/34. This inefficient and time-consuming process required the screening and selection of progeny viruses for the desired 6 + 2 genetic background, wherein the internal segments of the influenza virus are derived from the high-growth strain A/PR/8/34 and the HA and NA segments are derived from currently circulating strains. With this improved system, vaccine manufacturers could generate the desired recombinant virus using cDNA plasmids. A limitation, however, was that the cell lines approved for the generation of influenza virus vaccines, including Madin-Darby canine kidney (MDCK) and Vero cells, were not easily transfectable. Therefore, the chance of one cell receiving all twelve required plasmids and generating virus was low.

To help circumvent this challenge, a bidirectional approach was developed to express both vRNA and mRNA from the same plasmid (30, 31) (**Figure 2**). Here, a cDNA template encoding each IAV gene was flanked by a 5′ Pol II promoter and a 3′ Pol I promoter, resulting in the expression of both positive-sense mRNA and negative-sense vRNA. With this approach, cells would need to receive only eight plasmids simultaneously, rather than twelve, therefore increasing the odds an individual cell would have all the components required for virus rescue. Following transfection with all eight plasmids, cells displayed visible cytopathic effect, indicative of a lytic infection, and generated high titers of virus. In addition to rescuing a lab-adapted H1N1 strain, Hoffmann et al. (31) used this system to successfully rescue the avian influenza virus isolate A/Teal/HK/W312/97, demonstrating the utility of this approach to generate IAVs for natural isolates that would be difficult to acquire otherwise.

While this method was highly successful for generating IAVs, there are several fundamental differences between IAVs and IBVs that delayed the development of similar rescue systems for IBVs. First, consensus sequences for the noncoding regions, which are critical for the packaging of genomic segments into newly synthesized virions, were less well defined for IBV genomic

segments. Second, each virus uses different strategies to produce multiple proteins from comparable genomic segments. For instance, IAV segment seven encodes two proteins, M1 and M2, through splicing, while IBV segment seven encodes M1 and BM2 in separate open reading frames (ORFs). Finally, IBV expresses some genes with different kinetics, and it was not known if inauthentic gene expression patterns would impede rescue systems. However, two groups successfully established an eight-plasmid (32) and twelve-plasmid (33) rescue system in 2002 capable of generating IBVs of both the Victoria and Yamagata lineages.

Since the advent of the eight-plasmid system, several additional approaches to reduce the number of plasmids required for virus rescue have been established. In a three-plasmid system developed by Neumann et al. (34), cDNA cassettes encoding all eight influenza vRNAs were stitched together in one plasmid under a Pol I promoter. When transfected together with two plasmids encoding the replicase proteins, high viral titers were generated. Similarly, several groups have established one-plasmid or bacmid systems (35–37).

With reverse genetic systems in place, groups began to manipulate the influenza virus genome to express foreign proteins. To date, there have been many recombinant influenza viruses generated. However, for the purposes of this review, we focus on influenza viruses that express reporter proteins.

Development of Influenza Reporter Viruses

Reporter-expressing influenza viruses are influenza viruses that have been manipulated to express fluorescent, bioluminescent, or other proteins to allow for a qualitative or quantitative readout of influenza virus replication. In general, five strategies are used to encode reporter proteins in influenza viruses (**Figure 3**). First, a reporter may be inserted next to a viral gene. This strategy generally involves fusing the reporter to the viral protein or including 2A autoproteolytic cleavage sites to result in separation of the two proteins cotranslationally. Importantly, if the reporter is put on the 5′ or 3′ end of the viral gene ORF, the packaging signals must be duplicated so that they remain on the terminus of the segment (38). Second, a reporter can be inserted between two viral genes, typically between NS1 and NEP. This approach requires eliminating the splice donor/acceptor sites and incorporating at least one 2A cleavage site (39). Third, a segment encoding a reporter can be added by duplicating packaging signals and increasing the number of segments to nine or ten (40). Fourth, a reporter gene can replace a viral gene (41). However, it must be noted that these viruses generally cannot replicate without providing the missing viral gene in *trans*. In a fifth and final strategy, reporter proteins can be encoded in spliced intronic sequences, which requires introducing artificial introns (42).

Current Uses of Influenza Reporter Viruses

Reporter viruses have been used in a number of ways, including, but not limited to, identifying novel host factors (43–46), screening antiviral drugs (42, 47–56), and evaluating the efficacy of neutralizing antibodies (38, 41, 57–61). One area where reporter viruses have been useful has been the study of influenza-host interactions in vivo, particularly when used as tools to determine cellular tropism or viral replication kinetics, or to reveal mechanisms by which the host protects the lung or other sites of viral replication.

