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MiniReview

Reverse genetics of negative-stranded RNA viruses: A global perspective

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Abstract

The advent of reverse genetics technology has revolutionized the field of RNA viruses. It is now possible to manipulate even negative-stranded RNA viruses at will, and evaluate the effects of these changes on the biology and pathogenesis of these viruses. The fundamental insights gleaned from the reverse genetics-based studies over the last several years have provided a new momentum for the development of designed therapies for the control and prevention of these viral pathogens. The recombinant viruses have been exploited also as vectors for devising targeted therapies for non-viral diseases such as malignancies, and in gene therapy for inherited disorders. This review provides a brief summary of the stumbling blocks and the successes in the development of the technology for the negative-stranded RNA viruses. The many and varied applications of the recombinant vectors are also outlined.

Keywords: Reverse genetics; RNA viruses; pol I; Minigenome

1. Introduction

The reverse genetics technology makes it possible to manipulate RNA molecules through its complementary DNA (cDNA) copy so that it is now possible to study the effects of these changes on the biology of the virus at the phenotypic level. This technology was relatively easily applied to the modification of plus-stranded RNA virus genomes, and to the recovery (rescue) of infectious virus from cDNA, because the deproteinated genomes of these viruses are able to utilize the host cell machinery to initiate their life cycle. Thus, plasmid-encoded or in vitro synthesized genomic RNA of these viruses is infectious when introduced into permissive cells [13]. On the other hand, recovery of negative-stranded (NS) RNA viruses from either cDNA components, or synthetic RNA, was a challenge. This is because unlike the plus-stranded viruses, replication initiation of these viruses requires de novo protein synthesis mediated by their own RNA-dependent RNA polymerase (RdRp), and because the input genomic or antigenomic RNA would need to be encapsidated with the viral nucleoprotein before it can serve as a functional template to initiate transcription/replication [5,21,61]. The eventual successful development and application of the technology to manipulate the genomes of NS RNA viruses has had a dramatic effect on the field of RNA virology: much has been elucidated about the molecular characteristics and pathogenesis of these viruses and the insights obtained from such studies has provided new impetus for the development of rationally designed vaccines and antiviral agents.

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This review focuses on the NS RNA viruses only, and provides a brief overview of the hurdles and the successes in the development of the reverse genetics technology for making recombinant NS RNA viruses. The varied applications of the technology as well as future perspectives are also outlined.

2. Overview of replication strategies of negative-stranded RNA viruses

Many clinically significant human and animal viral pathogens belong to the NS RNA viruses and include the classical pathogens identified decades ago such as measles, mumps and rabies viruses. Also belonging to this category are many of the newly emergent and reemerging pathogens such as hendra, nipah, ebola, marburg, hantaan, andes, lassa, rift valley fever and crimean-congo hemorrhagic fever (CCHF) viruses. Most of these agents are capable of causing severe morbidity and significant mortality, some are identified as agents of bioterror, and several are on the NIAID (http://www.niaid.nih.gov/biodefense/bandc_priority.htm) and CDC (http://www.bt.cdc.gov/agent/agentlist.asp) Priority Pathogens list. The NS RNA viruses are classified into seven families [68]. Four of these, namely *Paramyxoviridae*, *Rhabdoviridae*, *Filovridae*, and *Bornaviridae* have genomes that are represented by a single RNA molecule (Mononegavirales). The remaining three families contain segmented genomes, *Orthomyxoviridae* with six to eight segments, *Bunyaviridae* with three segments, and *Arenaviridae* with two segments.

All the non-segmented negative-stranded (NNS) and the segmented negative-stranded (SNS) RNA viruses listed above have several features in common. They are enveloped viruses, they replicate in the cell cytoplasm, and they produce mRNAs that are not spliced. The two exceptions to this rule are orthomyxo and bornaviruses; they transcribe and replicate in the nucleus and some of their mRNAs are spliced. By definition, the polarity of the genomic RNA of NS RNA viruses is opposite (negative sense) to the conventionally designated messenger RNA (plus) sense. Unlike the mRNAs, genomic and antigenomic RNAs of these viruses are encapsidated co-transcriptionally with the nucleoprotein N or NP to form the encapsidated N–RNA template of helical symmetry, and associate with a variable number of polymerase proteins (large protein L and phosphoprotein P in the case of paramyxoviruses, rhabdoviruses and bornaviruses, L, VP30 and VP35 for the filovirus Ebola virus, just the L protein for the arenaviruses and bunyaviruses, PA, PB1 and PB2 for influenza viruses) to form a minimal replication initiation complex, also known as the ribonucleoprotein (RNP) complex. Transcription, replication and virus propagation initiates once a functional RNP complex is formed [5,21].

