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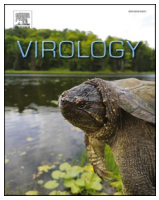

Invited Review

Multifunctional noncoding regions in the mammarenavirus genome

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ABSTRACT

Mammarenaviruses often cause long-term asymptomatic chronic infections in their natural hosts, primarily rodents, and include several human pathogens responsible for diseases ranging from mild febrile illnesses to life-threatening hemorrhagic fever. Mammarenaviruses encode two genes in each segment of their bisegmented RNA genome, with ambisense polarity. The multifunctionality of each gene product supports the optimal propagation of the virus. Moreover, the noncoding regions of the mammarenaviral genome have been shown to have multiple functions, beyond the control of viral transcription and replication. For instance, the noncoding intergenic region (IGR) is integral to the posttranscriptional regulation of viral protein expression. This mechanism underlies the efficient multiplication of the virus, which utilizes an ambisense coding strategy. Further clarification of the multifunctionality of the noncoding regions of the mammarenaviral genome will extend our understanding of the complex biology of these simple viruses and provide the basis for the development of novel medical countermeasures.

1. Introduction

Several members of the rodent-borne mammarenaviruses (family *Arenaviridae*, genus *Mammarenavirus*) cause hemorrhagic fever in humans, posing serious public health concerns in their endemic regions (Enria et al., 2008; Fichet-Calvet and Rogers, 2009). Lassa virus (LASV), the causative agent of Lassa fever, is a highly prevalent mammarenavirus that has been estimated, with a recent calculation model, to infect about 900,000 or more individuals annually in West Africa, with significant mortality (Basinski et al., 2021; Smith et al., 2024). Despite the significant impact of LASV endemicity on human populations, no licensed vaccine against this virus is available and the existing treatment options involve the nucleoside analogue ribavirin, which is only partially efficacious and has significant side effects (Carrillo-Bustamante et al., 2017; McCormick et al., 1986; Salam et al., 2021). This situation makes the development of a safe and effective vaccine and antiviral drugs directed against LASV a high priority. Therefore, Lassa fever has been identified as one of eight priority diseases by the Coalition for Epidemic Preparedness Innovations (CEPI), a global partnership accelerating vaccine development against emerging infectious diseases. However, the requirement for a biosafety level 4 (BSL-4) facility to handle live LASV has been a major obstacle to LASV research. Instead, studies using a prototypic mammarenavirus, lymphocytic choriomeningitis virus (LCMV), have provided novel insights into mammarenaviral multiplication mechanisms and pathogenesis, which can often be extrapolated to LASV (Cai et al., 2020a, 2020b; Cheng et al., 2015; Iwasaki et al., 2015). LCMV is genetically closely related to LASV and can be used in BSL-2 settings. Several other mammarenaviruses also cause severe disease in humans. Junín virus (JUNV) causes Argentinian hemorrhagic fever, a disease that is endemic to the pampas region of Argentina and has a high mortality rate (Harrison et al., 1999; Weissenbacher et al., 1987). Moreover, the identification in 2009 of the novel mammarenavirus, Lujo virus (LUJV), as the causative agent of a cluster of hemorrhagic fever cases in Zambia and the Republic of South Africa has raised concerns about the potential emergence of novel hemorrhagic fever-causing mammarenaviruses outside their present endemic regions (Briese et al., 2009; Paweska et al., 2009). A detailed understanding of mammarenaviral biology will provide rational strategies for developing medical countermeasures to combat human pathogenic mammarenaviruses.

Mammarenaviruses are RNA viruses that encode four genes. Each viral protein has multiple functions, allowing the virus to achieve

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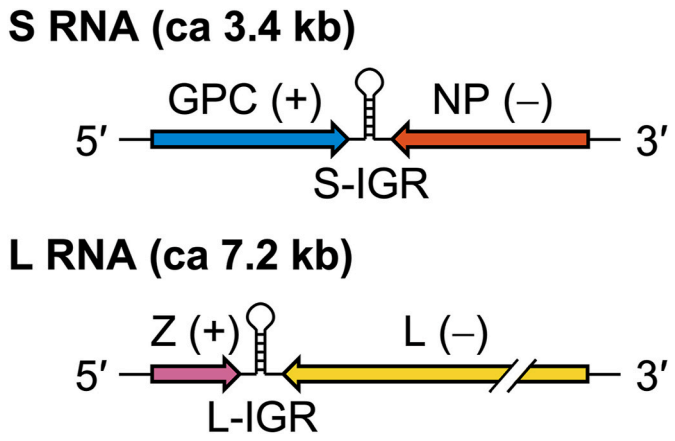

Fig. 1. Schematic diagram of the mammarenavirus genomic organization. +, sense polarity; –, antisense polarity.

optimal multiplication. Evidence indicates that noncoding regions in the mammarenaviral genome have additional functions beyond the regulation of viral gene transcription and genome replication. Reverse genetics and associated minigenome (MG) rescue systems have predominantly been used to study the functions of the noncoding regions of mammarenaviruses, because these experimental systems allow mutation–function analyses with single-nucleotide resolution. In this review, the biological features and reverse genetics systems of mammarenavirus are first briefly summarized. Our current understanding of the functions of their noncoding regions is then summarized and discussed in detail, focusing on their significance in viral multiplication and pathogenesis and their potential contribution to vaccine and drug development.