Prior to the development of reporter viruses, cellular tropism was determined by staining single cells or tissue sections for viral antigens or performing viral attachment assays (62, 63). However, inconsistencies in cellular tropism for the same virus have been reported, likely due to differences in ex vivo versus in vivo infections (64). Fluorescent or other enzyme-encoding influenza viruses have eliminated the need to perform time-consuming antigen staining and instead allow for direct

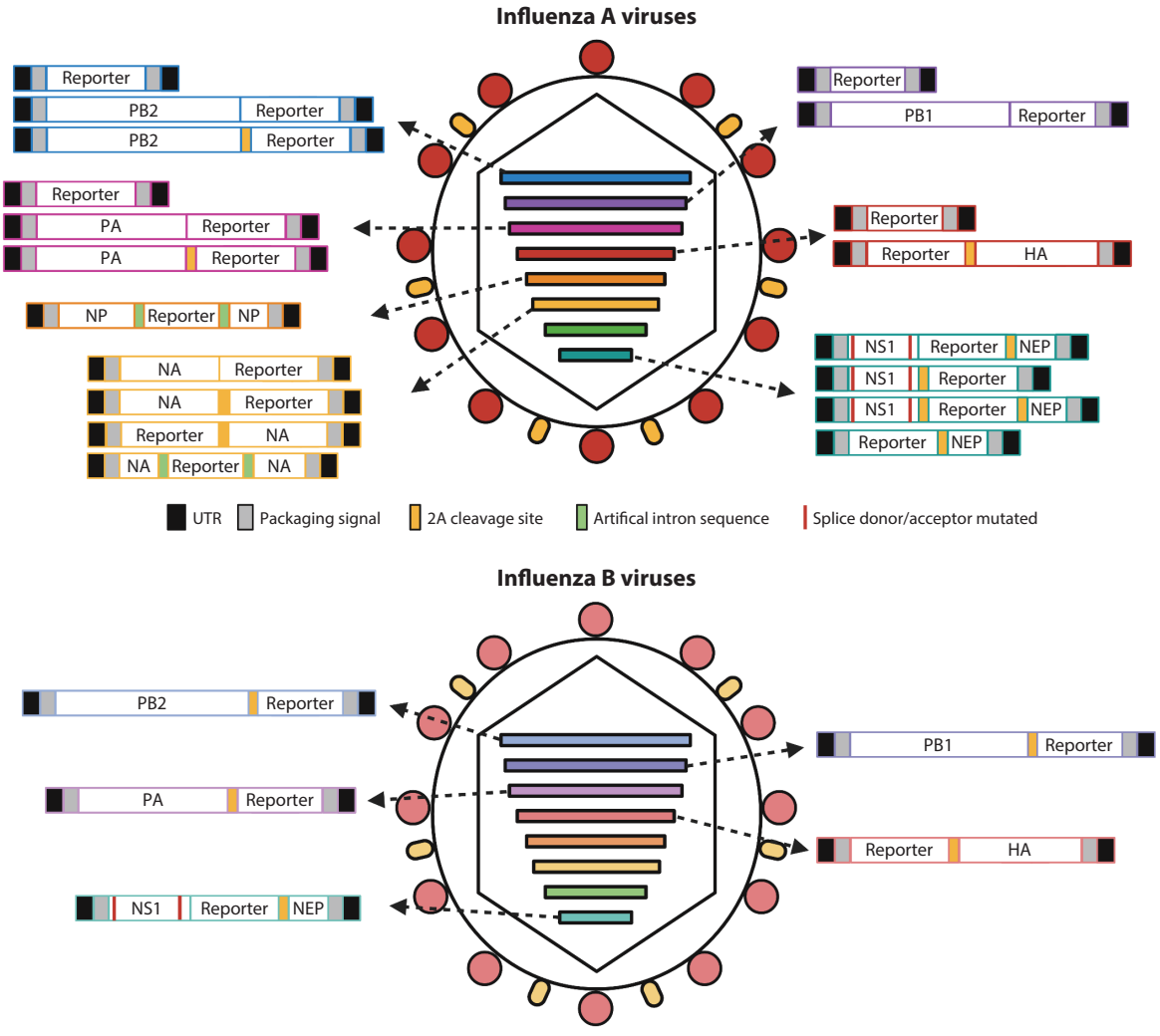

Figure 3

Genomic loci for influenza A and B viruses where reporter genes have been successfully inserted. Abbreviations: HA, hemagglutinin; NA, neuraminidase; NEP, nuclear export protein; NP, nucleoprotein; UTR, untranslated region.

