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REVIEW ARTICLE

Application of minigenome technology in virology research of the Paramyxoviridae family

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Minigenomes (MGs) are complementary DNAs of the synthetic analogs of genomic RNA. MGs are widely used to study the life cycle of the Paramyxoviridae family of viruses. MG-based studies have provided valuable insights into the mechanisms of viral replication and transcription in this family, including the roles of viral proteins, the location and boundaries of the *cis*-acting elements, the functional domains of *trans*-acting proteins, techniques for the measurement of neutralizing antibody, virus–host interactions, and the structure and function of viral RNA. This article provides a brief overview of the principle and application of MG technology in studies involving members of the Paramyxoviridae family. The advantages, potential limitations, and future scope of MG technology are also discussed.

Introduction

The minigenome (MG) system, also known as minireplicon or MG technology, is considered as a complementary and powerful tool for exploring the life cycle of a virus during infection. The Paramyxoviridae family includes viruses with a genome consisting of negative-sense, nonsegmented single-stranded RNA, some of which cause serious diseases in humans and animals.[1,2] Increasing numbers of MG systems specific to this family have been successfully constructed. In comparison with traditional experimental approaches such as single protein–protein interaction experiment, these dynamic MG-associated studies have expanded our knowledge for viruses belonging to this family. In this review, we will provide a brief overview of the design and execution of an MG assay as well as the related applications in studies involving members of the Paramyxoviridae family. The advantages, potential

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limitations, as well as use of MGs in combination with other experimental approaches are also emphasized.

Key elements of an MG construct

Understanding the essential elements for MG assay is a key step to designing a complete and efficient MG experiment. Fig. 1 shows a schematic of an MG assay and its replication and translation process. The MG plasmid DNA contains a promoter for T7 polymerase (T7) and for RNA polymerases I and II (pol I and pol II, respectively), a viral 5′-untranslated region (5′-UTR), reporter gene, viral 3′-UTR, and a terminator for T7, pol I, and pol II (Fig. 1A).

More specifically, an MG construct is an artificial DNA sequence consisting of a reporter gene flanked by the corresponding viral 5′- and 3′-UTRs. The UTRs contain all regulatory elements needed for encapsidation, transcription, replication, and packaging of the viral genome into a progeny virus.[3,4] During construction, an MG complementary DNA (cDNA) is inserted in the antisense orientation downstream of the associated promoter sequence to avoid its direct transcription into messenger RNA (mRNA) by RNA polymerase. The viral open reading frame itself is replaced by a reporter gene, which is an artificial DNA sequence. Genes encoding chloramphenicol acetyltransferase (CAT), firefly luciferase, or green fluorescent protein are often used as reporters in MG systems.[5,6] Of these, luciferase has a 100-fold greater sensitivity than that achieved with the CAT assay, as 10^{-20} mol of luciferase can be measured under optimal conditions.[7]

To generate an anti-MG RNA, MG constructs have to be inserted in the antisense orientation between RNA polymerase promoter and terminator sequences (Fig. 1A). Previously, the bacteriophage T7 pol promoter[8] and eukaryotic RNA polymerase promoter[9] had been widely used in MG-based studies. In addition, the hepatitis δ ribozyme (HDδ) sequence has also been placed between the viral UTR and the T7 terminator sequence (Fig. 1A). The HDδ catalyzes self-cleavage of a phosphodiester bond between the reporter gene and the HDδ sequence, creating a viral RNA strand-like negative RNA transcript.[10]

Exogenous bacteriophage T7 pol has also been used to transcribe the MG plasmid into an MG RNA from the T7 promoter in a T7-based system. Furthermore, the approach to generating T7 pol depends on the research purpose and availability of experimental material, among other factors and affects the level of target gene expression driven by a

