Chapter

Molecular Mechanisms for Norovirus Genome Replication

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Abstract

The genomes of positive strand RNA viruses often contain more than one open reading frame. Some of these viruses have evolved novel mechanisms to regulate the synthesis of the other open reading frames that in some cases involved the production of a subgenomic RNA or RNAs. Very often, the presence of the subgenomic RNA is used as indicator for active viral genome replication. Norovirus, a major cause for gastroenteritis as well as with all other caliciviruses follow a typical positive strand RNA viruses genome replication strategy. In addition, noroviruses also produce a subgenomic RNA during their replication in infected cells. Efficient and adequate synthesis of norovirus subgenomic RNA is crucial for successful viral replication and productive infection leading to the generation of infectious viral progeny. This chapter will dissect the significant findings on mechanisms involved in norovirus genome replication as well as focusing on subgenomic RNA production.

Keywords: RNA-dependent RNA polymerase, subgenomic RNA, replication, internal initiation, core promoter

1. Introduction

Noroviruses are often associated with outbreaks of gastroenteritis in hospitals, on cruise ships, schools, nursing homes and military camps where a close person to person contact cannot be avoided [1]. Infection is typically followed by a 24 to 48 hour incubation period before emergence of the clinical disease, symptoms of which include acute diarrhea and projectile vomiting, usually accompanied by several signs/symptoms such as abdominal cramps, myalgia, malaise, headache, nausea and low grade fever [2, 3]. Noroviruses are the most common cause of gastroenteritis infections due to their stability, low infectious dose, large host reservoir (humans), short term immunity, multiple transmission routes and large genetic diversity between strain [4]. The human norovirus (HuNv) infection is self-limiting and the symptoms typically last between 12 and 60 hours [3]. However, viral shedding appears to be prolonged up to several weeks after the symptoms are resolved, especially in persons with impaired immunity where persistent infection often occur by reinfection [5, 6]. More importantly, illness among the elderly and immunocompromised patient can be fatal due to the severe dehydration. The main transmission route for noroviruses is by fecal-oral, through the contaminated food, water or surfaces especially [1, 7]. Consumption of contaminated fresh produce food such as salads, fruits and sandwiches that requires no prior heating have also
been linked as a possible source of food-borne infections [8]. Furthermore, a high concentration of norovirus was also found within the gastrointestinal tissue of contaminated bivalves such as oysters and mussels that are filter feeders. Therefore, these contaminated bivalves are also considered as another important foodborne source of norovirus infection [9]. In addition, airborne transmission that involves the aerosolized vomit from an infected person has also been demonstrated [10, 11]. These findings are supported by the low infectious dose required for norovirus infection; less than 10 viral particles are sufficient enough to establish infection with Norwalk virus [12]. First described as Norwalk virus, which was responsible for a gastroenteritis outbreak at a school in Norwalk, Ohio US, in 1968 [13], human noroviruses (HuNv) are today recognized as the leading cause of viral gastroenteritis infections in human population. The United States Centre for Disease Control and Prevention (CDC) has estimated that noroviruses are responsible for at least 23 million cases of food borne illness each year in the United States with approximately 50 thousands hospitalization and 300 death [4, 14]. However, in one of the reviews which involves a period of study from 1996 to 2007, it is estimated to be nearly 110,000 hospitalization per epidemic years with the cost of approximately 500 million US dollar per year [15]. The recorded surveillance data from the Food Borne Viruses in Europe Network also indicates that more than 85% of viral gastroenteritis outbreaks that occurred between 1995 and 2000 could be attributed to these viruses [4, 16]. The cost to the United Kingdom National Health Service (NHS) in England and Wales as a direct result of the outbreaks occurring in hospitals has been estimated to be approximately 115 million pounds in 2002–2003 [17]. However, due to the acute nature of the infection, it is difficult to identify all the norovirus infection cases and therefore the real cost can be considered higher. Furthermore, the global impact of gastroenteritis caused by HuNv is hard to be estimated since most of the annually 3.5 to 5 million deaths are from developing world with inadequate healthcare, surveillance and diagnostic systems [1]. There is still no licensed vaccine against norovirus made available. However, there are few promising candidates in the pipeline with one already in phase 2 [18]. In addition, efforts in developing norovirus-specific antiviral drugs are also ongoing. To enable these efforts, our fundamental knowledge on norovirus biology needs to be enhanced especially with regards to norovirus genome replication. This chapter will emphasize on subgenomic RNA replication aspect of norovirus particularly focusing on works with MNV.