The genes of the NNS RNA viruses are arranged in tandem. The gene junctions include the conserved gene-end, the non-transcribed intergenic, and the gene-start signals. At the 3′ and 5′ genome ends, respectively, are short non-transcribed regions called leader and trailer. The leader and trailer nucleotides, and in the paramyxoviruses, the adjacent non-coding nucleotides together make up the 3′ genomic (GP), and 5′ antigenomic (AGP) promoters. The essential sequences (*cis*-acting elements) that control virus replication, encapsidation and packaging are embedded in both the GP and AGP nucleotides while mRNA transcription is exclusively initiated from the GP [5,21,61]. Transcription and replication of the NNS viruses initiate from the genomic promoter. During transcription, each gene is sequentially transcribed to make mRNAs following the gene start–stop signals. The 3′-promoter-proximal genes are transcribed more abundantly than the successive downstream genes (3′–5′ transcription gradient) because transcription initiates at the 3′-end and attenuation occurs at each gene-junction; the attenuation is believed to be due to polymerase disengagement at each gene stop signal and failure to reinitiate at the next start signal. Each gene is translated into a corresponding protein with the exception of the P cistrons of a majority of the paramyxoviruses; they encode for multiple proteins by accessing alternative open reading frames, and by an RNA editing mechanism which inserts one or more non-templated G residues during transcription by reiterative copying of a specific stretch of C residues. RNA editing and/or transcriptional frameshift mechanisms are also employed by some of the filovirus genes to increase their coding capacity. During the replication process, the polymerase ignores the gene-end and gene-start signals to make full-length copies called the antigenome (also referred to as complementary RNA, cRNA, particularly in the SNS RNA viruses); these in turn serve as replication intermediates to make further genome (viral RNA, vRNA, with reference to SNS viruses) copies [5,14,21,47,61]. Also, for a majority of paramyxoviruses, a functional nucleocapsid N–RNA template formation requires that the genome (and antigenome) length is divisible by six, i.e., it obeys the *rule of six* [8,35,54], and that the encapsidation is precisely end-to-end. Exact encapsidation of the genomic (and antigenomic) RNAs is thought to occur based on the stoichiometry of interaction between the N protein and the template RNA, where each protein subunit binds to exactly six nucleotides [18]: a model described by Kolakofsky et al. [35] proposes that the coordinated binding of the polymerase and the template hexamers to the N protein (phasing) enforces the *rule of six* in RNA synthesis of these viruses.

In many respects, SNS RNA viruses make a significant departure from the NNS viruses with respect to their replication and mRNA transcription strategies: each segment of the SNS viruses represents a separate transcription and replication unit. The terminal 3′ and 5′ nucleotides of each segment are conserved and show partial inverted complementarity. This results in base-paired terminal ends which together constitute a functional core promoter. Replication initiates from the 3′ end of the genome (vRNA) and antigenome (cRNA) to make full length copies. As with the NNS viruses, transcription in these viruses initiates only on the vRNA template; exceptions to this rule are the arenaviruses and some members of the *Bunyaviridae* (phlebo and tospoviruses) family which use ambisense coding strategy in one or more of their genome segments [5,21,44]. Unlike the mRNAs of NNS RNA viruses, which are thought to be co-transcriptionally capped by the viral polymerase [61], the initiation of mRNA synthesis in SNS viruses needs a short primer derived by viral polymerase-mediated cleavage of host cell RNA polymerase II transcripts in the nucleus (influenza virus) or the cytoplasm (arenaviruses and bunyaviruses). The mode of transcription termination varies in SNS viruses: in some (e.g., influenza viruses), a uridine stretch marks the end of transcription and the beginning of polyadenylation mediated by reiterative copying of the same stretch. In others e.g., the bunyaviruses, the process of transcription termination is not clear except that it stops somewhere in the 5′ non-coding region of the vRNA molecules; the status of mRNA polyadenylation also remains unclear: in the segments with a negative-sense coding strategy, sequence motifs analogous to the transcription termination–polyadenylation signals of other negative-stranded RNA viruses are obvious but the transcripts show no evidence of polyadenylation. Also, transcription termination without the homopolymeric U residues is seen. In the segments with ambisense coding strategy, a secondary structure or a G-rich like sequence motif in the intergenic region appears to mediate transcription termination [5,21,44].