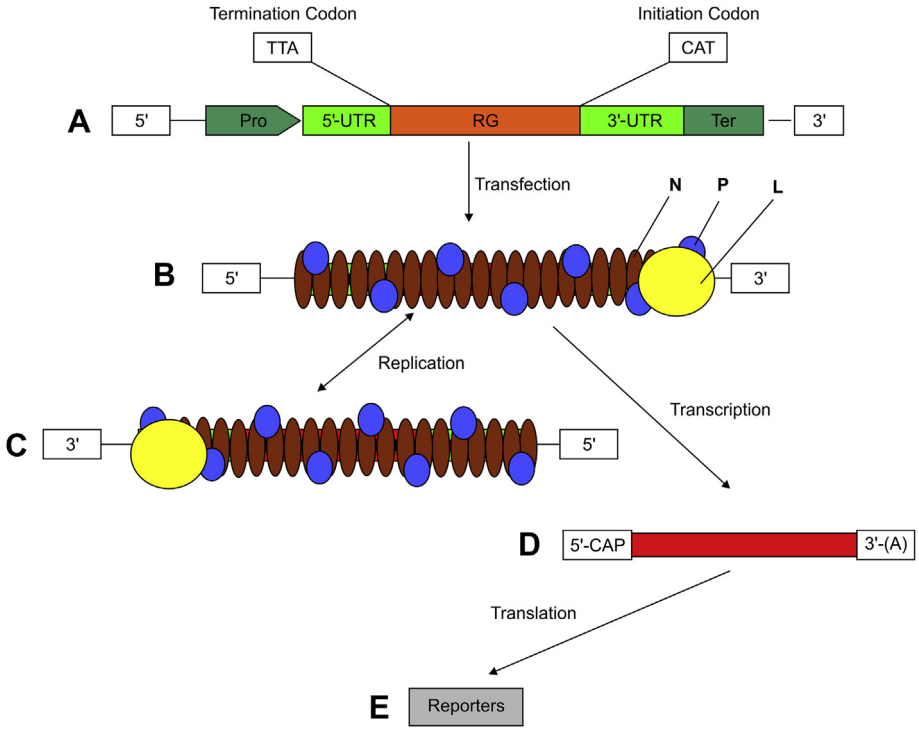

Figure 1. A schematic of a minigenome (MG) and its replication and translation processes. (A) The MG plasmid DNA. It contains a promoter for T7 polymerase (T7 pol), RNA pol I, and RNA pol II; a viral 5′-untranslated region (5′-UTR), a reporter gene (RG), a viral 3′-UTR, and a terminator (for T7, pol I, and pol II). (B) In the first step, after transfection of the plasmid DNA into recipient cells, the plasmid DNAs are transcribed by the corresponding RNA pol to generate a negative-sense (−) MG RNA, which is then encapsidated into an encapsidated MG RNA, in the presence of the corresponding nucleocapsid protein (N). (C) In Step 2, with the help of, for example, morbillivirus phosphoprotein (P) and large pol (L) proteins, an encapsidated sense (+) anti-MG RNA is produced using the negative-sense encapsidated MG RNA as a template. The positive strand is in turn used as template to generate the negative strand as a key step in viral replication. Both positive and negative RNA strands contain the full-length 5′- and 3′-UTR sequences. (D) In Step 3, the encapsidated negative RNA strand is transcribed into shorter sense MG transcripts [messenger RNA (mRNA)] lacking the complete 5′- and 3′-UTR. (E) Finally, the transcribed mRNA is translated by host-cell machinery to produce the reporter proteins. CAT = chloramphenicol acetyltransferase.

T7-based system.[11] Interestingly, when three G residues precede the T7 promoter sequence, the transcription efficiency of T7 pol is increased, and it has been reported that placing an encephalomyocarditis virus internal ribosome entry site sequence between the T7 promoter and the inserted open reading frame enhances protein expression.[12]

The pol I-based system is more attractive than the T7-based system in the development of some MG systems,[13] because it does not require exogenous T7 pol and ribozyme cleavage. Transcription of the MG plasmid is driven by RNA pol I that is present in the nucleolus of all eukaryotic cells. This system is therefore ideally suitable for studying viruses, such as influenza virus, which are transcribed in the nucleus. The many advantages of the pol I system over the T7 pol system have been reviewed previously.[14] Recently, the Vero cell line-derived pol I promoter was verified to be more efficient than that of human pol I in rescuing recombinant influenza A virus using the Vero cell line.[15,16]

These results indicated that the high efficiency of the eukaryotic RNA pol I promoter may be tightly related to host-cell specificity. However, the cytomegalovirus promoter could not rescue a Sendai virus (SeV) MG, whereas a T7-based system could.[13] In short, the T7-based system has a wider application compared with the eukaryotic RNA pol system; this may be due to the lack of cell specificity of the T7-based system, and the cell or virus specificity of the eukaryotic RNA pol.

Reporter gene expression

A successful MG experiment is characterized by MG reporter gene expression. The MG RNA mimics the process of viral RNA replication, transcription, and translation. Therefore, the first step in forming a ribonucleoprotein (RNP) complex in an MG assay is encapsidation of the artificial antisense RNA by N protein into an RNase-resistant nucleocapsid (encapsidated genomic RNA; Fig. 1B). The (−) MG-based RNP (M-RNP) complex of the Paramyxoviridae family has previously been demonstrated to function as a template for MG RNA transcription and replication, whereas the naked RNA does not.[17,18] The L protein then uses (−) M-RNP (−RNP analogy) as a template to generate MG mRNA, which is capped at the 5′ end and also contains a poly(A) tail at the 3′ end (Fig. 1D). Recipient cell machinery can then translate this into a reporter protein (Fig. 1E), which can then be used to verify a functional MG system.