2. Building of norovirus particle

The first norovirus virion to be observed by immune-electron microscopy was Norwalk virus in 1972 by Albert Kapikian. The virions are icosahedral, with a diameter ranging from 27 to 39 nm and a buoyant density of 1.36 ± 0.04 g/cm³ [4, 13, 19]. The virus’s capsids are composed of 180 copies of a major protein VP1 (formed into 90 dimers) and one or two copies of the minor capsid protein VP2 [20, 21]. Studies using Norwalk virus-like particles (VLPs) revealed that the major protein VP1 is structurally divided into two domains referred to as the ‘shell’ (S) and ‘protruding’ (P) domains, with the P domain being further divided into P1 and P2 subdomains [21]. The inner S domain sub-units interact each other to form a continuous ‘shell’ structure for capsid while the P domain emanates from the S domain surface and forming cup-like structure. Furthermore, the outer P2 subdomain has been recognized as the most variable region of the calicivirus capsid and the region that determines the species-specific binding of these viruses to the respective cell receptor [22, 23]. Molecularly, norovirus particle capsid encloses the viral genome,
a positive-sense single stranded RNA molecule of about 7.4 to 8.3 kb in size. The genome has a virus encoded protein covalently linked to the 5′ end (VPg) and a poly A tail at the 3′ end (Figure 1). This genomic RNA (G RNA) encodes three open reading frames (ORFs) flanked by two short untranslated regions (UTRs) and with a small degree of overlap at the 5′ and 3′ junctions between ORF1 and ORF2 [24, 25]. In addition, within the Norovirus genus, only murine norovirus (MNV) contains a fourth alternative open reading frame (ORF4) which overlaps ORF2 in a +1 frameshift.

3. The norovirus life cycle

Like all viruses, the life cycle of noroviruses begins with the attachment of the viral particles to their specific receptor on the membrane of the host cell. Susceptibility to norovirus infections in humans, specifically Norwalk virus, is associated with ABO histo-blood group antigens (HBGA) and individual secretor status [26]. HBGA are carbohydrates found on the surface of gut epithelial cells [4, 20, 27]. These carbohydrate molecules are involved in the attachment of noroviruses but are unlikely to be the main receptor as co-receptor may also be involved [27]. In addition, the secretor status of individuals also determines the susceptibility to norovirus infections [28]. Individuals who are non-secretors of H type 1 were found to be resistant to norovirus infections due to a mutation in the α-(1,2)-fucosyltransferase (FUT2) gene, involved in the production of H-type 1 antigen in saliva and mucosa [29]. Study using murine macrophages which support the propagation of the MNV had found that terminal sialic acid moieties present on gangliosides can act as a receptor for MNV attachment [30]. However, MNV entry into permissive cells has been shown to be pH independent [31]. Relatively recently, genome-wide CRISPR screens have identified CD300lf as the receptor for MNV attachment to host cells [32]. CD300lf is a type I integral membrane protein with a single extracellular Ig-like domain. CD300lf is part of a larger family of CD300 molecules that function as cell death sensors, as they recognize phospholipids typically found on the inner leaflet of cells [33]. After a successful attachment, the norovirus particles are believed to get internalized via endocytosis mechanisms such as cholesterol- and dynamin-dependent [34, 35]. At this stage, the viral genome is released from the capsid and translocated to the endosomal membrane in order to enter the host cell cytoplasm. However, relatively little is known about detailed mechanisms of norovirus entry into cell’s cytoplasm to date.
After entry of the positive sense viral genome into the host cell cytoplasm, it can immediately act as mRNA for protein synthesis. The subsequent event of norovirus lifecycle is a pioneer round of viral proteins translation from the positive strand viral genomic RNA. The norovirus VPg (viral protein genome link) protein is a 13–15 kDa non-structural protein covalently linked to the 5’ end of the viral genomic (G RNA) and subgenomic RNA (SG RNA) and acts as a cap substitute (Figure 1). The VPg protein recruits host cells translation initiation factors in initiating the translation process to produce viral proteins [36, 37]. This mechanism is a unique strategy employed by noroviruses to ensure the preferential translation of their RNA over host cell mRNA which possess a classical 5’ cap structure. In fact, all caliciviruses use this translational strategy since their 5’ UTR is relatively short (only 5 nucleotides in MNV) compared to the closely related picornavirus genome which contains a much longer 5’ UTR. Even though the picornavirus genome also possesses a VPg at the 5’ end, this smaller protein (~22 amino acids) does not have any sequence homology with the calicivirus VPg and is not involved in picornavirus translation. Indeed, picornavirus translation is driven by the presence of an internal ribosomal entry site (IRES) structure within its 5’ UTR [38]. Translation of the first open reading frame of noroviruses typically yields a large polyprotein, representing the non-structural proteins. This large polyprotein is subjected to further processing by the virus encoded 3C-like (3CL) protease at five specific protease cleavage sites yielding six mature forms of the non-structural proteins [39, 40]. Sosnovtsev et al. have demonstrated that the proteolytic processing of MNV non-structural proteins in an in vitro system closely correlates to the products observed in infected RAW264.7 cells [25]. Uncleaved precursor proteins like NS6/NS7 (Pro-Pol) and NS1/NS2 can also be detected [25]. However, unlike the FCV protease and polymerase that functions as a fusion protein called p76 in infected cells, these proteins in noroviruses must be separated in order to be functionally active [25, 41].

The NS1/2 protein is the first non-structural protein in noroviruses (Figure 1) and is predicted to have a similar function to the picornavirus 2B protein, which is involved in membrane rearrangement and results in a modification of membrane permeability [42]. The enterovirus 2B protein which is a member of the Picornaviridae family is localized to the endoplasmic reticulum (ER) and the Golgi complex, reduces ER and Golgi complex calcium ion levels, and further inhibits protein trafficking through the Golgi complex [43]. Studies using Norwalk virus revealed that expression of the NS1/2 protein, also referred to as the N-Term protein, leads to Golgi disassembly, indicating a potential role for this protein in replication complex formation [42, 44].