2. General biology of *mammarenavirus*

Their geographic distributions, antigenic properties, and genetic classification divide mammarenaviruses into two groups: the Old World (OW) mammarenaviruses, including LASV, LUJV and LCMV, and the New World (NW) mammarenaviruses, including JUNV (Radoshitzky et al., 2015). Mammarenaviruses are enveloped viruses with a bisegmented, single-stranded RNA (ssRNA) genome (Radoshitzky et al., 2023). Each RNA segment encodes two genes with negative or positive polarity, in a coding strategy referred to as 'ambisense' (Fig. 1). The two genes on each RNA segment are separated by a noncoding intergenic region (IGR) (Auperin et al., 1984a). The S segment encodes the viral nucleoprotein (NP) and the glycoprotein precursor (GPC) (Auperin et al., 1984b). GPC is cotranslationally processed by cellular signal peptidases to generate a stable signal peptide (SSP), and is then post-translationally processed by membrane bound transcription factor peptidase, site 1 (MBTBS1) to generate subunits GP1 and GP2 (Beyer et al., 2003; Kunz et al., 2003; Lenz et al., 2001; Rojek et al., 2008a). GP1 and GP2, together with SSP, form the glycoprotein (GP) complex, which is responsible for receptor recognition and membrane fusion. The L segment encodes the viral RNA-dependent RNA polymerase (L) and the small matrix RING finger protein (Z) (Salvato and Shimomaye, 1989). NP encapsidates the viral genomic RNA to configure the nucleocapsid in a form that makes it available as the template for viral RNA synthesis by the L protein. The nucleocapsid recruits the L protein, generating the viral ribonucleoprotein (vRNP), the minimum unit of viral gene transcription and genome replication.

The mammarenavirus life cycle occurs entirely within the cell cytoplasm (Fig. 2). The LASV virion attaches to a cell-surface receptor, primarily α-dystroglycan (Cao et al., 1998), and enters the cell via an atypical macropinocytosis pathway (Iwasaki et al., 2014; Oppliger et al., 2016). When the virus reaches the acidic environment of the endosome, GP1 undergoes a conformational change that allows it to bind to the intracellular receptor lysosomal associated membrane protein 1 (LAMP1) (Jae et al., 2014). This is followed by the fusion of the viral and

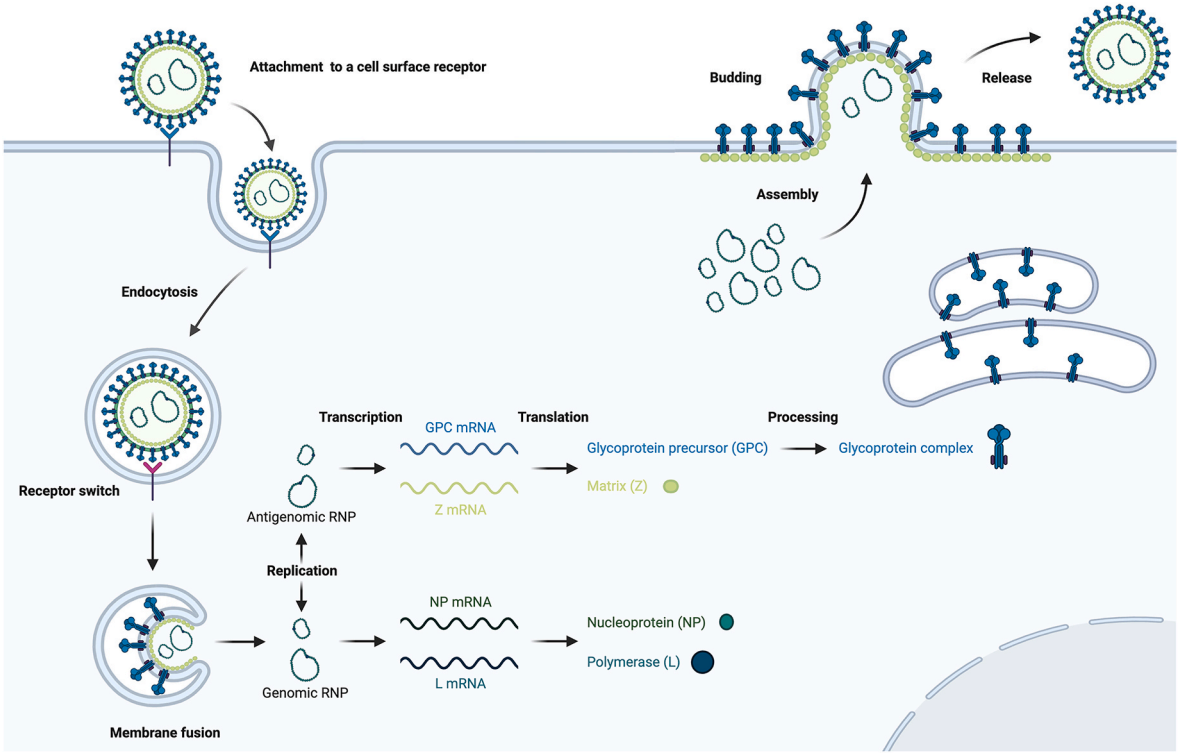

Fig. 2. Schematic diagram of the mammarenavirus life cycle. Processing of the glycoprotein precursor occurs in the endoplasmic reticulum and Golgi. This figure was created in BioRender.com.