The N, P, and L proteins are termed helper proteins. In the MG system, these proteins are generated from host cells infected by homologous viruses and/or transfected with recombinant plasmids harboring these helper protein genes. Virus-free plasmid-based MG assays are much safer and controlled. More important, this approach eliminates the potential danger of using live viruses, and thus is a useful method for studying the mechanism associated with viral transcription and replication in a dynamic state. In addition, the ratio of the helper plasmids is critical in determining the efficiency of MG reporter gene expression. The optimum ratios, as well as the amounts of helper plasmids and MG constructs, should be adjusted based on the expression level of the reporter gene. For example, the amount of the plasmid encoding RNA-dependent RNA pol (RdRp) should be less than that of the other helper plasmids.[8] By contrast, the efficiency of the MG system also depends on both protein−protein and RNA−protein interactions. It is obvious that the plasmid-based approach has the disadvantage of requiring transfection of more than four types of plasmids into each recipient cell, which can limit the efficiency of transfection and target gene expression.

Applications of MG technology

Roles of viral protein

Understanding the roles of viral proteins will greatly contribute to the understanding of the life cycle of the Paramyxoviridae family. The MG technology has rapidly developed into a novel tool for studying viral proteins involved in viral replication and transcription. The roles of structural and nonstructural proteins are discussed in the following sections.

Structural protein

Viral structural proteins are structural components of a mature virus. The N, P, and L proteins and the matrix (M) protein are the main structural proteins associated with viral RNA replication, transcription, and translation. The N protein serves several functions in the viral life cycle, including encapsidation of the genomic RNA, association with the P−L polymerase during replication and transcription, and, most likely, interaction with the M protein during virus assembly.

The N protein has been intensively studied with MG technology. The soluble N protein of human respiratory syncytial virus (hRSV) is required for hRSV MG replication,[19] and increased expression of N protein did not alter the balance between MG RNA transcription and replication. Moreover, optimal levels of cytoplasmic N protein are required for efficient replication, whereas an excessive amount may be deleterious.[20] In addition, two regions of the N protein, a central portion of 244−290 amino acids (aa) and a C-terminal portion of 338−364 aa, have been demonstrated to be key regions for association with the P protein, based on a bovine RSV MG assay.[5]

The P protein itself is essential for viral RNA synthesis. In the case of the Paramyxoviridae family, the P protein is an intrinsically disordered polypeptide, which forms tetramers through a central α-helical coiled-coil region and is regarded as being responsible for the recruitment of the large polymerase L onto the viral N-RNA template through direct interactions with N and L.[21] Using a human parainfluenza virus type 3 (hPIV3) MG system, it was confirmed that the N terminal of hPIV3 P protein is primarily involved in viral replication, whereas the C terminal is involved in both transcription and replication.[22] Based on an RSV MG assay, two P protein regions, an internal 161−180 aa and a C terminal 221−241 aa, have been shown to be independent N protein-binding sites, and residues 121−160 were shown to be the L protein-binding site.[23] Furthermore, the effect of P protein on viral replication and translation occurs

mainly through interaction with both N and L proteins. The P protein associates with the N protein for viral RNA encapsidation, and with the L protein for transcriptional activity.

The L protein is the largest and least abundant of the structural proteins, and is considered to be the viral polymerase, probably due to its location to transcriptionally active viral cores. It is thought that L protein performs multiple functions, including RNA synthesis, capping, and methylation, as well as polyadenylation.[24,25] Measles virus (MV) and canine distemper virus (CDV) MG constructs have been used for investigating heterologous N, P, and L protein combinations. The results of such studies revealed that there is no heterologous restriction in either MG replication assay.[4] Some heterologous helper plasmids actually generate a higher level of reporter gene product than that of their homologous counterparts. Sequencing indicated that MV N, P, and L proteins may have the same motifs as CDV N, P, and L proteins, respectively. The *cis*-acting signal of MV and CDV may also be similar in part in both UTRs. However, this has not been experimentally verified. Conceivably, the broader specificity of the L protein is in part due to a variation in the polymerases of vaccine and wild-type strains of morbilliviruses.[26] It has been established that, phenotypically, MV polymerases from vaccine strains have greater transcriptional processing capacity than their wild-type equivalents,[27] a fact that may contribute to their overall attenuated phenotype. A peste des petits ruminants virus (PPRV) MG was constructed to confirm the function of L protein, and three domains (A, B, and C) of the L protein were predicted from multiple sequence alignments.[28] These three domains could interact with N protein, P protein, and genomic RNA, respectively.