The norovirus NS3 protein is a nucleotide triphosphatase (NTPase) (Figure 1). A study using a human norovirus (Southampton virus) showed that NS3 has NTPase activity that functions to hydrolyse nucleotide triphosphate [45]. In MNV infected cells, the NS3 has been shown to associate with the viral replication complex [46]. In addition, the equivalent protein in FCV called p39, was found to co-localize with viral replication complexes suggesting a possible role in replication [41, 47]. Little is known about the NS4 protein. However, it is thought that NS4 may play a role in tissue culture adaptation of MNV since repeated passage of MNV-1 in RAW264.7 cells give rise to attenuated viruses in part caused by sequence changes in NS4 [48]. Furthermore, NS4 is also thought to recruit VPg to membranous replication complexes during replication [46]. Targeted mutations in poliovirus 3A, the NS4 equivalent, resulted in viruses defective in RNA synthesis [49] indicating that by analogy, the norovirus NS4 may also contribute to viral RNA synthesis.

The NS5 encodes the viral VPg protein that plays a multifunctional role in the viral life cycle. The main role of VPg has been identified to be in translation initiation. This 13–15 kDa protein is covalently linked to the 5’ end of the G RNA and
SG RNA of caliciviruses [50]. VPg has been shown to be essential for viral RNA infectivity as treatment of viral RNA with proteinase K rendered the viral RNA non-infectious [51]. In vitro translation and infectivity of RNA are also abolished upon the removal of FCV and MNV VPg from viral RNA [50, 52]. However, in vitro transcribed capped FCV and MNV RNA generated from cDNA clone were infectious when transfected into cells [53, 54]. These observations indicate that the VPg plays a role as a cap substitute during the typical mRNA translation process. Using in vitro assays, MNV, FCV and Lordsdale virus VPg have been shown to bind the cap-binding eIF4F component, eIF4E [37, 52, 55]. Glutathione S-transferase (GST) pulldowns using Norwalk virus VPg demonstrated that other eIF4F components such as the eIF4A helicase and the scaffold protein eIF4G also associate with the translation complex [36]. Although both the FCV and MNV VPg proteins bind to eIF4E, only this interaction in FCV is essential as inhibition of eIF4E activity was found to severely affect FCV VPg linked RNA [37]. The same inhibition in MNV did not affect in vitro translation of MNV VPg linked RNA [52]. Differences were also observed in the requirement for eIF4A in vitro, where an increased requirement of MNV translation for eIF4A had been demonstrated [52]. Encoded by NS5 in the ORF 1 of the viral G RNA, the calicivirus VPg protein has also been shown to interact with the viral polymerase and capsid protein indicating a multifunctional role for this protein in the calicivirus life cycle [56].

The NS6 encodes the viral 3C-like protease and is thought to play a role in inhibition of cellular protein synthesis in infected cells. In vitro studies using recombinant norovirus 3CLpro demonstrated that it cleaved polyA binding protein (PABP) [57] and the eukaryotic initiation factor eIF4G [58], both of which are required in mRNA translation of host cells. In FCV, the protease is present only in its active state when fused to the polymerase. The calicivirus 3C-like protease is released from the ORF1 polyprotein by autocatalytic cleavage, subsequently cleaving the other proteins in ORF1 with high specificity [41].

The NS7 protein, located at the C-terminus the norovirus ORF 1, encodes the RNA-dependent RNA polymerase (RdRp), which is a key enzyme in viral replication. This protein will be elaborated further in the subsequent subsection of this chapter because it plays a major role in viral G RNA and SG RNA replication.

The ORF2 and ORF3 of noroviruses code the structural proteins VP1 and VP2 respectively. Both of these proteins are expressed from the viral VPg-linked SG RNA that is 3′ co-terminal with the G RNA (Figure 1). However, in lagoviruses, sapoviruses and neboviruses, the capsid protein may also be produced from the G RNA as the capsid genes for these viruses are in frame with ORF1 giving rise to a polyprotein that contains both the non-structural proteins and the major capsid protein [59, 60]. ORF2 of norovirus encodes the 58.9 kDa major capsid protein (VP1) and ORF3 encodes the 22.1 kDa minor capsid protein (VP2) [61]. The expression of VP1 protein with or without co-expression of VP2 allows dimer formation that can be further assembled to produce VLPs in the absence of RNA genome [62–64]. Since the HuNv is currently not efficiently propagated in tissue culture, VLPs have been used to study a variety of virus-host interactions as they are morphologically and antigenically indistinguishable from real virus particles [4]. In FCV, the capsid protein contains a leader peptide (leader capsid or LC) at its N terminus that is cleaved by p76 to give rise to the mature capsid protein VP1. The VP2 protein has been shown to stabilize and protect VLPs from proteolytic degradation when this protein is co-expressed with VP1 in the baculovirus system [65]. The very basic character of VP2 suggests an interaction with nucleic acid and it may contribute to the encapsidation of the viral RNA. However, this hypothesis has yet to be examined and confirmed. Furthermore, at least for FCV, the VP2 protein is essential for the production of infectious particles and for virus replication [66]. In addition to
the ORF2 and ORF3, there is another alternative ORF, namely ORF4, which was found in MNV, overlapping with the VP1 coding region and encoding the virulence factor 1 protein (VF1) [67]. This VF1 protein has been demonstrated to play a role in infection and virulence in vivo. Infection of STAT1<sup>−/−</sup> mice with a mutant virus lacking the ability to express ORF4 resulted in a delayed onset of clinical signs compared with WT virus infected mice. Using a reverse genetics system, VF1 has been shown to function as a classical viral accessory protein that is not required for replication in tissue culture [67].