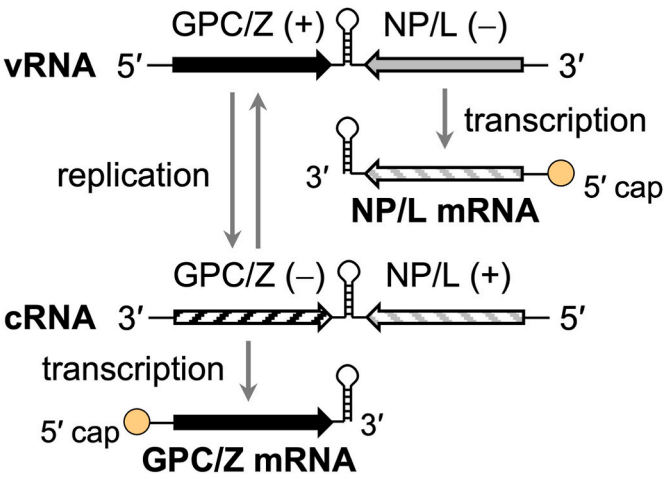

Fig. 3. Schematic diagram of mammarenavirus transcription and replication. +, sense polarity; –, antisense polarity; vRNA, genomic RNA; cRNA, antigenomic RNA.

cellular membranes, which is induced by a conformational change in GP2, releasing vRNP into the cell cytoplasm. Recently, CD164 was identified as an essential entry factor for LCMV (Bakkers et al., 2022; Liu et al., 2022). CD164 may be an analogue of LAMP1 that facilitates LASV cell entry, because CD164 is required to trigger LCMV glycoprotein-mediated membrane fusion at low pH, which mimics the acidic milieu of the endosome, where LCMV membrane fusion occurs (Bakkers et al., 2022). Likewise, the LUJV glycoprotein attaches to the ectodomain of neuropilin 2 at neutral pH, and CD63 stimulates LUJV glycoprotein-mediated membrane fusion at low pH (Raaben et al., 2017). The cell entry of human pathogenic NW mammarenaviruses, such as JUNV, is initiated when their glycoprotein binds to the transferrin receptor of each host species or the human orthologue (Abraham et al., 2009; Radoshitzky et al., 2007, 2008). This is followed by the internalization of the virus via clathrin-mediated endocytosis (Martinez et al., 2007; Rojek et al., 2008b). Although an acidic pH is required to induce membrane fusion, whether NW mammarenaviruses use intracellular receptors remains to be determined. The L protein, which is associated with the vRNP released into the cell cytoplasm upon membrane fusion, initiates transcription using a 5′-capped RNA fragment for priming, which is obtained from a cellular mRNA by cap-snatching (Polyak et al., 1995; Raju et al., 1990). Transcription begins at the 3′-termini of the genomic S and L RNAs and terminates within the IGR, generating the NP and L mRNAs, with 5′-capped and 3′-nonpolyadenylated untranslated regions (UTRs) (Iapalucci et al., 1991; Meyer and Southern, 1993) (Fig. 3). The L protein then produces the antigenomic S and L RNAs, from which the GPC and Z mRNAs and the genomic S and L RNAs are generated. Ultimately, infectious progeny virions are assembled and released from the plasma membrane by Z-mediated budding (Perez et al., 2003).

3. Reverse genetics

The mammarenavirus MG rescue system was first established for LCMV (Lee et al., 2000). The LCMV MG RNA encodes no viral genes but contains an open reading frame (ORF) for a reporter gene at the NP locus and the *cis*-acting regulatory sequences of the LCMV S segment—the 5′- and 3′-UTRs and the IGR—required to control viral gene expression and replication. The formation of the active LCMV MG ribonucleoprotein (RNP) by the T7-polymerase-mediated intracellular synthesis of the LCMV MG RNA, together with the expression of *trans*-acting factors NP and L, results in the synthesis of anti-MG RNA and the reporter gene mRNA. Reporter gene expression can be used to monitor the efficiency of viral transcription and replication. An LCMV MG system that depends on murine polymerase I (Pol-I)-mediated MG RNA synthesis has also been developed (Lee et al., 2002). The first LCMV MG study demonstrated that the NP and L proteins are the minimum *trans*-acting factors required for viral gene expression and genome replication, which is consistent with other mammarenaviral MG systems, including those for LASV (Hass et al., 2004), JUNV (Albarino et al., 2009; Emonet et al., 2011), and NW Tacaribe virus (TCRV) (Lopez et al., 2001).

The rescue of infectious mammarenavirus entirely from cloned cDNAs (plasmids) was also first described for LCMV (Sanchez and de la Torre, 2006). The transfection of BHK-21 cells with two plasmids that directed the synthesis of the full-length LCMV S and L RNA species with antigenomic polarity under the control of the T7 promoter and three plasmids expressing *trans*-acting factors NP, L protein, and T7 polymerase, reconstituted the active vRNPs for the S and L segments within the cell cytoplasm. This allowed subsequent viral gene expression and genome replication mediated by the L protein, followed by the release of infectious virus into the cell culture medium. In addition to T7 polymerase, reverse genetics systems have been developed based on the murine or human Pol-I-mediated synthesis of LCMV antigenome RNA species (Flatz et al., 2006; Ortiz-Riano et al., 2013). A series of reverse genetics systems has been established for mammarenaviruses LASV (Albarino et al., 2011; Cai et al., 2020b; Carnec et al., 2011; Yun et al., 2013), LUJV (Bergeron et al., 2012), OW Mopeia virus (MOPV) (Carnec et al., 2018), JUNV (Albarino et al., 2009; Emonet et al., 2011), TCRV (Foscaldi et al., 2020; Ye et al., 2020), NW Guanarito virus (GTOV) (Taniguchi et al., 2024), NW Chapare virus (CHAPV) (Jain et al., 2023), NW Machupo virus (MACV) (Jain et al., 2023; Patterson et al., 2014), and NW Pichinde virus (PICV) (Lan et al., 2009).