qualitative readout of infection. Indeed, in vivo cellular tropism has been determined for a number of IAV and IBV strains using reporter viruses (39, 51, 65–69). Single-cycle reporter viruses have also been used to evaluate how cellular tropism is altered throughout the course of infection (69). This study revealed that respiratory epithelial cell populations induce different magnitudes of response to interferon signaling, and cell types with a strong interferon-induced antiviral response are more protected from later rounds of infection.

Reporter viruses have also been used as tools to track disease progression in animal models of viral infection. Influenza viruses encoding bright fluorescent proteins, such as mCherry or enhanced green fluorescent protein (eGFP), have been used to measure the kinetics of viral replication in the lungs of infected mice (45, 53, 56, 70–75). Additionally, reporter viruses encoding bioluminescent proteins have enabled real-time, nonlethal imaging of viral infection in vivo. These

bioluminescent influenza viruses have been used to evaluate the efficacy of candidate vaccines and antiviral therapeutics, determine host tropism, and study transmission dynamics (48, 58, 59, 76–79).

In addition to revealing cellular tropism and replication kinetics, reporter viruses have facilitated the detailed study of the host innate and adaptive immune responses to influenza virus infection. Multiple groups have used reporter viruses to reveal the contribution of various immune cell populations, and the underlying mechanisms, to antigen presentation (80–82). Using multiple single-cycle reporter IAVs incapable of progressing past various stages of the viral life cycle, Fay et al. (83) revealed specific innate immune responses are induced by different viral replication stages in multiple populations of epithelial cells. Additionally, single-cell RNA sequencing analyses were used to interrogate the innate immune response to IAV infection in single reporter-positive (infected) and reporter-negative (bystander) ciliated cells (66). This study revealed that a seemingly homogenous population of infected cells can elicit a number of distinct responses to IAV infection, even when isolated from the same host.

Finally, reporter viruses have furthered our understanding of infected cell fates in the lung and upper airway. Using Cre-expressing influenza viruses along with transgenic mice, multiple groups have demonstrated respiratory epithelial cells are capable of surviving direct infection with IAV or IBV in vivo (65, 67, 68, 84–86). These survivor cells provided short-term protection against infection with a variety of respiratory viruses, likely by inducing a prolonged inflammatory state in the lungs (85). Further, survivor cells have been found to display distinct gene expression patterns and alter their morphology after infection (65). These changes are hypothesized to provide critical support for the respiratory barrier and lung function, as mice depleted of survivor cells experienced delayed recovery from infection. Additional studies using this model system have also revealed unique mechanisms by which the brain likely protects itself from IBV infection (68). Thus, a number of fundamental insights into influenza virology are the direct result of experimentation with reporter-expressing strains. However, as with all modified virus studies, care in interpretation of the results must be taken when extrapolating to parental wild-type viruses.

ARENAVIRUSES

Mammarenaviruses are a large family of enveloped, bisegmented, negative-sense RNA viruses that chronically infect rodents worldwide and are capable of infecting and causing disease in humans (87). Severe infections in the immunocompromised population, including deaths among transplant acquired infections and neonates, have led to the classification of the prototypic arenavirus LCMV as a neglected human pathogen of clinical significance and an emerging fetal teratogen (6, 87). LCMV has proved a useful tool in the fields of virology and immunology, with many discoveries being relevant for other viral infections (88, 89). One feature of LCMV infections that makes them especially useful for research is that LCMV infections in mice are variable depending on host factors (including age, strain, and immune status), thus allowing for a system in which researchers can probe areas pertaining to various facets of virus-host interactions.