The pioneer round of viral proteins production is proceeded with G and SG RNA replication once the viral replication-related proteins are made available in the infected cell’s cytoplasm. This particular process will be further elaborated in separate section below. When all the viral proteins become available and the replication has occurred, the viral RNA progeny is then packaged into viral particles. As mentioned earlier, the VP2 protein may contribute to this event. The mechanism of calicivirus encapsidation has yet to be studied in great detail. Present evidence suggests that the SG RNA could be encapsidated separately in the case of RHDV as well as in FCV [68, 69]. However, little is known about the mechanisms of viral release, but since norovirus infections induce apoptosis, it is speculated that apoptosis-induced membrane collapse releases the virus particles from the infected cells [70–72].

4. The norovirus genome replication

Once the translation of the norovirus non-structural proteins has begun, their presence in infected cells induces the formation of cytoplasmic membrane-bound replication complexes, enabling the viral genome replication process to take place [73]. These replication complexes, which contain the viral RdRp, viral RNA (single and double-stranded intermediates) and other viral enzymes and host cell factors, act as a surface or platform for the viral replication. The rearrangement of intracellular membranes (particularly the endoplasmic reticulum and Golgi apparatus) of MNV-1 infected RAW264.7 cells has been observed whereby membrane vesicles start to appear at twelve hours post infection [74]. The elaboration of norovirus genome replication in this chapter will be done interchangeably with the function of the central replication enzyme, RdRp.

The RdRp, also known as the RNA replicase, is an enzyme that catalyzes the synthesis of RNA from RNA templates. This particular virus enzyme is therefore distinct from the typical eukaryotic DNA-dependent RNA polymerase that catalyzes transcription of mRNA from a DNA template. All RNA viruses carry an RdRp gene in their RNA genome since this viral replication enzyme is pivotal for genome replication in infected cells. In addition, the virions of negative strand and double-stranded genome viruses must contain the RdRp as a ribonucleoprotein component since the incoming RNA genome cannot be translated or copied directly by the cellular machinery. The first viral RdRp was discovered in the early 1960’s from poliovirus (PV). The poliovirus RNA polymerase (PV3D) is one of the best-studied viral RdRps and is often used as a reference for other newly identified RdRps. Studies including structural, RNA binding, nucleoside triphosphate (NTP) binding, polymerization of nucleotides, RNA strand displacement, and interactions with other viral proteins have been thoroughly investigated for PV3D [75–78].

Most of our understanding on the properties of viral RdRps comes from in vitro studies using purified proteins. This includes the initiation of RNA synthesis that is driven by RdRps. The mechanism of RNA synthesis initiation is divergent between RdRps from different viruses. However, common mechanisms have been
determined to be the de novo and the primer-dependent initiation. The presence of an RdRp, an RNA template, the initiating NTP (NTPi) and a second NTP is required in order to achieve the de novo initiation. The initiating NTPi, sometimes known as the one-nucleotide primer provides the 3′-hydroxyl (OH) group for the addition of the next nucleotide and elongation usually follows immediately [79]. The de novo initiation normally occurs at 3′ end of viral RNA. However, internal initiation may also appear as in the case of SG RNA synthesis. For RdRps that employ a primer for primer-dependent initiation, the primer can be a protein-linked oligonucleotide (i.e; VPg-pU-pU, as in the case of picornavirus) or oligonucleotides with a 5′ end capped structure that is cleaved from the cellular mRNA in a process called ‘cap-snatching’ (as used by many segmented negative strand RNA viruses such as influenza virus) [79]. Some viral RdRps also exhibit the terminal transferase activity that confers an ability to incorporate NTPs at the 3′ end of viral RNA template. RdRps with this property can initiate RNA replication by ‘copy-back’ or ‘template-primed’ synthesis mechanism. Incorporation of NTPs at the 3′ end of RNA template forms a loop structure able to fall back onto the RNA template and eventually serve as a primer for the RdRp to carry on with elongation. Terminal transferase activity for hepatitis C virus [80], poliovirus [81] and more significantly for HuNv RdRp [82] has been reported in vitro whereby the detection of double length RNA as a predominant product compared to the template RNA used in the reaction is often observed. However, this ‘copy-back’ synthesis by RdRp could theoretically be an artifact of in vitro reactions [79].

The RdRp gene of noroviruses is located at the C-terminal of non-structural polyprotein. With an approximate size of 57.5 kDa (in MNV), this virally encoded non-structural (NS7 in MNV) protein plays a key role in norovirus G RNA and SG RNA replication. Generally, the replication of G RNA is achieved through a negative sense RNA intermediate which serves as a template for the production of nascent positive sense viral G RNA. This general mechanism also applies to the caliciviruses where the presence of negative sense G RNA as well as SG RNA has been shown by Northern blot analysis during the infection of FCV in tissue culture. Currently, four main mechanisms for the initiation of RNA synthesis by recombinant calicivirus (including norovirus) RdRps have been demonstrated in vitro. They are: a de novo initiation and primer-independent initiation [82–84], back-priming base initiation [79, 85, 86] and a protein-primed initiation via VPg nucleotidylylation [84, 87]. The biochemical features of bacterially expressed recombinant RdRp noroviruses (HuNV and MNV) have been well characterized and in vitro enzymatic activity has been described [87–90]. Out of these four established mechanisms however, the de novo initiation is the proposed model to be employed by norovirus for the synthesis of both the G RNA and SG RNA by direct interaction between viral RdRp with its’ VP1 (at the shell domain). A cell-based assay supported this proposed model through indirect measurement of 5′-triphosphorylated RNA production by the RdRp [91].