Reverse genetics systems provide rational strategies for investigating the roles of the four viral gene products and the *cis*-acting regulatory sequences in these genomes in the context of natural infections, based on the phenotypic characterization of recombinant mammarenaviruses with predetermined mutations. However, some mutations may have a deleterious effect on the viability of the virus, which complicates studies that use recombinant viruses with those mutations. The MG rescue system compensates for this limitation. The coexpression of the GPC and Z protein in the presence of the active MG RNP results in the incorporation of the MG RNP into virion-like particles (VLPs), driven by the Z protein (Perez et al., 2003). This MG RNP-containing VLP is decorated with the GP complex and is therefore infectious to susceptible cells. When *trans*-acting factors NP and L protein encoded on plasmids or the live, homologous parental virus are provided to the infectious VLP-infected cells, MG gene expression and replication take place in the passaged cells. This approach, referred to as an 'infectious VLP assay', allows us to evaluate the activities in the steps proceeding from cell entry through to the egress of the progeny virions without the generation of a viable recombinant virus. Therefore, this strategy is less demanding and expands the mutation repertoire. It is noteworthy that the mammarenaviral Z protein exerts an inhibitory effect on the transcription and replication mediated by the L protein (Cornu and de la Torre, 2001, 2002; Lopez et al., 2001). The optimization of plasmid-supplied Z levels is required to balance the efficiencies of MG activity and infectious VLP production.

4. The IGR

4.1. Role of the IGR in transcription termination

Mammarenaviral transcription terminates within the IGR, generating 5′-capped and 3′-nonpolyadenylated mRNA species (Singh et al., 1987). The nonpolyadenylated 3′-UTR is complementary to part of the IGR. The 3′ ends of four mRNAs of TCRV were determined by nuclease S1 mapping (Iapalucci et al., 1991). The unencapsidated mRNAs present in TCRV-infected cells were prepared by recovering the pellet after the centrifugation of a cytoplasmic extract on a CsCl or sucrose gradient. The mRNAs were hybridized with a radioactively 3′-labeled cDNA probe

covering the region across the IGR, and then digested with nuclease S1, which digests ssDNA and ssRNA. The 5′-end sequences of the cDNA probes protected by the nuclease S1 treatment were determined by polyacrylamide gel electrophoresis. Using the cDNA probe for each mRNA, the 3′ ends of the L and Z mRNAs were mapped to the distal (relative to the coding regions of each mRNA) regions within the IGR, with the sequences of their 3′ ends overlapping. In contrast, the 3′ ends of the NP and GPC mRNAs were mapped inside the IGR, specifically to the distal side of one (proximal, relative to the coding region) of the two hairpin structures comprising the IGR, without an overlapping region. The mapped 3′ end positions of each mRNA showed slight variations. Intriguingly, the 3′-UTRs of all TCRV mRNA species were predicted to fold into stable hairpin structures (Iapalucci et al., 1991), which are postulated to be involved in transcription termination based on their similarity to self-complementary hairpin structures found at the 3′ ends of transcripts of prokaryotes (Platt, 1986). The 3′ ends of the NP and GPC mRNAs of LCMV were analyzed by the intramolecular circularization method (Meyer and Southern, 1993). The mRNA fraction was separated from the encapsidated genomic RNA by CsCl gradient centrifugation and the mRNA was intramolecularly circularized with RNA ligase. The circularized RNA was converted to cDNA and used as a template for PCR across the 3′–5′ junction. A population (length) analysis of the PCR product showed that the 3′ ends formed two (GPC mRNA) or four (NP mRNA) clusters within the IGR. The 3′ ends of the JUNV NP and GPC mRNAs were mapped by an RNase protection assay (Tortorici et al., 2001), similar to the approach used to determine the 3′ ends of TCRV (Iapalucci et al., 1991). The unencapsidated mRNA fraction was recovered from the pellet sedimented by the CsCl centrifugation of JUNV-infected cell lysate and hybridized with radioactively labeled, genome- and antigenome-polarity RNA probes containing regions across the IGR, followed by digestion with RNase T1 and RNase A. The lengths of the protected RNA probes were analyzed on a sequencing gel by electrophoresis. The 3′ ends of the NP and GPC mRNAs were mapped within the IGR with slight positional variations and overlapping sequences. Whether the different degrees of distribution of the viral mRNA (vmRNA) 3′ ends in TACV, JUNV, and LCMV are attributable to differences in the NW and OW mammarenaviruses remains to be clarified. This heterogeneity at the ends of the 3′-UTRs and their predicted stable hairpin structures indicate that transcription termination is mediated by the secondary structure of the IGR, rather than by a specific terminator signal sequence. Intriguingly, the L protein of LCMV was unable to transcribe reporter mRNA with a length similar to that transcribed from an MG with an intact IGR from an MG lacking the IGR (MGΔIGR), but generated RNA species with lengths similar to that of the full-length anti-MG (Pinschewer et al., 2005). Because reduced, but significant, reporter protein expression was detected in the MG assay with MGΔIGR, those RNA species may have contained full-length anti-MG RNA and 5′-capped reporter mRNA species. The role of the secondary structure in transcription termination was analyzed further with TCRV MG constructs containing a series of deletions in the IGR, which generated different hairpin configurations of the IGR (Lopez and Franze-Fernandez, 2007). A northern blotting analysis of total RNA from cells expressing MG constructs with mutant IGRs and high ΔG values detected significantly reduced or negligible levels of reporter mRNA at the size expected if the transcription terminated within the IGR. Furthermore, an MG construct with a mutant IGR with a ΔG value similar to that of wild-type IGR but containing a ‘bulged’ stem (the stem region contained several unpaired bases) significantly reduced the expression of reporter mRNA with a length expected if it were terminated within the IGR. These results suggest that a stable hairpin structure with a highly complementary stem is important for the termination of transcription.