3. Development of reverse genetics technology and rescue of infectious viruses

For the NS RNA viruses, the minimal replication initiation complex required to synthesize viral RNA is the encapsidated genomic or antigenomic RNA template associated with core proteins (polymerase complex proteins, support proteins, *trans*-acting proteins) to form a functional RNP complex (Fig. 1). During the course of developing reverse genetics technologies, several different strategies were developed to reconstitute biologically active RNP complexes from cDNA components. Two of these methods are widely used: in one, each of the components necessary to reconstitute an enzymatically active RNP complex is generated inside cells infected with a recombinant vaccinia virus expressing T7 RNA polymerase [30] and transfected with cDNA plasmid constructs under T7 phage polymerase control to start de novo viral synthesis. Alternatively, T7 mediated in vitro transcribed viral RNA is transfected into cells infected with a homologous helper virus which provides all the support proteins necessary to reconstitute a biologically active complex. Both these approaches were successfully used during the late eighties and early nineties to rescue individual segments of a segmented virus, or to recover reporter gene-bearing synthetic RNAs (genome analogs or minigenome constructs). Early successes at rescue also included several other strategies. For example, In 1989, a minigenome RNA was transcribed in vitro, encapsidated in vitro to make the N–RNA template, and also complexed with the purified polymerase proteins in vitro. Transfection of this RNP complex in cells infected with the helper virus resulted in CAT rescue [40]. In 1990, using a similar protocol, modification of the influenza virus genomic NA segment via its cDNA copy was demonstrated [17,47].

A similar approach for the in vitro formation of a N–RNA template for the NNS viruses was not successful and this meant that in these viruses, all the components including the RNA template had to be generated from plasmid DNAs in the cytoplasm where the encapsidation of the nascent RNA and the downstream processes would take place. Alternatively, a helper virus-driven system (described above) had to be used. The latter approach was applied to demonstrate reporter gene rescue from a Sendai virus (SeV) and respiratory syncytial virus (RSV) minigenome constructs [10,48]. In earlier studies based on recombinant helper plasmids, a vesicular stomatitis virus (VSV) genome analog was shown to replicate and be packaged into progeny particles [52].

The recovery of complete virus from cDNA proved to be far more complex and technically demanding however, and functional minigenomes were established for a majority of the NNS and SNS viruses well before cDNA mediated infectious viruses were generated [6,10,12,16, 24,26,27,38,48, and others reviewed in 44]. Minigenomes are smaller in length and easier to manipulate because as described above, they usually only carry a single reporter gene open reading frame flanked by the viral non-coding termini. They proved to be invaluable surrogates for full-length virus in establishing the requirements, and optimizing the conditions for the ultimate rescue of infectious recombinant viruses. Many factors were identified which could compromise rescue. In plasmid driven systems, the ratio of the support plasmids is critical [7,16,52], just as it is in the virus life cycle (transcription gradient). Precisely encapsidated viral ends are required for the rescue of many viruses possibly because of the stoichiometry of interaction between the N (NP)