5. Production of subgenomic RNA in other viruses relative to norovirus

The genome organization and strategies for gene expression of positive strand RNA viruses are diverse. In addition to the occurrence of specific proteolytic cleavage sites which mediate the translational processing of the large polyprotein and give rise to several mature proteins encoded by one large ORF, many viruses often express their downstream ORFs through the transcription and translation of a SG RNA. Generally, SG RNAs of positive strand RNA viruses are identical to the 3′ ends of their parental G RNA. However, they vary in length where the 5′ end of
these SG RNAs are in proximity with the start codon of respective ORF. In most cases, these viral SG RNAs carry the ORFs that code for proteins required in the intermediate and late stages of infection, such as the structural proteins. Animal positive stranded RNA viruses that produce SG RNA include the Coronaviridae and Arteriviridae family of Nidovirales order, Togaviridae, Nodaviridae, Astroviridae and Caliciviridae families. However, the vast majority of plant viruses have been demonstrated to produce SG RNAs. These viruses are from the Luteoviridae, Bromoviridae, Tombusviridae and Closteroviridae families and the Tobravirus, Carlavirus, Tymovirus, Potexvirus, Hordeivirus, Tobamovirus, Sobemovirus and Furovirus genera [92]. The mechanism of SG RNA synthesis has been studied in more detail in plant viruses than in animal viruses. Therefore, most of our understanding of the mechanisms of how SG RNA synthesis is achieved comes from established models for plant viruses.

There are currently two well-characterized and one additional mechanism for positive strand RNA virus SG RNA synthesis. The first described mechanism and the most widely recognized model is internal initiation, which has been clearly demonstrated in studies involving brome mosaic virus (BMV) (Bromoviridae family). In this instance the viral RdRp initiates (+) strand SG RNA4 transcription internally at a specific promoter region on the full-length (−) strand template of G RNA3 [93]. The BMV genome is composed of three positive sense, capped RNAs. RNA1 (monocistronic) encodes protein 1a with capping and putative RNA helicase activities. RNA2 (monocistronic) encodes protein 2a, a putative RNA-dependent RNA polymerase. RNA3 (bicistronic) encodes for two proteins: 3a, which is required for cell-to-cell movement, and the capsid protein. The capsid is translated from a subgenomic RNA, RNA4 [94]. The transcription of SG RNA4 is driven by the interaction of the replicase with the promoter sequence which functions on the minus-strand RNA3 and is situated directly upstream of the SG RNA4 initiation site. Initial studies showed that at least four key nucleotides in the core promoter are recognized by the viral replicase prior to the initiation of SG RNA4 synthesis highlighting the importance of primary RNA sequences in the SG RNA promoter [95]. Subsequent studies however, showed that a short RNA hairpin in the core promoter serves as the replicase binding site and that some of the key nucleotides help to form a stable hairpin structure in this core promoter region [96, 97]. Eventually, Sivakumaran et al. [98] concluded that the key nucleotides in the core promoter as reported previously act by directing replicase recognition. Whilst the formation of stem-loop is only required at a step after the binding of replicase to this promoter region [98].

Animal viruses such as Sindbis virus (alphavirus) and Rubella virus (rubivirus) from the Togaviridae family have also been extensively studied as models for internal initiation of SG RNA synthesis [99]. The Sindbis virus genome consists of an 11.7 kb positive strand RNA which is capped at its 5’ end and is polyadenylated at the 3’ end (Figure 2) [100]. The four alphavirus non-structural proteins (nsP1234; which involve in catalysis the genome replication) are translated from the 5′ ORF, and are synthesized as a polyprotein, which are subsequently processed into individual proteins. The 3′ ORF codes for the three structural proteins; capsid (C) and envelope proteins (E123) are translated from the SG RNA. Synthesis of SG RNA is mediated via an internal promoter on the (−) strand viral RNA. The minimal sequence on the (−) strand RNA which has SG promoter activity in vivo corresponds to a region from −19 to +5 on the viral genome, using the initiation nucleotide of the SG RNA (nucleotide 7598 of the viral genome) as +1 [101]. Further studies have shown that a longer nucleotide sequence from −98 to +14 is required to obtain a more efficient SG RNA transcription [102]. On the other hand, the in vitro synthesis of SG RNA using a cell-free system proved that the internal initiation
mechanism is employed, where the critical component which is a minus-strand promoter-template corresponding to the region of the Sindbis virus genome from nucleotide 7441 to nucleotide 7772 (−157 to +175 relative to the SG RNA transcription initiation site at nucleotide 7598) [103]. Therefore, it could be concluded that sometimes, the minimal promoter requirements in vitro are generally insufficient in vivo. Additional sequences are required in vivo to allow the replicase complex to come into proximity with the core promoter. Such requirements may not be critical in highly purified in vitro systems [92].