4.2. Role of the IGR-derived 3′-UTR of viral mRNA in the regulation of translation

The S- and L-IGRs differ significantly in both their sequences and their predicted structures. These differences are associated with functional differences because the replacement of the S-IGR with the L-IGR resulted in nonviable LCMV (Iwasaki et al., 2015). The functional differences in the S- and L-IGRs were further examined with the LCMV MG system. A series of MG constructs containing the IGR and 3′- and 5′-UTRs from the S and L segments in various combinations was examined for the expression of reporter proteins and mRNAs. In the wild-type MG constructs, reporter protein expression was significantly higher when the reporter ORF was inserted into the NP locus than into the GPC locus, or was inserted into the Z locus than into the L locus. In contrast, the reporter mRNA levels were significantly higher when the reporter ORF was inserted into the GPC locus than into the NP locus, or into the Z locus than into the L locus. Swapping the IGRs between the S- and L-segment MG constructs significantly altered the reporter protein levels expressed from each gene locus, with only a slight impact on the reporter mRNA expression patterns. This suggests that the IGR-derived, non-polyadenylated 3′-UTRs of vmRNAs regulate the efficiency of translation. A reporter assay using *in vitro*-transcribed vmRNA mimics that encoded a reporter gene flanked by the chimeric or mutated UTRs of LCMV NP and GPC mRNAs, in various combinations, identified a short (8- or 10-nt) sequence within the 3′-UTR proximal to the ORF as the major determinant of translation efficiency. Additionally, the small stem–loop structure formed by the ORF-proximal 10-nt sequence within the 3′-UTR of the NP mRNA was shown to promote translation (Hashizume et al., 2022). Intriguingly, a mutated TCRV NP mRNA mimic containing an unstructured 3′-UTR produced about fourfold more reporter protein than the TCRV NP mRNA mimic in which the wild-type 3′-UTR formed a major stem–loop structure (Foscaldi et al., 2017). This indicates the predominant suppressive role of the major stem–loop structure in vmRNA translation. In contrast, the small stem–loop structure may act as a crucial vmRNA translation enhancer in the context of the intact 3′-UTR sequence where the major stem–loop structure is present (Hashizume et al., 2022). Moreover, the LCMV mRNA mimics showed no clear binding to poly(A)-binding protein (PABP), and the small interfering RNA (siRNA)-mediated knockdown of PABP did not significantly affect the expression of the reporter protein. Similarly, the siRNA-mediated knockdown of eukaryotic initiation factor 4E reduced the reporter protein expression of a TCRV NP mRNA mimic significantly less than that of the cellular mRNA mimic (*in vitro*-transcribed mRNA containing the 5′-cap structure and 5′- and 3′-UTRs from human β-globin mRNA) (Foscaldi et al., 2017). These findings suggest that as-yet-unidentified host cell machinery regulates vmRNA translation.

Whereas a recombinant LCMV (rLCMV) with the L-IGR in the S segment was nonviable, rLCMV containing the S-IGR in both segments [rLCMV(IGR/S-S)] was viable (Iwasaki et al., 2015). rLCMV(IGR/S-S) showed a modest reduction in fitness in cultured cells. In contrast, rLCMV(IGR/S-S) was severely attenuated *in vivo* insofar as this mutant virus failed to cause mortality in a normally lethal meningitis mouse model of LCMV infection. Moreover, mice inoculated with rLCMV (IGR/S-S) were fully protected from lethal challenge with wild-type LCMV. Therefore, rLCMV(IGR/S-S) presented the ideal characteristics of a live-attenuated vaccine. The LCMV MG assay results described above suggest that the mechanism of rLCMV(IGR/S-S) attenuation involved the stable reorganization of the control of viral protein expression. This attenuation strategy was applied to LASV, generating rLASV containing the S-IGR in both segments (rLASV[IGR/S-S]) (Cai et al., 2020a). Similar to rLCMV(IGR/S-S), rLASV(IGR/S-S) showed slightly reduced fitness in cultured cells, but a severely attenuated phenotype in a highly sensitive guinea pig model of LASV infection. Guinea pigs initially exposed to the rLASV(IGR/S-S) virus were fully protected from lethal challenge with wild-type LASV. These studies suggest that this translation-regulation mechanism involving the

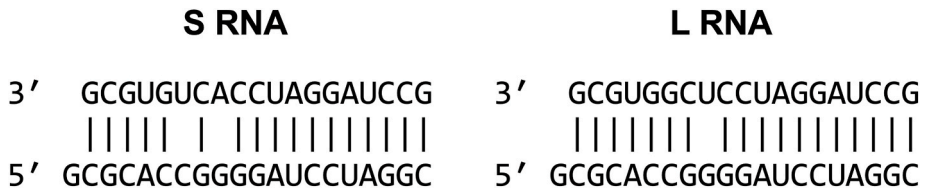

Fig. 4. Complementarily conserved 3′- and 5′-end sequences of the S (left) and L (right) genomic RNAs.

3′-UTRs of viral mRNAs may be a common feature across the mammarenaviruses, although the IGR sequences of these viruses are highly variable. Likewise, an MACV variant (Car^{91}) containing a 35-nt deletion and a 1-nt substitution in the L-IGR was avirulent in the guinea pig infection model, whereas the parental wild-type MACV caused 100% mortality (Golden et al., 2017). Moreover, Car^{91} afforded a high level of protection to guinea pigs against lethal challenge with a distantly related NW GTOV, indicating that Car^{91} can act as a universal NW mammarenavirus vaccine. However, the precise mechanism behind Car^{91} attenuation remains to be determined.