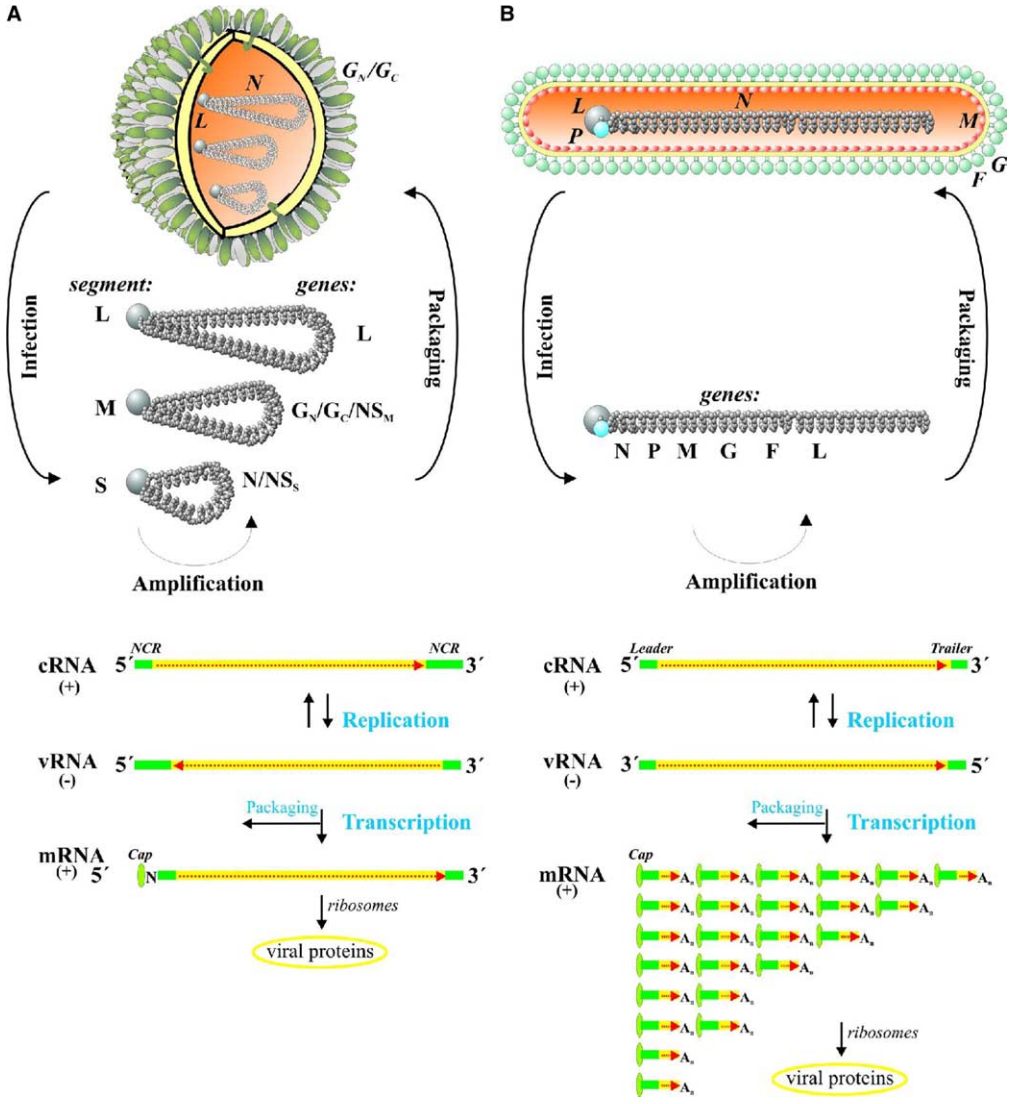

Fig. 1. The life cycle of SNS (A) and NNS (B) viruses: the figure represents the overall schema of the lifecycle of SNS and NNS viruses for illustration purposes only, since the viruses may differ from each other with respect to the number of genes, and with reference to the specific details of their life cycle. Note that for the SNS as well as the NNS groups, the mRNAs are sub-genomic deproteinated transcripts that undergo end modification; they are capped at the 5′ end and polyadenylated at the 3′ end. The full length genomic (vRNA) and the antigenomic (cRNA) RNAs are complete and unmodified complementary copies of each other; they are cotranscriptionally enwrapped with the N protein, and serve as functional templates only in this encapsidated form. For further details, see text, and Refs. [3] and [13].

protein and the RNA template [18]. The efficiency of rescue is reduced, or is even eliminated in constructs under the control of a wild type T7 promoter, which results in the addition of three non-viral G residues at the 5′ end of the transcript. Major improvement in rescue efficiency occurred when transcripts with authentic viral ends were achieved with the use of a truncated T7 promoter sequence which prevented the addition of non-viral G residues [34] and by the exploitation of the exact 5′ end self-cleavage property of the hepatitis delta virus ribozyme to produce precise 3′ viral ends [57]. A further insight was provided with the finding that to be functional, the genome (or the genome analog) of a majority of paramyxoviruses needed to obey the *rule of six* [8,35,54]. Yet another consideration was the length of the RNA template being rescued, the longer the template, the lower the rescue efficiency [12]. Heat shock is reported to enhance cDNA rescue [49]. The use of recombinant vaccinia virus to deliver the T7 polymerase added a further complication because of virus-mediated cytotoxicity. This problem has been ameliorated to a great extent with the use of a variety of approaches such as the use of a replication deficient host range mutant of vaccinia virus (MVA-T7), or treatment with cytosine arabinoside, rifampicin or UV cross-linking to prevent or reduce replication of vaccinia-T7 virus and the consequent host-cell damage [reviewed in 42]. Expression plasmids under the control of CMV immediate early en-