The second mechanism for SG RNA synthesis is termed as a premature termination and occurs during the (−) strand template synthesis from the full length (+) strand G RNA. This premature termination gives rise to a subgenomic-length (−) strand RNA that then serves as a template for subsequent end-to-end (+) strand SG RNA synthesis. The generation of this smaller subgenomic-length (−) strand complementary RNA is due to the early disengagement of the RdRp when it reaches a RNA secondary structure in the (+) strand viral genome template (known as a termination signal). These RNA structures are normally comprised of either local secondary structures or long-distance RNA interactions that form a highly ordered structure. The plant virus tomato bushy stunt virus (TBSV), the prototype member of the Tombusviridae family, provides the best-studied and complex example for premature during SG RNA synthesis. This virus was first isolated from tomato plants in 1935 where it causes stunting of growth, leaf mottling, and deformed or absent fruit. The size of the (+) strand TBSV genome is 4.8 kb in length and it contains five functional ORFs [104]. The 5′-terminal ORF encodes p33 and a read through product p92. These two proteins are the only viral proteins required for viral RNA synthesis, and both are translated directly from the viral G RNA [105]. The translation of the other three proteins (p41, p22 and p19) is supported by the production of two SG RNAs. The coat protein, p41 is translated from the SG RNA1 while the p22 (cell to cell movement) and the p19 (suppression of host defense mechanism) proteins are translated from SG RNA2 via overlapping ORFs [106]. The employment of a premature termination mechanism for TBSV SG RNAs synthesis is mediated by the formation of two different sets of long-distance RNA–RNA interactions, both present in the positive strand genomic RNA. The first one involves an RNA sequence located immediately 5′ to the site of transcriptional initiation of SG RNA1 called receptor sequence (RS1) and partner segments positioned ~1000 nucleotides upstream called activator sequence (AS1) which mediate the transcription of SG RNA1 [107]. The second interaction which mediates the synthesis of SG RNA2 involves the distal element (DE) which is located ~1100 nucleotides upstream from the initiation site of SG RNA2 transcription. This DE must base pair with a
portion of the core element (CE) located just 5′ to the SG RNA2 initiation site [108]. Furthermore, another long distance interaction between AS2/RS2 has been identified and is essential (along with DE/CE) for regulating the production of SG RNA2 [104]. It is possible that the AS/RS structure could be bound by a protein factor that stabilizes them, therefore facilitating the premature termination step of RNA copying by the viral replicase. In another plant virus that utilizes the premature termination mechanism for SG RNA synthesis, a more complex RNA–RNA interaction has been demonstrated. In red clover necrotic mosaic virus (RCNMV), an AS/RS-like interaction is also essential for SG RNA transcription from RNA1. However, this interaction forms in trans between the two G RNA segments called RNA1 and RNA2 [109].

In addition to the two well-characterized SG RNA synthesis mechanisms described above, there is another more unusual mechanism employed by members of the families Coronaviridae and Arteriviridae from the Nidovirales order, known as discontinuous transcription. Viruses from these families contain a very large positive sense RNA genome (between 15 and 31 kb) and produce a nested set of seven 3′ co-terminal SG RNAs. Uniquely, all these SG RNAs contain a 90 nucleotide leader sequence derived from the 5′ end of the G RNA. These SG RNAs are synthesized from non-contiguous sequences at the 5′ and 3′ ends, respectively, of the viral (+) strand genome. The leader and body of SG RNAs are separated by a conserved sequence found in the intergenic regions (IG) that can be found at the 3′ end of the leader and at the 5′ end of the SG RNA body. Discontinuous transcription occurs during (−) strand RNA synthesis. Most of the (+) strand RNA template is not copied, perhaps because it loops out as the polymerase completes the synthesis of leader RNA. The resulting (−) strand RNAs with leader sequences at the 3′ ends, are then copied to form the various length SG RNAs.

6. The norovirus subgenomic RNA transcript and its translational products

All noroviruses produce a SG RNA during their replication cycle in infected cells. This SG RNA is 3′ co-terminal with the full-length G RNA, has VPg linked at the 5′ end and carries a poly-A tail at the 3′ end. Typically, the SG RNAs of noroviruses contains ORF2 and ORF3 (and ORF4 in the case of MNV and some sapoviruses) which code for viral structural proteins (VP1 and VP2). The production of a SG RNA message may act to delay the production of structural proteins until the initial rounds of viral replication have taken place. Both positive and negative sense SG RNA intermediates (~2.5 kb in length) can be detected by northern blot analysis of purified FCV replication complexes [73].