4.3. Role of the IGR in infectious virion formation

The IGR is not strictly required for viral transcription or replication because IGR-deficient LCMV MG can be amplified normally by the L protein (Pinschewer et al., 2005). In the same study, the authors also examined the contribution of the IGR to viral assembly and budding, with an infectious VLP assay. The deletion of the IGR from the LCMV MG resulted in the very low expression of reporter protein in the VLP-infected cells, suggesting that the IGR is involved in a critical step during the formation of infectious particles. The IGR forms stable stem−loop structures (Auperin et al., 1986; Gonzalez et al., 1996; Wilson and Clegg, 1991), which may act as packaging signals, analogous to those in several other RNA viruses that use stable secondary RNA structures in genome packaging (Hagey et al., 2022). This notion is supported by the observation that the impaired infectious VLP production caused by the depletion of the IGR in an LCMV MG was rescued by the insertion of the IGR of LASV, which differs significantly from that of LCMV in sequence but forms a similar hairpin structure (Iwasaki et al., 2016). How viral proteins, particularly the matrix Z protein, are involved in IGR-dependent particle formation remains to be determined. JUNV NP is reported to specifically bind to the IGR (Tortorici et al., 2001), implying that the IGR–NP–Z interaction contributes to the formation of the JUNV particle. However, experimental verification of the potential role of the IGR–NP–Z interaction in JUNV particle formation is required.

5. The 3′- and 5′-UTRs

5.1. Role of the conserved 19-nt 3′-end sequence in viral RNA synthesis

The 19-nt sequences at the 3′-termini of the mammarenavirus S and L genomic segments share a high degree of sequence identity (Auperin et al., 1986), suggesting that these regions contain a promoter sequence commonly used by the mammarenaviral polymerase L protein for transcription and replication. Furthermore, the 3′- and 5′-termini of the S and L segments have a high degree of complementarity, and can therefore anneal to form a panhandle structure (Fig. 4). This is supported by the electron-microscopic observation of a closed circular configuration of a vRNP-like structure in the nucleocapsid fraction prepared by centrifuging purified PICV lysate on a Urografin gradient (Young and Howard, 1983). Any pattern of 1–19-nt deletions introduced into the 19-nt sequence at the 3′-end of the LCMV S MG completely abolished the expression of the reporter gene (Perez and de la Torre, 2003). Moreover, 2-nt substitutions within either the 3′- or 5′-terminal 19-nt sequence that disrupted their complementarity also abolished

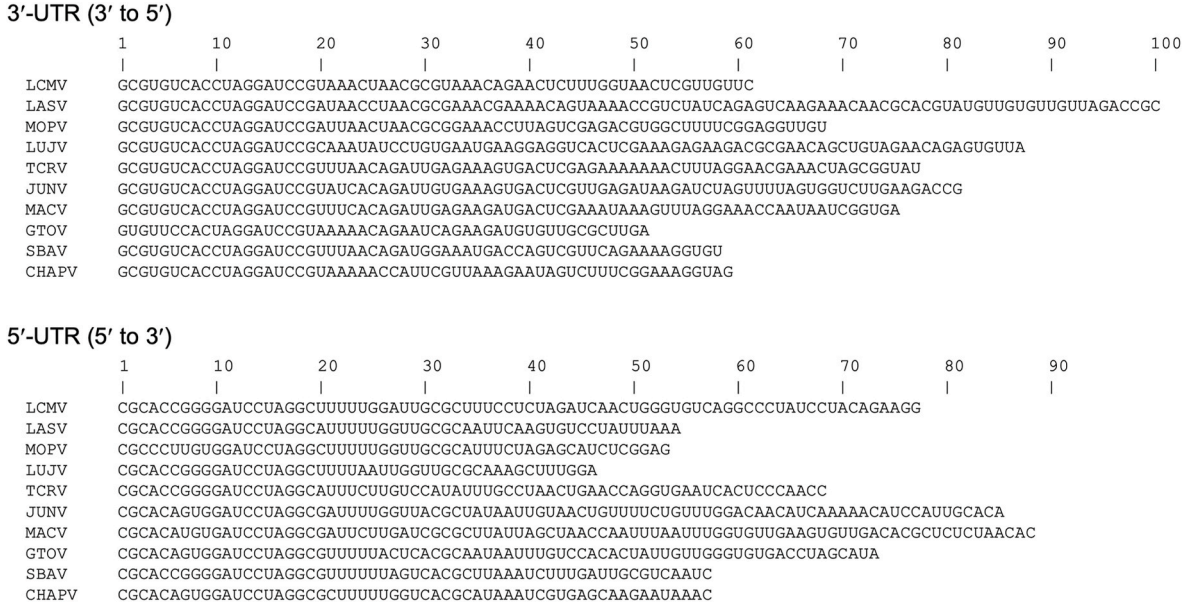

Fig. 5. Sequence alignments of 3′- and 5′-UTRs of mammarenavirus S segment genomic RNA. Sequences are referenced from the National Center for Biotechnology Information: lymphocytic choriomeningitis virus (LCMV), AY847350.1; Lassa virus (LASV), J04324.1; Mopeia virus (MOPV); AY772170.1; Lujo virus (LUJV), JX017360.1; Tacaribe virus (TCRV), MT081316.1; Junin virus (JUNV), AY358023; Machupo virus (MACV), AY129248.1; Guanarito virus (GTOV), AY129247.1; Sabia virus (SBAV), U41071.1; Chapare virus (CHAPV), EU260463.1. A residue (usually a G residue), if present at the 3′ and 5′ ends of the referenced sequences on the 3′- or 5′-terminal side of the conserved 19-nt sequence, was omitted and the first residue of the 19-nt sequence at the 3′ and 5′ ends was set as the position 1.

reporter gene expression. This was not rescued by additional substitutions within the other side of the terminal region that restored their complementarity, suggesting that the sequence specificity of the 3′-terminal 19-nt sequence and the formation of the 3′−5′-terminal duplex are both strictly required for viral gene expression and genome replication. An intensive mutation−function analysis of the 3′-terminal 19-nt sequence using an LASV S-segment-based MG system further showed that the base specificity in the first 12 positions (with some level of tolerance at unpaired positions 6 and 8) and the complementarity at positions 13–19 are important for promoter activity (Hass et al., 2006). Consistent with these studies, a biochemical assay using purified NW MACV L protein identified a sequence motif at positions 2–5 of the 3′-terminus that is strictly required for the L protein to bind to the 3′ promoter region (Kranzusch et al., 2010).