hancer/promoter elements [29], or chicken β-actin promoter [45] have been used for T7 expression, and cell lines stably expressing T7 polymerase have been developed [58] as alternatives, but none have found broad acceptance because T7 expression is generally lower in these systems than that generated by recombinant T7-vaccinia virus. A RNA polymerase I (Pol I) transcription system has been applied more recently [75] and overcomes a majority of the limitations of the T7-driven systems (see below). Yet another factor impacting on rescue efficiency has been the orientation of the template; although transfection of plasmid DNAs generating genomic RNAs has resulted in rescue, recovery is generally more efficient from constructs producing antigenomic RNA as the start template [34]. In fact, the first successful rescues of NNS [60], and SNS [7] viruses required antigenomic constructs.

Finally, in 1994, all the factors necessary to make a complete recombinant virus came together, resulting in the rescue of the first NS RNA virus, rhabdovirus rabies virus (RV), starting entirely from cDNA [60]. Rescue of other full-length RNA viruses soon followed, and included vesicular stomatitis virus (VSV) [39,72], measles virus (MV) [58], and many others [5,44].

Notwithstanding the early success in the rescue of individual influenza virus segments from cDNAs, the successful construction and rescue of minigenomes of a majority of the SNS RNA viruses [1,6,16,24,26,27,37,38,56], and the continuous improvement in rescue technology, so far, only three of SNS viruses have been recovered from cDNA constructs. The first report in this category of viruses was the rescue in 1996 of the bunyamwera virus [7]. In this case, rescue was dependent on T7 RNA polymerase based cDNA clones for the genome segments as well as the support plasmids. In 1999, the eight-genome-segmented influenza A was rescued by Neumann et al. [43] from cDNA constructs carrying for the first time, the Pol I promoter and terminator sequences to drive viral RNA transcription. Fodor et al. [28] also generated recombinant influenza A virus but used a system in which the cDNA constructs contained Pol I promoter sequences while maintaining the use of hepatitis delta virus ribozyme sequences for transcription termination. Hoffman et al. [33] subsequently described a more efficient ambisense expression system in which the RNA Pol I cassette was cloned between an RNA Pol II promoter sequence and polyadenylation signal. The vRNA and mRNA was generated simultaneously from these constructs, thereby reducing the total number of plasmids required for rescue to just eight instead of 15. The six segmented Thogoto virus was rescued in 2001 by using a combination of T7 and Pol I driven systems for protein expression and vRNA transcription, respectively [69].

The Pol I system, initiated by Hobom and colleagues, [75] have several advantage over the T7 polymerase system: The Pol I enzyme is expressed in the nucleolus of all eukaryotic cells and therefore does not need to be provided in *trans*. Transcripts generated by the Pol I constructs have precise viral ends, i.e., they lack the 5′ cap structure and the 3′ poly A tail. Also, the Pol I system does not have the potential inherent disadvantage of vaccinia virus-mediated recombination. The Pol I system is ideally suited for viruses like the influenza virus or the bornavirus that transcribe in the nucleus. However, the majority of RNA viruses replicate in the cytoplasm, and in this case, it is necessary for the plasmids to enter the nucleus to undergo transcription and then the transcripts need to exit to the cytoplasm to complete the downstream processes. Nevertheless, the location of the enzyme does not appear to be a factor since Flick et al. and others [24–27,37,56] have successfully and extensively applied the Pol I based minigenome rescue systems for several of the bunyaviruses namely CCHF, Uukuniemi and Hantaan viruses, arena viruses and bornaviruses Whether the Pol I system would be effective for full-length rescue of the cytoplasmic RNA viruses remains to be demonstrated.

4. Applications of the reverse genetics systems

4.1. Molecular characterization of NS RNA viruses

Reverse genetics-based studies have had a dramatic effect in expanding our knowledge of the molecular biology and pathogenesis of RNA viruses and has put to rest many issues that were impossible to address by conventional virological or biochemical procedures. Minigenome-based studies have been the main stay in providing the numerous insights into the many aspects that control the virus life-cycle and the following are a few of the many noteworthy contributions: The location and boundaries of the *cis*-acting elements that control virus transcription, replication, encapsidation and packaging; the secondary structure of promoter regions; the functional domains of the *trans*-acting proteins; the relative strength of the genomic and antigenomic promoters; the gene stop–start signals; the function and characteristics of the intergenic regions; the *rule of six* obeyed by the majority of paramyxoviruses, and its implications on the template encapsidation and virus replication; the mechanism of reiterative copying resulting in mRNA editing and polyadenylation; cap-snatching mechanism for transcription initiation of SNS viruses; the presence of a transcription elongation factor which controls the processivity of RSV transcripts; the role of the non-structural genes in virus biology and pathogenetic mechanisms; virus–host interactions and many others [5,13,14,21,42,44,47,59,61].