Arenavirus Genomic Structure

The arenavirus genome is composed of two segments, large (L) and small (S), that each encode two proteins oriented in an ambisense coding pattern (**Figure 4*a***). These proteins are separated by a noncoding intergenic region (IGR). The L segment encodes the RING protein Z and the RdRp, also known as the L polymerase protein, on the 5′ and 3′ ends, respectively (90). The Z protein (analogous to other RNA virus matrix proteins) is required for virion assembly and budding

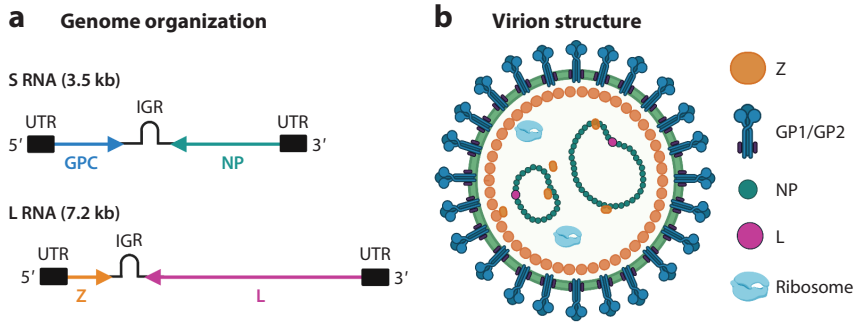

Figure 4

Arenavirus genomic and particle organization: (*a*) arenavirus genome and (*b*) virion structure. Abbreviations: GPC, glycoprotein precursor; IGR, intergenic region; L, large; NP, nucleoprotein; S, small; UTR, untranslated region.

while the L polymerase protein is necessary for viral replication and gene transcription (90). The S segment encodes on the 5′ and 3′ ends of the viral glycoprotein precursor (GPC) and the NP, respectively. The GPC is post-translationally processed to yield glycoproteins 1 and 2, which form the spikes on the virion surface and are necessary for host receptor binding and cell entry (91–93). The NP encapsulates the vRNA and along with the L protein forms the vRNPs, which are the basic components necessary for arenavirus genome replication and transcription (94, 95). The NP also interacts with the Z protein to incorporate the vRNPs into infectious virions (96) (**Figure 4*b***).

Arenavirus Reverse Genetic Systems

The development of reverse genetic systems began with the development of an arenavirus minigenome rescue system (94). Lee and colleagues (94) utilized an LCMV minigenome consisting of the negative-sense copy of the chloramphenicol acetyltransferase (CAT) reporter gene flanked upstream by S 5′ UTR and IGR and downstream by the S 3′ UTR, which allowed for the identification of *cis*-acting signals and *trans*-acting factors involved in the transcription and replication of LCMV. Subsequently, others developed minigenome systems for additional arenaviruses (97–100). Importantly, these early minigenome systems identified that the L and NP proteins represent the only viral *trans*-acting factors required for efficient RNA synthesis by the arenaviral polymerase (94, 97, 99).

As with influenza viruses, due to the negative-stranded nature of LCMV, the deproteinized genomic and antigenomic RNAs cannot function as mRNAs and are therefore not infectious. Thus, the generation of infectious recombinant LCMV (rLCMV) from cDNA requires *trans*-complementation of all viral proteins necessary for virus replication and transcription. Not long after the development of the minigenome system, Pinschewer and colleagues (92) developed the first molecularly engineered recombinant arenavirus. The GP gene of LCMV was replaced by the vesicular stomatitis virus G gene (rLCMV/VSVG). This system relied on the transfection of cells with the rLCMV S RNA segment followed by infection with wild-type LCMV as a helper virus and subsequent selection of rLCMV/VSVG by passage in S1P-deficient cells. Shortly thereafter, the generation of infectious virus entirely from cloned cDNAs, and without the need for a helper virus or time-extensive selection processes, represented another major step in arenavirus reverse genetic systems. Importantly, it was demonstrated that the cDNA-derived arenaviruses are comparable to wild-type virus in vitro and in vivo (101, 102). With the development of rLCMV from cDNA and the subsequent generation of rLCMV containing selected mutations, researchers have