The M protein is the most abundant protein in the virion and is peripherally associated with the viral membrane.[29,30] In MV MG assays,[31] M protein has been verified as being a regulator of viral RNA synthesis and assembly through interaction with N protein; MG RNA synthesis is inhibited by interaction between M protein and C-terminal residues of the N protein. These results indicated that the M and C proteins regulate viral RNA synthesis by different means. By contrast, MV M protein could not affect the efficiency of reporter gene expression in SeV and PIV5 MG assays.[31] The interaction between N and M proteins is probably specific to each virus of the family; however, to our knowledge, it is not yet known which domain(s) are involved in the M—N protein interaction. Although M protein plays an important role in regulating the rate of viral replication, protein synthesis, and viral assembly, it remains unclear whether the M protein of all members of the Paramyxoviridae family and other virus families possesses the same regulatory function. In a previous study using an RSV MG system,[32] coexpression of the M2 gene at high levels of input plasmid inhibited transcription and replication, but resulted in efficient production of full-length RNAs at a high level, especially for genes larger than approximately 1000 nt. These findings suggested that the amount of RSV M2 protein could have a very important effect on the balance between RNA transcription and replication. Furthermore, it was shown that RNA and P binding to RSV protein M2-1 can be uncoupled, and that both are critical for the transcriptional antitermination function of M2-1.[33]

In short, an MG system could possibly be established for each virus, and then various studies could be conducted to research viral replication and transcription. More important, virus-free technologies that would not pose biosafety issues could be developed. In addition, the MG technology has been approved as a promising tool for research of protein interactions and associations within the RNP or RdRp complex. Using an MG assay together with other experimental approaches, much information has been accumulated in terms of the function and structure of viral proteins. However, MG technology could not be directly applied to studies of the envelop glycoprotein or attachment protein of the family, as these are not components of the RNP or RdRp complex.

Nonstructural proteins

Nonstructural proteins are proteins encoded by viral genes, but which do not form part of the viral particle. The C, V, and W proteins are known nonstructural proteins of many Paramyxoviridae viruses and play multiple roles in the viral life cycle. The V protein of most viruses in the Paramyxoviridae family is known to bind to intracellular melanoma differentiation-associated gene 5, which is an interferon (IFN)-inducible box helicase, and therefore interrupts the signal transduction pathway activating the IFN-β promoter by forming double-stranded RNA.[34] In an MV MG assay, mutations in the V protein caused protein—RNA binding to occur again, and therefore, to function as a template for RNA synthesis.[35] Furthermore, MV C, V, and W proteins inhibited Nipah virus (NiV) MG RNA replication; NiV C and V proteins inhibited MV MG RNA replication.[6] However, the mechanisms by which MV V and C proteins inhibit their own and NiV MG RNA replication are poorly understood. By contrast, sequencing indicated that MV C and V proteins may have a close relationship with NiV C and V proteins, respectively, with respect to their roles in viral replication. The hRSV MG experiment was performed, and revealed that nonstructural protein NS1 can inhibit RNA transcription and replication and that it functions as a negative regulator in the viral life cycle.[36] These studies collectively illustrated that one of the roles of these nonstructural proteins is to regulate viral RNA synthesis, although the mechanism is not fully clear. These experimental findings further showed that MG technology is a useful tool for exploring the roles of nonstructural proteins of this virus family at the level of transcription and replication. The domain locations or functional amino acid sites of those nonstructural proteins related to the virus life cycle could be further mapped using the MG platform.

Viral noncoding regions

The MG system is a powerful biotechnology tool for investigating noncoding regions of the viral genome that function as viral *cis*-acting signals or indeed other regulatory roles involved in the virus life cycle. In an experiment using a Newcastle disease virus (NDV) MG, the reporter activity in cells transfected with a single helper plasmid, expressing L protein, was higher than that of the blank and negative controls, as a result of a possibly unknown eukaryotic promoter-