Reverse genetic analysis has been particularly successfully applied to the identification and

characterization of the *cis*-acting elements of RNA viruses. A large body of minigenome-based studies of several NNS and SNS viruses indicate that the *cis*-acting elements that control transcription, replication, encapsidation and packaging are embedded in the 3′ and 5′ end non-coding nucleotides; that the terminal 12–13 nucleotides of each segment of influenza A virus interact with each other to constitute the basic core promoter required for virus propagation but that the adjacent non-coding nucleotides seem also to be required for optimal replication efficiency [22], that the sequence control elements of VSV, the first NNS virus to be analyzed, are confined to the extra-cistronic leader and trailer nucleotides and do not extend into non-coding nucleotides of the adjacent genes [53], that the same does not apply to the paramyxoviruses, since additional regulatory sequences beyond the leader and trailer core promoter residues are also essential [36,41,65,70]. A selected group of some of the more revealing studies is reviewed below in more detail.

Experimental evidence for extended promoters in the paramyxoviruses was first reported in a SeV study [65] and subsequently confirmed for paramyxovirus simian virus 5 (SV5) [41] and MV [70]. The impact of the experimental design and procedures used in the characterization of *cis*-acting sequences was underscored from the results of the MV investigation: mutational analysis of the 18 nucleotide region of MV AGP internal replication control element clearly demonstrated that mutating combinations of some nucleotides had a profound effect on replication efficiency although when individually substituted, they appeared not to be required. Results of mutational analysis in the same study showed further, that the nucleotides at only some of the positions of the internal element were essential for functionality. However, the use of an in vivo nucleotide selection procedure revealed that with continued replication, a preference for the conservation of wild type nucleotides across the entire element was clearly evident. These results indicated that all the nucleotides in this region were required for replication, although to varying degrees; the essential nucleotides may be involved in sequence specific binding with precise regions of the homologous polymerase while the preferred nucleotides may be contributing to the energetics of binding [73]. A replication model described in this study [70] proposes that to initiate and maintain optimal replication efficiency, the polymerase complex proteins need to make multiple contacts with the terminal and internal nucleotides of the promoter; some of these are essential and while others are preferred. Precise spacing of the sequence elements with reference to each other may be needed for correct positioning of the polymerase to initiate replication; the need for precise spacing between the *cis*-acting sequences was indicated from experimental data in SeV [65] and SV5 [41] studies, and from our unpublished data, which showed that nucleotide deletions in the region intervening the terminal and internal elements were not tolerated.

Whether substitution of the essential nucleotides in a promoter element disrupts a required structure in the RNA template or destabilizes a preferred nucleotide specific interaction between the template and the polymerase is not clear. The elucidation of the mechanism(s) of RNA selectivity/recognition by the homologous polymerase has been particularly difficult because of the limited knowledge of the three-dimensional structure of the promoter regions. Despite such limitations, detailed analysis of the influenza A virus promoter, which consists of the 3′ and 5′ ends of vRNA terminal nucleotides forming a partially base-paired element, has provided new insights into the intra-strand structure, and template–polymerase interactions required to initiate virus replication: based on a complete set of nucleotide substitutions, Flick and colleagues [22,23] have described a unique ‘‘corkscrew’’ conformation of the promoter region of the viral RNA. The proposed structure consists of a six base-pair rod structure in the distal element and two stem–loop structures of two intra-strand short-range base pairs; the latter support an exposed tetranucleotide loop within each branch of the proximal element in an overall oblique organization. A complete set of single substitutions and double complementary mutations showed that the number of invariant nucleotides in this structure was few and retention of structural integrity of the region was paramount. Interestingly, the invariant nucleotides are located within the tetranucleotide loop structure which is likely to facilitate its interaction with the polymerase complex proteins. The corkscrew conformation, verified in recent [15] and previous studies, is more complex than the two other promoter models predicted previously for this virus, the panhandle and the fork structure [reviewed in 44]. Most of the viruses in the *Bunyaviridae* are also likely to have a corkscrew conformation based on computer generated secondary structure predictions of the promoter regions of these viruses [25], but this needs experimental evaluation.