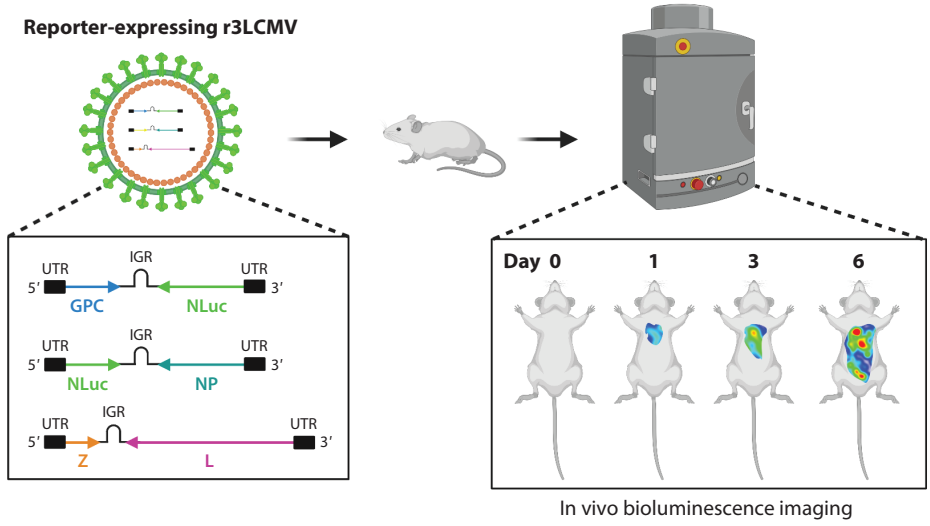

Figure 5

Arenavirus reporter strain usage: schematic representation of a bioluminescence reporter-expressing r3LCMV with the NP and GPC replaced by NLuc and its application for in vivo imaging of LCMV infection. Abbreviations: GPC, glycoprotein precursor; IGR, intergenic region; LCMV, lymphocytic choriomeningitis virus; NLuc, nanoluciferase; NP, nucleoprotein; r3LCMV, recombinant trisegmented lymphocytic choriomeningitis virus; UTR, untranslated region.

identified arenaviral elements important for viral assembly (92, 93, 95), infection (92, 93, 95), and arenavirus-host defenses (96, 103).

Trisegmented Reporter Lymphocytic Choriomeningitis Virus Systems

The development of recombinant trisegmented LCMV (r3LCMV) represented the next major advancement in arenavirus genetic systems. To generate a r3LCMV reporter-expressing virus, susceptible cells are cotransfected with expression plasmids that encode the NP and L segments. The resulting r3LCMV contains 1L and 2S segments whereby each of the S segments have either GPC or NP viral ORFs replaced by a gene of interest (GOI) (104) (**Figure 5**). Importantly it has been repeatedly shown that both GPC and NP can be replaced with a GOI without the threat of attenuation in cell culture (104–106); however, reporter genes are expressed in higher levels from the NP locus compared to the GPC locus (104–107). Various types of GOIs have been introduced into r3LCMV including genes useful for studying other viruses (92, 93, 108) and genes encoding host molecules (106), as well as tumor (109, 110) and vaccine-antigen specific genes (111, 112).

Over the years other variations of the r3LCMV system have also been generated including a r3LCMV where the viral NP and GPC ORFs replace one another (r3LCMV/TransS) (107), a r3LCMV with GPC under the 3′ UTR control to generate viruses with an artificial genome organization (109), and a r3LCMV/eGFP-Strep that expresses a C-terminal Strep-tag of eGFP that can replace either the GPC or NP (113). There has also been the recent generation of a bicistronic reporter-expressing LCMV that allows for production of both the green fluorescent protein (GFP) and NP proteins from the same mRNA (114). These variations in r3LMCV have allowed for the development of models suitable for arenavirus live-attenuated vaccines (107), a potential viral vaccine delivery system (109), a model to study the interaction of viral NP with host cell proteins (113), and surrogate measures for virus multiplication (107, 114). Together these

studies demonstrate the use of trisegmented arenavirus platforms to express a multitude of GOIs in vitro and in vivo and offer a way to assess various facets of arenavirus biology, infection, and pathogen-host interactions.

Lymphocytic Choriomeningitis Virus Reporter-Expressing Systems

In addition to the aforementioned type of genes that have been introduced into rLCMV systems, an important usage of rLCMV, especially the r3LCMV systems, has been the development of a variety of rLCMVs that stably express nonfluorescent reporters such as CAT (104, 115, 116) as well as fluorescent reporter genes. These include the replacement of wild-type viral GPC or NP segments with eGFP (104, 106, 107, 114–124), teal fluorescent protein (125), mCherry (126, 127), or ZsGreen (128–130). Not only do these reporters allow for assessment of viral replication in less time than is needed for traditional plaque assays, but also they have been a powerful tool in high-throughput screening approaches used to identify small-molecule antivirals (131), identification of receptors required for host cell infection (126), and the discovery that LCMV can cause long-term immune dysfunction months after infection (125), among many other important discoveries.