Following the transcription of MNV SG RNA, the expression of this messenger transcript via VPg-dependent translation initiation is achieved as described for the G RNA. The 5′ proximal ORF2, which encodes the major capsid protein is first translated when the scanning ribosomal complex encounters the first AUG codon, a typical strategy for translation. However, in viruses with polycistronic SG RNAs, the translation of their 3′ terminal ORF is not as efficient as the preceding ORF. Therefore, many viruses employ several strategies to provide sufficient access for ribosomes to downstream ORFs. These strategies include leaky scanning of 40S subunits past the start codon of the first ORF, the possession of intercistronic internal ribosome entry signal, programmed ribosomal frame-shifting during elongation and stop codon suppression at the termination step [110]. All noroviruses SG RNA are bicistronic messages. The translation of the 3′ proximal ORF in this case is achieved by a unique mechanism called termination reinitiation. In this
mechanism, a proportion of the 40S ribosomal subunit remains associated with the mRNA following the translational termination at the preceding stop codon. This enables reinitiation at the AUG of a downstream ORF, which is in close proximity. This characteristic has been observed for different caliciviruses where the initiation codon of VP2 (overlapped with VP1) is only 2 nucleotides away from the stop codon of VP1 for RHDV. Meanwhile for Norwalk virus, FCV and MNV, the start codon of VP2 is overlapped with the stop codon of VP1 [111]. Other than the close proximity between the stop and start codon, the efficiency of termination-reinitiation translation is also determined by a stretch of 70 to 80 nucleotides upstream of the stop-start window which facilitates the transit of the ribosome through the stop codon of VP1. This region of conserved sequence is termed TURBS (termination upstream ribosome binding site motif). The translation of VP2 from the FCV, RHDV and MNV SG RNA is dependent on this TURBS region, which is located immediately upstream of the VP1 stop codon [110–112]. The TURBS contain two important sequences; the 5’ sequence (termed as Motif 1) is proposed to function in binding the 18S rRNA (through complementary sequence) whilst the other sequence is thought to be important in tethering the ribosome to enable translation of VP2 at the correct site [110, 113]. Alternatively, the TURBS may also act by interacting with eIF3 or eIF3/40S ribosome complexes preventing disassembly of the ribosome following VP1 translation termination. This alternative mechanism is supported by the fact that purified eIF3 is able to stimulate translational re-initiation [114].

7. The replication of norovirus subgenomic RNA

The presence of SG RNA of norovirus in infected cells is often used as indicator for active viral genome replication. Importantly, the mechanism that is used by noroviruses to achieve their SG RNA transcription is poorly understood until very recently. Initial evidence from in vitro studies using RHDV RNA transcripts suggests that the internal initiation mechanism is employed [115]. In vitro promoter mapping analysis using a panel of nested negative sense RNA templates that included the region before the start of ORF2, demonstrated that the RHDV RdRp requires 60 bases upstream of the start of the SG RNA transcription start site in order to produce SG RNA [115]. At this point, this finding indicates the existence of a promoter site upstream of the SG RNA start site that enables the binding of RdRp and internally initiates the SG RNA synthesis on the negative strand G RNA. Subsequently, in another study involving MNV, mutational analysis of an evolutionarily conserved RNA stem loop structures using the available reverse genetics system has highlighted that the stability of a specific RNA structure is critical for MNV replication [116]. This specific RNA stem loop structure was consistently detected exactly 6 nucleotides upstream of the SG RNA start site in all caliciviruses on both the positive (SL5018) and negative strand RNA (SLa5045) (Figure 3) [116]. This observation implies that these RNA secondary structures may accommodate a functional role in viral SG RNA synthesis. Furthermore, the stem loop structure was generally found to be more stable on the negative strand genome (SLa5045) than the positive strand. Thus, such a structure was initially hypothesized to play a role as the putative SG RNA promoter for the synthesis of MNV SG RNA via internal initiation mechanism. It is also possible that after internal initiation has occurred to produce newly synthesized VPg-linked SG RNA, this RNA may then be picked up by the viral replication machinery and replicated in a similar manner to the G RNA, effectively producing a negative strand SG RNA molecule.

As reported by Simmonds et al. [116], a mutant cDNA clone containing a series of non-coding mutations called m53 that destabilized the RNA structure was
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These mutations were designed to destabilize the stem loop structure by weakening the base pairing without affecting the NS7 coding sequence. This mutated cDNA clone was used in the DNA-based reverse genetics system and reported to cause a lethal phenotype effect, whereby no infectious virus can be detected by TCID50 in the recoveries. However, by compensating the initial m53 mutations to restore the base pairing within the stem loop structure (called m53r), a viable virus was recovered with a titer close to that of the wild type virus. This series of experiments concluded that RNA stem loop structure is important for viral replication and might function as part of the SG RNA promoter. Even though the m53 mutation disrupting SLa5045 caused a lethal phenotype, serial “blind” passage of the recoveries (from the DNA based reverse genetics system) in RAW264.7 cells often produced viable viruses. Sequence analysis revealed that these viable viruses contained two types or classes of mutation. The first class were phenotypic-revertant viruses where nucleotide changes were identified that resulted in partial reformation of the SLa5045 stem loop structure. The ability to isolate phenotypic revertant viruses that repaired the defective RNA structure was not unexpected as the stem loop structure is predicted to play an important role in viral replication. This observation indicates that m53 mutation in the viral genome results in poor viral genome replication in tissue culture. Phenotypic-revertant mutations arise in tissue culture and those that promote replication are favored and amplified during the serial “blind” passage until they become dominant. Another type of mutation observed were suppressor mutations, whereby the m53 mutation in the SLa5045 was still present, but changes outside the stem loop structure, within the NS7 coding region, were also identified [117]. Further characterization of this suppressor mutant viruses in cell culture revealed that they possess a slower growth kinetics, lower-level proteins production and lower-level of G RNA and SG RNA transcripts synthesis compared to WT virus [117]. More importantly, these data indicate that nucleotide changes were responsible for the suppression phenotype rather than any amino acid change, suggesting the potential involvement of long-range RNA–RNA interactions between SLa5045 and a region ~100 nucleotides upstream of this Sla5045 stem loop structure [117]. However, this hypothesis is yet to be proven with