4.2. Recombinant viruses as vectors

The successful generation of recombinant viruses and the ability to manipulate their genomes at will has made it possible to ask questions pertaining to the molecular biology and disease pathogenesis of RNA viruses and as a result, our understanding of these aspects of the RNA viruses continues to grow. The ability of the recombinant viruses to stably express foreign genetic material has also resulted in the conception of novel therapeutic [4] and disease prevention paradigms [5,11], and the potential of these viruses in such applications is being tapped at an ever increasing rate. The

paramyxo- and rhabdoviruses are particularly suited for the stable insertion of foreign genetic information for several reasons: the encapsidated genomes of these viruses make elimination of the inserted sequence by homologous recombination a very unlikely event; genome integrity is likely to be further enforced by the requirement by a majority of the paramyxoviruses for the *rule of six*: deletions that disrupt this requirement would result in non-viable genomes. The genomes of these viruses are able to accept and stably express up to 5 kb of introduced genetic material so that making bivalent or even multivalent vaccines would be a reality. Moreover, introduction of extra transcription units is achieved with relative ease by the introduction of additional gene stop and start signals. Finally, the levels of expression of the inserted gene can be controlled by careful selection of the point of insertion [71]. This is because of the 3′–5′ gradient of mRNA abundance in these viruses as described earlier. Most importantly, recombinant viruses widely used as vectors, like the measles vaccine virus, already have a long safety record as live attenuated vaccines; other viruses such as SeV and the VSV vaccine virus which are considered non-pathogenic to humans are also widely used as vectors. Importantly, the life cycle of these viruses is confined to the cytoplasm, and therefore such vectors carry no accompanying risk of unwanted chromosomal integration.

Segmented viruses have also been used as vectors of foreign genetic material [reviewed in 44]. The simple approach of introducing additional genome segments in these viruses is not successful however, because segments which are not essential for virus survival are rapidly lost by re-assortment or become non-functional through mutation. Creative approaches have been used to circumvent this problem: the segmented genomes have been successfully used as vectors by making fusion genes mediated through insertion of internal ribosomal entry sites (IRES) or by the insertion of the 17aa long protease 2A sequence of foot and mouth disease virus between the viral gene and the foreign genetic material. Alternatively, selected regions of foreign genetic material such as the T cell specific epitope(s) have been incorporated into the stalk region of NA, or the antigenic sites of the influenza virus HA genes.

4.3. Recombinant viruses for vaccine development

A variety of approaches has been undertaken to use recombinant viruses to generate live attenuated vaccines. For example, attenuating point mutations corresponding to host range, cold adaptation, temperature sensitive phenotypes or others have been identified and some have been inserted into vaccine or wild type backgrounds to generate vaccine candidates [11,50,51]. Mutations which alter enzyme cleavage specificity, e.g., by substitution of a cleavage site recognized by a universal protease with that which is recognized by a specific one, would result in attenuation that could be exploited for vaccine purposes [reviewed in 42]. A class of live attenuated recombinant virus involves the alteration or elimination of the non-structural interferon antagonist genes. Such altered viruses are attenuated because they have reduced, or no capacity to counteract host interferon responses [14,19,63,67]. Rearrangement of genes also results in attenuation [71]. Replacing the promoter region(s) of a virus with that of a closely related virus has the same effect. This was demonstrated by us using MV and CDV minigenome chimeras (our unpublished data), and the validity of this approach for vaccine purposes was reported by Chapman et al. [9]. Mice vaccinated with full length Coxsackie B and poliovirus 5′ non-translated region chimeric virus were protected from disease on challenge with wild type Coxsackie B virus. Recombinant viruses carrying the glycoprotein gene of a related or an unrelated virus instead of its own have been used for vaccine purposes [5,11,14,59].