The development of bioluminescent reporter-expressing rLCMV, including reporters with firefly luciferase (104), Gaussia luciferase (107, 115, 117, 120), Renilla luciferase (124), and nanoluciferase (NLuc) (132), offers additional ways to study virus infection and replication not only in vitro (115, 124, 132) but importantly in vivo as well (104, 132) (**Figure 5**). The use of bioluminescent reporter tracking with in vivo imaging has proven useful for the identification of antiarenaviral drugs (124, 132) and analysis of viral tropism during infection (132). Recently, using a NLuc expressing rLCMV, bioluminescent virus was able to be detected as early as one day postinfection, which was up to twelve days prior to obvious signs of disease or death (132). This rapid identification of viral replication and tropism will be useful for investigating real-time virus infection and could help identify tissues important for infection that may be missed by more traditional tissue harvest methods. Importantly, antiviral therapeutic and vaccine efficacy can be monitored in real time and quantitatively evaluated in vivo using this system. However, it will be important to consider the size limits of bioluminescent reporters that can be introduced into the rLCMV, as some reporter-expressing rLCMV has been attenuated in vitro and in vivo, suggesting there may be a limit in the acceptable length of introduced genes (104).

BUNYAVIRUSES

RVFV is a mosquito-borne, negative-stranded, segmented arbovirus and member of the *Bunyavirales* family that causes acute hemorrhagic fever in both human and animal hosts. Over the past 30 years, endemic and epidemic outbreaks of RVFV in humans and livestock have mainly been localized to Africa and the Arabian Peninsula. However, studies into suitable vectors for the virus have suggested that it can be transmitted through the dominant mosquito species of a given geographical location, in addition to bodily fluids and aerosol transmission. This wide breadth of transmission pathways and zoonotic host diversity presents RVFV as a threat to global human health and livestock biosecurity. RVFV reporter viruses have been developed and utilized to not only inform our understanding of the fundamental molecular biology and pathogenesis of the virus but also be leveraged as useful tools in identifying existing and novel antiviral compounds.

Genetic Organization of Bunyaviruses

Bunyaviruses are composed of three genomic segments—large (L), medium (M), and small (S)—that encode for four structural and three nonstructural proteins. The L and M segments encode for the RdRp and glycoproteins G_c and G_n, as well as the nonstructural protein NSm1/2,

respectively. While the L and M segments are entirely negative sense, the S segment is ambisense, allowing for expression of the nucleocapsid (N) from its negative sense and a nonstructural (NSs) protein from a subgenomic mRNA expressed from its positive-sense orientation (133). Bunyaviral replication takes place entirely in the cytoplasm, in which the viral genomic RNAs (vRNAs) are first transcribed into mRNAs by the RdRp (L) protein during an immediate early transcription step (134–136). These mRNAs are capped with host cellular oligonucleotides obtained through a cap-snatching mechanism similar to that used by orthomyxo- and arenaviruses, and the presence of this cap allows for initiation of mRNA translation into viral proteins (137). To replicate the viral genome, a complementary full-length antigenomic RNA is transcribed to serve as a template for generation progeny vRNA segments (136). Translated viral proteins and replicated vRNA segments are then packaged into budding virions through the Golgi apparatus (136).

The History of Bunyavirus Reverse Genetic Systems

Before the rescue of fully infectious viral particles, many groups used T7- and Pol I–driven minigenome rescue systems to investigate various aspects of bunyaviral biology. Dunn et al. (138) first paved the way for T7-based bunyaviral minigenome rescue systems by developing a reporter system in which viral transcription requirements could be investigated through transfection of a plasmid expressing the negative-sense reporter gene CAT, flanked by Bunyamwera virus 5′ and 3′ UTRs into cells expressing bunyaviral proteins. This work was then built upon by Flick and Pettersson (139) to develop a Pol I–based minigenome rescue system similar to the one reported by Dunn et al. for the bunyavirus Uukuniemi virus. In this study, the minigenome assay relied on either cotransfection of a CAT or GFP reporter gene flanked by 5′ and 3′ UTR sequences of Uukuniemi virus into cells with plasmids expressing the L, N, or NSs proteins or superinfection with the virus itself.

Moving forward, the Flick group was able to adapt this system to additional bunyaviruses such as Hantaan virus and Crimean-Congo hemorrhagic fever virus (140, 141). These T7- and Pol I–based minigenome rescue systems have since been utilized by numerous other groups to investigate bunyavirus biology such as key transcriptional start and termination signals within the 3′ and 5′ terminal genomic regions, *cis*-acting trafficking and localization signals, and functional *trans*-roles of individual viral proteins during the viral life cycle (134, 135, 139–151). These noninfectious minigenome technologies have also been proven beneficial in developing quantifiable, high-throughput reporter systems to screen antiviral compounds in lower biosafety level environments for traditionally high-containment bunyaviruses (57, 137, 152–155).