Figure 3. Conserved RNA secondary structures upstream of the subgenomic transcript predicted by Alifold programme for the 5 calicivirus genera. The stable small secondary stem loop structure was consistently found 6 nucleotides upstream of the MNV SG RNA initiation site. The stem loop is shown in its antisense orientation (SLa5045). Gray filled boxes represents the SG RNA start site and black boxes represents the ORF2 initiation codon. The unpaired 6 nucleotides sequences between the predicted structure and the subgenomic start site are underlined (figure is taken from Simmonds et al. [116]).
scientific experimental and the current readily available bioinformatics tools are not adequate to accurately predict such long range RNA–RNA interaction. On the other note, such long range RNA–RNA interactions between promoter regions are well established with some sequences being up to ~1500 nucleotides apart e.g. the nodavirus Flock House virus (FHV) and tombusvirus tomato bushy stunt virus (TBSV) have been documented to contain such interactions even though these viruses employ a premature termination mechanism for their SG RNA synthesis [118, 119]. In the case of MNV however, it is worth to note that this long-range RNA–RNA interaction presumably occurs on the negative strand RNA to produce a suppression effect on m53 mutation of the SLa5045.

Utilizing the MNV reverse genetics system, virus recoveries using series of modified cDNA with additional copy of SLa5045 in cis within the noncoding region upstream the capsid gene (SLa5045Dup) showed that only m53r mutation and two nucleotide changes at the terminal loop in the second copy stem loop of m53 backbone construct (m53/m53r and m53TL-Dis) produced detectable viable virus [117]. The other construct namely SLa5045Dup m53/SLa WT + 8, whereby the nucleotide spacing of the stem loop and initiation site of the SG RNA was increased from 6 to 8 nucleotides, failed to produce any viable virus. In addition, total sequence modification that retained or totally disrupt the stem structure in the additional SLa5045 also caused a debilitating effect to the virus [117]. These constructs were designed with the aim to introduce a more synonymous mutations since the second copy of SLa5045 is located at the noncoding region compared to the first stem loop which positioned at the NS7 coding region. Any extensive mutations introduced in the first copy of the structure would affect the NS7 coding capacity. These set of data demonstrate that the sequence, exact location in the norovirus genome and stability of SLa5045 are mandatory for virus replication [117].

Even though the presence of low levels of negative sense SG RNA have been argued for the premature termination of negative sense G RNA during elongation by RdRp that produces SG-length negative sense RNA transcript (act as template for positive sense SG RNA) [73, 120], a more detailed study suggests that norovirus SG RNA replication follows the internal initiation mechanism. Employing genetics and biochemical tools, a recent study demonstrates that accurate norovirus SG RNA synthesis is depend on a sequence and genotype-specific interaction of the viral RdRp with a stem-loop sequence (SLa5045) on the minus-strand RNA [117]. In that study, the investigators performed an in vitro RNA synthesis assay involving series of chemically synthesized RNA templates containing the SLa5045 sequence (from MNV and human GII.4 norovirus) that called proscripts and recombinant MNV RdRp. The outcomes of that specific experiment indicate that the norovirus RdRp is capable of recognizing the stem loop sequence and subsequently direct the RNA synthesis. Therefore, the role of stem loop structure as core promoter for norovirus SG RNA synthesis has been established. However, whether there was any direct interaction between the RNA stem loop with RdRp remained unclear until a more detailed biochemical study came out later in 2015. Using a reversible crosslinking peptide fingerprinting analysis (RCAP) in one of the mapping studies, the investigators identified that 17 peptides originating from MNV RdRp were associated with RNA proscripts that contained the noroviruses SG RNA core promoter sequences (from MNV and HuNv GII.4) [121]. Based on the MNV-1 crystal structure, most of these cross-linked peptides are precisely located in the central cavity of the enzyme which is critical for RNA synthesis [121]. A more detailed mutational and functional analysis also revealed that residues R411 (arginine at position 411) and R416 (arginine at position 416) of amino acid sequence in MNV RdRp contributed to the binding towards subgenomic promoter hairpin [121]. These series of studies concluded that the noroviruses are highly likely employing an internal initiation mechanism for their SG RNA synthesis.
8. Conclusion

The synthesis of norovirus SG RNA is a clear signal for the existence of genome replication since the production of this smaller RNA (that is 3′ co-terminal with the full length viral genome) is dependent on efficient genome replication in infected cells. Furthermore, the transcription of SG RNA at the middle and latter stages of infection is also thought to regulate the production of infectious virions. Since the capsid proteins of noroviruses are translated from the SG RNA messenger, the encapsidation process is initiated once the viral RNA replication begins. Investigations on the involvement of functional RNA elements in regulating the synthesis of the MNV SG RNA were carried out extensively to determine the mechanism employed by noroviruses in their genome replication accurately. Based on the established data available recently, now clear that we could confidently presume that norovirus follows the internal initiation mechanism for the synthesis of SG RNA. The studies also proved the crucial role of small stem loop/hairpin structure within the coding region of NS7 in the viral replication.

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Conflict of interest

The author declares no conflict of interest.

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