4.4. Other applications of recombinant viruses

The recombinant RNA viruses have also been exploited for many other preventive, therapeutic and investigative strategies: in one approach, RV and VSV pseudo-types were engineered to express HIV-1 CD4 and CXCR-4 cellular receptor genes for targeted destruction of HIV infected cells [reviewed in 42]. Similar tactics are being investigated for targeted killing of malignant cells by incorporating the appropriate ''bullet'' into the vector envelope. For example, in one study, virus binding was redirected by creating MV H gene-anti CD38 fusion gene, a myeloma cell marker, to create a recombinant measles virus with altered tropism, with the ultimate aim of targeted destruction of the malignant cells [55]. Another area of active investigation is the oncolytic potential of some NS RNA viruses [5]. VSV in particular has several advantages over other viruses as antitumor agents [2,3,5] and recombinant VSV engineered to contain genes that modulate immune response, and/or contain suicide cassettes, augment their oncolytic capability [20]. Pseudo-type viruses have been used as substitutes for investigations involving highly pathogenic viruses which otherwise would require biocontainment: for example, a G gene deletion mutant of VSV carrying Ebola virus glycoprotein was used to probe the receptor usage of this virus [62]. Similarly, a VSV pseudo-type carrying Hantaan or Seoul virus envelope protein was used as an effective replacement for the complete virus to develop a safe neutralization assay for Hantaan virus [46]. Selected cytokine genes have been inserted in VSV and other viral vectors to modulate immune responses [59]. Recombinant paramyxoviruses are likely to be valuable for gene therapy because these vectors have several obvious advantages over the

conventional retroviral and adenoviral vectors used for this purpose. For example, SeV vectors have been used to express foreign genes efficiently in non-dividing cells such as neurons [32]. Gene transfer to murine lung epithelium mediated by a replication competent SeV vector appeared also to be effective [74]. The exploration of recombinant viruses for therapy and prophylaxis has just begun, and innovative and novel applications continue to grow and show exciting promise for the future.

5. Current limitations

The above discourse underscores the undisputed importance of reverse genetics in improving our understanding of RNA viruses at the molecular level. However, in many instances, a cautionary note needs to be introduced in the interpretation of data generated by this approach since the results may depend on the systems used. The following are some examples. Tissue culture based studies may produce information that may not reflect that which results from interaction of the virus and its human or animal host. A case in point is the studies investigating the role of non-structural proteins such as the C and V, encoded by the P cistron of MV and SeV. In tissue culture based studies, these proteins appear to be dispensable since recombinant viruses lacking these genes were able to replicate and produce plaque sizes and virus titer comparable to those obtained from wild type viruses. However, growth of the deletion mutants was highly restricted in both the upper and lower airways of chimpanzees indicating the role of these non-structural proteins in pathogenesis [reviewed in 5, 42, 59]. Evaluation of the role of the *cis*-acting elements by mutational analysis should also be viewed with a caveat because it is conceivable that compensatory changes may be introduced elsewhere to restore the effects of the designed mutations. Experimental design and procedures used may also alter the interpretation of data [70]. Minigenome based data of the sequence characteristics or the viral proteins may need to be validated in full length recombinant viruses because the interactions of the different viral proteins with one another and with the host milieu are likely to play a role in the outcome. Importantly, majority of the evaluations currently undertaken are based on tissue culture adapted laboratory strains such as Edmonston B measles virus, and the results obtained may not apply to the circulating wild-type viruses.

6. Future perspectives

Knowledge of RNA viruses has grown exponentially since the availability of reverse genetics technology, and has provided insights into the biology and pathogenesis of RNA viruses which were previously impossible to address. However, it also has raised many questions and produced conflicting data, some of which may relate to the differences in the viruses or experimental systems used, or may be due to factors that we have failed to fathom yet. Many questions still remain to be answered. These include the mechanism(s) that confer the exquisite recognition and selectivity between the nucleoprotein encapsidated RNA template and the homologous polymerase; the mechanisms and the role of the secondary RNA structure vs. nucleotide in the recognition process; the yet unresolved issue of factor or factors that trigger the transcription to replication switch; and the role of host proteins in the virus life cycle; the mechanism that favors packaging of genomic over antigenomic RNAs; the question of random versus selective packaging of the genome segments in progeny virions of the SNS viruses. Also, aspects that were considered to be well elucidated until recently may need to be re-visited on the basis of new information: for example, a recent report indicates that the packaging signal for influenza A virus extends into the coding regions of that virus [31]. This suggests that although the non-coding viral termini of NS RNA viruses may be sufficient to drive transcription, replication, encapsidation and packaging [22,36,42,52,65,66,70], the presence of additional signals enhancing/modulating these functions cannot be ruled out. The tenet that transcription and replication initiate from a single 3′ proximal promoter is also challenged based on a recent VSV study which suggests that transcription initiates internally at the N gene start site without prior transcription of the leader RNA [64]. These are only a few examples of the many as yet unresolved issues, and the field promises many challenges and exciting possibilities.

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