Reverse genetic systems for fully infectious clones of bunyaviruses are largely based on similar technologies as those described for orthomyxoviruses and arenaviruses. The bunyavirus Bunyamwera virus was the first segmented, negative-stranded RNA virus to be rescued helper independently from cloned cDNAs by Bridgen and Elliott and colleagues (142, 156) and then by Lowen et al. (157). Full-length cDNA copies of the three bunyavirus genomic segments were cloned into transcription plasmids flanked by the T7 promoter and hepatitis delta virus ribozyme sequences (142). Upon transfection of these plasmids and expression plasmids encoding for the Bunyamwera viral proteins and the T7 polymerase into mammalian cells, infectious progeny virions were rescued (142). This same T7-based methodology was then used to successfully rescue RVFV and La Crosse virus (158–163). Additionally, further exploration into viral rescue strategies by Billecocq et al. (164) and Habjan et al. (165) revealed that a Pol I–based rescue system in which cotransfection of Pol I–driven plasmids encoding the L, M, and S genes with L and N expression plasmids was an equally successful system with which to rescue RVFV. Together, these studies have solidified multiple methods of reverse genetic systems for bunyaviruses and opened a new door to the breadth of applications of recombinant viruses.

Rift Valley Fever Virus Reporter Viruses and Their Uses

Since the development of robust reverse genetic systems to rescue myriad bunyaviruses, numerous RVFV reporter viruses have been developed and utilized (143, 145, 161, 166–176). RVFV reporter viruses similarly rely on inserting a fluorescent gene such as GFP or a nonfluorescent reporter gene such as CAT into one of the viral gene segments; however, the insights they have been used to reveal can range broadly. Gauliard et al. (143) utilized a CAT-expressing RVFV minigenome assay to investigate the influence of 5′ and 3′ UTRs on transcription functionality, while Gommet et al. (167) used a GFP-expressing infectious RVFV to reveal further insights into tissue tropism in vivo and overall virus pathogenesis (143, 167).

While these RVFV reporter viruses have been utilized to uncover basic mechanisms of viral biology and critical host-pathogen interactions to better design both vaccines and antivirals, they can also be used as valuable tools to screen for currently existing and novel antiviral drugs. RVFV remains an emerging threat to human and livestock health, for which we have extremely limited vaccines and antiviral drugs. Fluorescent RVFV reporter viruses present an efficacious and high-throughput system in which to screen for compounds that can inhibit virus replication (**Figure 6**). Furthermore, cell-based assays in which a rescued virus can be used for infection provide increased benefits over similar assays utilizing purified protein binding as they better mimic the complete environment in vivo. Islam et al. (166) have rescued and utilized a GFP-expressing RVFV virus and screened more than 20,000 small molecules in human lung epithelial cells, from which they identified more than 600 compounds that largely inhibited RVFV infection. Numerous other groups have developed similar assays, including fluorescent minigenome assays, in which a quick and