5.2. Roles in viral RNA synthesis and pathogenesis of the 3′- and 5′-UTR sequences downstream from the conserved 19-nt sequence

Although the 19-nt 3′- and 5′-end sequences have been well studied, the roles of other regions of the 3′- and 5′-UTRs are less well defined (Fig. 5). An LCMV S segment MG construct with a 10-nt deletion introduced into the 3′- or 5′-UTR next to the highly complementary 23-nt sequence at the 3′- or 5′-end showed reporter gene expression similar to that of the corresponding wild-type MG construct. This suggests that the contribution of this 10-nt sequence to L-protein-mediated RNA synthesis is limited (Perez and de la Torre, 2003). Furthermore, a 19- or 21-nt deletion within the 3′- or 5′-UTR next to the highly conserved 19-nt sequence of the LCMV S segment reduced MG activity (Taniguchi et al., 2020). Given that the 4 nt next to the 19-nt sequence are not necessary for LCMV MG activity (Perez and de la Torre, 2003), the short sequences in the 3′- and 5′-UTRs (5 and 7 nt, respectively) located 15 nt downstream from the 19-nt sequence may play a critical role in viral transcription and replication. In contrast, the depletion of the 3′-UTR downstream (relative to 3′ end of the genome) from the 19-nt sequence of the TCRV S segment MG construct resulted in a modest reduction in reporter protein levels, but produced similar levels of anti-MG RNA species. Moreover, any pattern of 10–47-nt deletions introduced into the 5′-UTR downstream (relative to 5′ end of the genome) from the 19-nt sequence significantly reduced MG activities, indicating that the 5′- (or 3′-) noncoding region neighboring the 19-nt sequence in the S segment is strictly (or less strictly) required for TCRV replication (D'Antuono et al., 2023).

In an analysis of a variety of LCMV mutants with deletions or substitutions in the 3′- and 5′-UTRs downstream from the 19-nt sequence in the S segment (assuming that both the 3′ and 5′ ends were upstream from the 19-nt sequence), a mutant rLCMV containing a 22-nt deletion starting 20 nt downstream from the 19-nt sequence in the 3′-UTR of the S segment genomic RNA showed a severely attenuated phenotype in a mouse model of lethal LCMV infection, whereas the mutant had growth properties similar to those of the wild-type parental LCMV in cultured cells (Taniguchi et al., 2020). Moreover, this deletion did not significantly reduce the MG activity or packaging efficacy. These findings suggest the presence of an as-yet-unknown function in these deleted regions that is necessary for LCMV pathogenesis *in vivo*. In TCRV, replacing the 28-nt sequence downstream from the conserved 5′ sequence in the S segment genomic RNA with its reversed sequence caused a modest cell-type-specific reduction in fitness, with a limited impact on virulence *in vivo*, suggesting some degree of plasticity in this region in terms of TCRV pathogenesis (D'Antuono et al., 2023).

5.3. Role of the additional nontemplated G residue at the 5′ end of the genome in circumventing the induction of type I interferon (IFN-I)

The 5′-terminus of the mammarenaviral genome has a nontemplated G residue (Garcin and Kolakofsky, 1990; Polyak et al., 1995; Raju et al., 1990). One proposed model for the addition of this extra G residue is a prime-and-realign mechanism that initiates genome replication (Garcin and Kolakofsky, 1992). In this model, antigenome replication is initiated by the GTP at position +2 of the template, which is next to the exact 3′ end (+1) and extends to dinucleotide $_{ppp}G_pC_{OH}$. This $_{ppp}G_pC_{OH}$ is then realigned to allow the G at position +1 of the template to pair with the C residue of the dinucleotide, and is then extended to copy the entire genomic RNA, except for the G residue at the 5′ end of the template. The same concept can be applied to genomic RNA synthesis. The annealing of the 3′ and 5′ ends of the genome generates a single 5′-pppG overhang. A double-stranded RNA (dsRNA) with an uncapped 5′-triphosphate ($_{5'ppp}$dsRNA) can be the ligand for the cytoplasmic pattern recognition receptor RIGI, leading to the strong induction of IFN-I (Kato et al., 2006). Remarkably, several variations of an *in vitro*-transcribed $_{5'ppp}$dsRNAs containing a 5′-pppG overhang, mimicking the base pairing of the mammarenavirus at its 3′ and 5′ ends, lacked the ability to induce IFN-I, whereas $_{5'ppp}$dsRNA with a blunt end strongly induced IFN-I in a RIGI-dependent manner (Marq et al., 2010). Moreover, various model $_{5'ppp}$dsRNAs with a 5′-ppp-nucleotide overhang competitively bind RIGI, interfering with IFN-I induction by blunt-ended $_{5'ppp}$dsRNAs (Marq et al., 2011).

6. Conclusions and future perspectives

The noncoding regions of mammarenaviruses play multiple, crucial roles in different steps of the viral life cycle and are associated with viral pathogenesis. Therefore, the noncoding regions are attractive targets for medical interventions. For instance, the stringent requirement for sequence specificity and duplex formation in the highly conserved 3′- and 5′-terminal sequences is a feasible target for broad-spectrum antiviral drugs directed against human pathogenic mammarenaviruses. Accordingly, a series of siRNA duplexes targeting the 3′- and 5′-terminal sequences reduced the production of infectious viruses, including LASV, LCMV, and MOPV, by up to 90% in cultured cells (Muller and Gunther, 2007). The improved *in vitro* efficacy of such strategies and an appropriate delivery system will allow us to evaluate their *in vivo* efficacy during the further development of oligonucleotide-based therapeutics targeting the highly conserved 3′- and 5′-terminal sequences of mammarenaviruses.