like sequence in the downstream region of the NDV-MG RNA.[7] Postulated vaccine-related changes at Positions 5 and 26 in the rinderpest virus (RPV) genome promoter (GP) may account for the inability of PPRV helper proteins to recognize and interact efficiently with RPV GP.[37] A mutation from A to G at the 7th position was shown to inhibit accumulation of RSV anti-MG RNA, but did not inhibit mRNA synthesis in an RSV MG assay.[38] This finding indicated that the 7th position plays key roles in the genome and anti-GP sequences. As in other non-segmented negative-strand RNA viruses, although RSV intergenic regions also function to separate the viral genome into nine genes, these regions do not modulate the efficiency of subsequent transcription in the dicistronic MG system.[39] This supported the idea that the various intergenic regions do not affect the efficiency of recognition of adjacent, upstream gene-end signals and do not affect the levels of read-through mRNAs produced by the polymerase. Furthermore, combining selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE), antisense-interfered SHAPE, and Pb^{2+}-induced cleavage analysis with a dengue virus MG RNA assay not only verified long-range interactions between the 5′- and 3′-terminal regions, which involved hybridization during 5′—3′ cyclization, the 5′—3′ upstream of AUG region as well as 5′—3′ downstream of AUG region, but also revealed that communication between the 5′—3′-terminal regions strongly depends on the structure and sequence composition of the 5′-cyclization region.[40] Structure-directed and site-directed mutagenesis analyses of viral MG RNA are novel approaches for elucidating viral RNA replication and synthesis.

A tool for high-throughput screening of potential antiviral compounds

Screening antiviral drugs using infected cell models require a laboratory with a high biosafety level. However, virus-free MG systems have been widely used for screening antiviral drugs. To date, large numbers of compounds have been tested for potential antiviral activity using an MG platform. In brief, after development, characterization, and optimization, MG-transfected cells are treated with potential antiviral compounds for a limited period and are then assayed for reporter gene activity.[8] Six of 960 compounds were found to inhibit luciferase activity by >70% in an Ebola virus MG reporter system.[41] However, it is important to keep in mind that antiviral drugs screened by MG assay would not inhibit other stages of the virus life cycle. Therefore, the viral and cellular proteins/pathway targets of those compounds will require further investigation.

Although rescuing newly recombinant viruses from a full-length cDNA clone has provided important advantages in the development of vaccines, vectors, and the study of viral gene function, it raises new concerns that the recombinant virus could be pathogenic to humans and animals. By contrast, direct studies of wild-type viruses are cumbersome. In such cases, these agents, classified under biosafety level 3 or 4, regardless of whether they represent the original virus or a new recombinant virus, constitute a threat to human and animal safety, and may even be bioterror agents.

The MG methods have several attractive advantages, compared with traditional experimental approaches. An MG system does not only avoid the threats posed by a live virus to laboratory workers and the environment, but also facilitates study of the molecular biology of the virus, whether or not it has been rescued from full-length cDNA clones.[26,42] Furthermore, prior to rescuing a recombinant virus from its full-length cDNA, an MG system is first useful to verify the validity of the experimental process.[43] Moreover, recombinant MG RNA can be packaged into virus-like particles; these pseudotype viruses containing a foreign gene could be passaged several times in the cells.[44] Therefore, an MG system could be a multifunctional platform for the research of viruses, as previously reviewed.[45] In addition to the Paramyxoviridae family, MG technology has also been used to study other virus families, including viruses from the Rhabdoviridae,[46] Filoviridae,[20] Bornaviridae[47] families, and other minus- or even plus-stranded RNA viruses, such as the hepatitis C virus.[48] Furthermore, MG technology revolutionized the approaches to screen candidate compounds against viruses, without requiring infectious viruses, and also facilitated detection of neutralizing antibodies. Another potential use of the MG system is in the study of gene products of nonviral origin, such as cytokines, cytokine antagonists, or other immune modifiers, on the virus. In contrast to immunoprecipitation, Western blot analyses, yeast two-hybrid assays, among others, MG technology could allow interaction of more than two proteins in a dynamic system, and the downstream reaction could also be observed by detection of expression of targeted proteins or mRNAs.[4,27] Moreover, a recent study that combined MG technology with crystallization and structure determination assays identified more than 3 aa residues in influenza A virus N proteins that are critical for N protein dimer formation, which is required for viral RNA replication.[49] Therefore, the MG system as a model of viral replication has its own unique features and represents a breakthrough in the *in vitro* research of viral replication and transcription, and of antiviral drugs, etc.

Despite the wide use of the MG system, there are still many limitations to this approach. Because of a lack of viral proteins in *trans*, MG can also not be used to study viral primary transcription. The intracellular environment for the plasmid-based expression of helper proteins is very different from the normal infectious cycle in terms of the stoichiometry of the helper proteins present in the system. Furthermore, a viral MG is considerably shorter than its full-length genome. These limitations should be considered when planning an MG assay.

As our understanding in MG technology and the virus life cycle deepens, there will be new opportunities for the development of antiviral agents that specifically target pathways associated with viral replication, transcription, and translation, such as successful antiretroviral protease inhibitors.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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