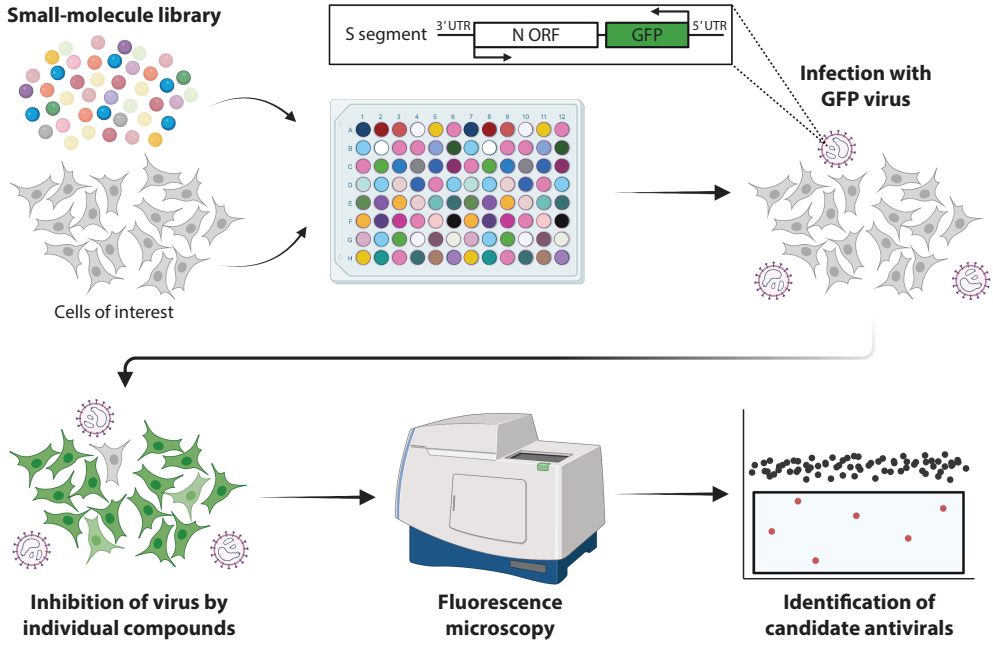

Figure 6

Schematic representation of a high-throughput screen for antiviral compounds using a fluorescent reporter virus. To identify new viral replication inhibitors, small molecules are arrayed onto permissive cells. A reporter virus is then used to infect the cells and compounds that decrease fluorescence are selected for further study. Abbreviations: GFP, green fluorescent protein; ORF, open reading frame; S, small; UTR, untranslated region.

quantifiable readout using a plate reader can be obtained to identify existing compounds that have antiviral activity against bunyaviruses, as well as inform future novel antiviral drug design (153, 177). Certain limitations to high-throughput screening do include the risk of false positives due to off-target effects; however, this can be curbed by employing thorough hit validation following the screen.

CONCLUSIONS

In this review, we have selected representative orthomyxoviruses, arenaviruses, and bunyaviruses and described their general genetic organization, the development of their reverse genetic systems, and the experimental uses of reporter encoding strains. While rational design of reporter viruses has facilitated both basic and translational studies, there remain open questions and clear areas for future investigation. For example, despite new strains of influenza viruses being isolated every year, the vast majority of reporters have been encoded in lab-adapted viruses. While using well-characterized laboratory strains as a genetic background is less technically challenging, understanding the breadth of new antivirals or evaluating the effect of novel vaccines would be significantly facilitated by generating reporter viruses in more contemporary strains. Additionally, the simultaneous use of multiple reporter viruses to understand the dynamic of viral reassortment and resolving the tropism and spread of viruses after authentic aerosol transmission remain important open questions.

For arenaviruses, the advancement in reverse genetic systems over the last two decades has provided numerous insights into arenavirus biology and pathology. The continued use of reporter-expressing rLCMV will be important for addressing areas of LCMV research that remain incompletely defined. Notably, bioluminescent reporter systems will be especially useful for investigating antiarenaviral drug effects in real time and in creating arenavirus therapeutics, of which there are currently none. Additionally, the ability to monitor arenavirus infections in live mice opens the door to better understand LCMV spread, viral tropism, and immunopathology, all of which will be important in the quest to create the first LCMV-specific vaccine.

Finally, not only has the use of recombinant RVFV reporter viruses allowed for valuable insight into virus biology but also the use of similar genetic strategies may represent new methods of generating vaccines and identifying effective antivirals. The continued development of recombinant RVFV viruses as live-attenuated or otherwise modified vaccines for livestock will likely be critical in enzootic regions in order to curb outbreaks within livestock. Additionally, high-throughput screening assays of large compound libraries that can be performed with reporter-minigenome assays at low-biocontainment levels provide quick, robust, and sensitive methods to identify compounds with antiviral activity.

As evidenced by recurrent seasonal, epidemic, and pandemic outbreaks, viruses pose significant threats to human health, and the family of viruses discussed in this review contributes to that burden. Our increased understanding of their biology and the mechanisms of pathogenesis is critical as we evaluate how best to reduce and eventually eliminate human disease burden. Reverse genetic systems and the development and use of reporter strains represent powerful tools to interrogate viral biology, and the continued development of such approaches will likely be at the forefront of next-generation efforts to combat viral disease.

DISCLOSURE STATEMENT

N.S.H. is a named inventor on intellectual property filings related to the development of influenza virus reverse genetic systems and viral genomic organizations for biotechnology applications. The Heaton laboratory is also funded by federal and private organizations for the continued

development and use of recombinant influenza virus strains. The authors are not otherwise aware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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