The diverse functions of the noncoding regions of the mammarenaviral genome have been summarized in this review, focusing on the regions that contribute most clearly to viral regulation. However, different parts of the noncoding regions may coordinately regulate these functions to different degrees. For instance, the replacement of the 5′-UTR of *in vitro*-transcribed TCRV NP mRNA mimics, which encoded the reporter firefly luciferase ORF instead of the NP ORF, with a pyrimidine-rich unstructured sequence with a higher free energy than the wild-type 5′-UTR or with human β-globin mRNA, slightly but significantly reduced the firefly luciferase levels (Foscaldi et al., 2017). This implies that the specific sequence or structure within the 5′-UTR of TCRV mRNA favors its translation. Furthermore, an infectious VLP assay using LCMV S- and L-segment MG constructs showed that regardless of the type of IGR, infectious VLP production was more than twofold higher when the MG constructs contained the 3′- and 5′-UTRs from the L segment rather than those from the S segment (Iwasaki et al., 2015). Enhancement of infectious VLP production by the 3′- and 5′-UTRs from the L segment may help to overcome the size disadvantage of the L segment, which is about twice as large as the S segment, during its incorporation into virions.

As with other negative-strand RNA viruses, mammarenaviruses generate significantly higher levels of genomic RNA species than complementary antigenomic RNA species (Iwasaki et al., 2015), suggesting that antigenomic RNA species have strong promoter activity and genomic RNA species have weak promoter activity. Their ambisense coding strategy indicates that mammarenaviruses use the same promoter sequence for replication and transcription, and may not regulate these processes differently (Hass et al., 2006), leading to the higher expression of GPC mRNA than NP mRNA and higher expression of Z

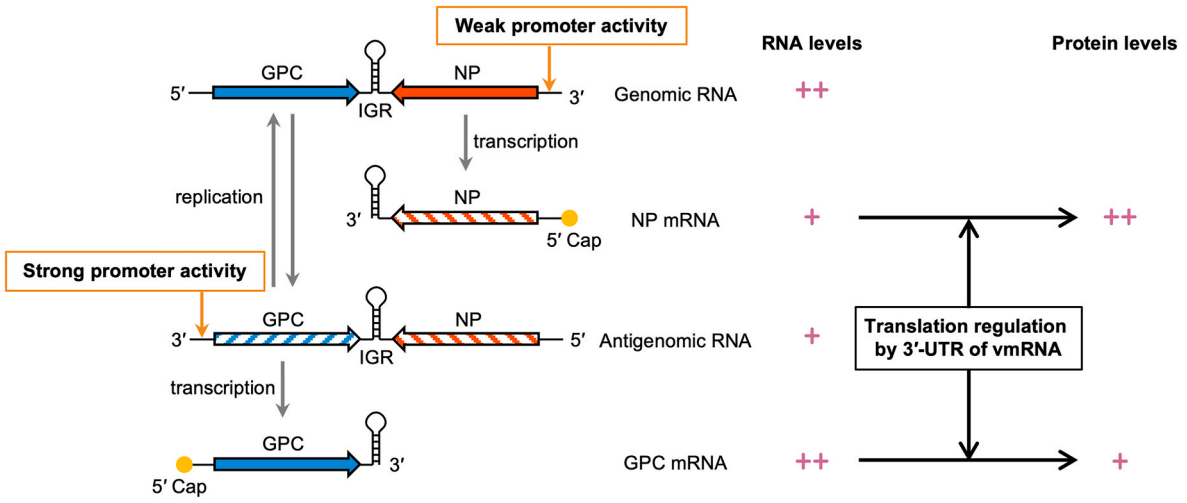

Fig. 6. Conceptual representation of the influence on translation of the nonpolyadenylated 3′-UTR of the mammarenavirus mRNA (vmRNA), illustrated for the S segment.

mRNA than L mRNA. However, low NP and high GPC levels are lethal, and low Z and high L levels cause severe attenuation of the virus, as demonstrated by the LCMV rescue system (Iwasaki et al., 2015). The regulation of protein levels by the IGR-derived nonpolyadenylated 3′-UTR sequences of vmRNAs, rather than by the amount of mRNA, would be essential to overcoming this constraint and may underlie the ambisense coding strategy (Fig. 6).

Live-attenuated vaccines have been the primary approach to LASV vaccine development because virus-specific cell-mediated immunity plays a critical role in controlling this virus (Baize et al., 2009; Fisher-Hoch et al., 2000; McCormick et al., 1992). The attenuation of mammarenaviruses by the manipulation of noncoding regions offers the advantage that the resulting virus contains the same antigenic composition as the parental hemorrhagic fever-causing mammarenavirus. A well-defined molecular mechanism of attenuation mitigates the concern about the emergence of a live-attenuated vaccine mutant with increased virulence. Combinational attenuation strategies will improve the safety profiles of candidate live-attenuated vaccines, because attenuated LCMV containing the S-IGR in both segments and the codon-deoptimized GPC gene showed marked resistance to the acquisition of virulence (Sakabe et al., 2023). Further clarification of the roles of the noncoding regions in the mammarenaviral genome will allow us to rationally design live-attenuated vaccines against human pathogenic mammarenaviruses based on multiple attenuation mechanisms, with solid genetic stability.

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Declaration of competing interest

The author declares that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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