Introduction

The prototype norovirus, Norwalk virus, was first described in 1972 as the aetiological agent responsible for an outbreak of acute gastroenteritis in an elementary school in Norwalk, OH, USA (Kapikian, 2000). Noroviruses are now accepted as a leading cause of gastroenteritis in developed and developing countries (Glass et al., 2009; Hall et al., 2013). Spread primarily via the faecal–oral route, norovirus infections are typically an acute self-limiting gastrointestinal infection. Norovirus gastroenteritis has recently been identified as a significant cause of morbidity and mortality in the immunocompromised, and can result in long-term persistent disease (reviewed by Bok & Green, 2012). Norovirus infection has also been associated with a number of more significant clinical outcomes: necrotizing enterocolitis (Turcios-Ruiz et al., 2008), seizures in infants (Medici et al., 2010), encephalopathy (Ito et al., 2006), pneumatosis intestinalis (Chan et al., 2010; Kim et al., 2011) and disseminated intravascular coagulation (CDC, 2002), to name but a few. In developing countries, an estimated 200 000 deaths in children <5 years of age are thought to be due to norovirus infections (Patel et al., 2008) and they have recently been reported as the second leading cause of gastroenteritis-related deaths in the USA, typically resulting in 797 deaths per annum (Hall et al., 2013). Despite their significant impact, noroviruses remain one of the most poorly characterized groups of RNA viruses, due largely to the fact that, despite numerous attempts (Duizer et al., 2004; Papafragkou et al., 2013; Takanashi et al., 2013), human noroviruses (HuNVs) have yet to be cultured efficiently in cell culture.

Noroviruses are members of the family Caliciviridae of small, positive-sense RNA viruses, which is divided currently into five genera: Vesivirus, Lagovirus, Nebovirus, Sapovirus and Norovirus. Members of the genera Norovirus and Sapovirus are able to infect humans and cause gastroenteritis. The genus Norovirus is subdivided into at least five genogroups (GI–V). Genogroups GI, GII and GIV infect humans and cause acute gastroenteritis, but noroviruses have also been isolated from numerous other species including pigs (GII), cattle and sheep (GIII), and mice (GV). More recently, a novel norovirus identified in domestic dogs with diarrhoea has been proposed to represent a new genogroup, GVI (Mesquita et al., 2010). To date, zoonotic norovirus infections have not been reported, but there is clear evidence of the potential for transmission. For example, HuNVs can infect gnotobiotic piglets (Cheetham et al., 2006) and there is serological evidence of HuNV in pigs (Farkas et al., 2005). Whilst antibodies to GVI noroviruses have been identified in veterinarians, it has yet to be determined whether infection results in clinical disease (Mesquita et al., 2013). Additional studies are required to further elucidate the zoonotic potential of noroviruses and whether animals represent a reservoir from which new strains may emerge.

Norovirus genome organization

The norovirus genome is a compact, positive-sense ssRNA molecule, ranging in size from 7.3 to 7.5 kb across the genus (Fig. 1). The 5' end of the genomic RNA is covalently attached to a virus-encoded protein known as VPg, whilst the 3' end is polyadenylated. The UTRs at either end of norovirus genomes are typically short, e.g. the 5' and 3' UTRs of murine norovirus (MVN) are 5 and 78 nt, respectively, and the 3' UTR of HuNV is typically 48 nt (Gutiérrez-Escolano et al., 2000a; Karst et al., 2003;
Pletneva et al., 2001). The UTRs contain evolutionarily conserved RNA secondary structures that extend into the coding regions and can be found throughout the genome (Simmonds et al., 2008). These structures are important for viral replication, translation and pathogenesis (Bailey et al., 2010b; McFadden et al., 2011; Simmonds et al., 2008).

The norovirus genome is organized into three conserved ORFs (Fig. 1a), with the exception of MNV, which has a fourth alternative ORF (Fig. 1b). The fourth ORF is unique to the MNV cluster in GV and has not yet been identified in any other norovirus; the only member of the family Caliciviridae known to have an equivalent fourth ORF is human sapovirus (Clarke & Lambden, 2000; McFadden et al., 2011). For all noroviruses, ORF1 is translated as a large polyprotein, which is co- and post-translationally cleaved by the virus-encoded protease NS6 to produce the NS proteins. ORF2 and ORF3 are translated from a subgenomic RNA. 2C-L, 2C-like; 3A-L, 3A-like. (b) MNV shares a similar genome organization, but has an additional alternative fourth ORF. ORF4 overlaps with ORF2 and is also translated primarily from the subgenomic RNA into the virulence factor 1 (VF1) protein.

**Model systems for the study of norovirus gene expression and replication**

As highlighted above, the inability of HuNV to be cultured in vitro has hampered the characterization of the viral life cycle. Prior to the discovery of MNV in 2003, numerous other members of the family Caliciviridae were used widely as model systems with which to study norovirus biology (reviewed by Vashist et al., 2009). Feline calicivirus (FCV), a member of the genus Vesivirus, was the first calicivirus for which a cell culture and reverse-genetics system was available (Sosnovtsev & Green, 1995), and has been used widely to study various aspects of the calicivirus life cycle. More recently, however, studies have indicated that HuNV RNA isolated from faecal samples is infectious in cell culture, as transfection into cells results in RNA replication, viral protein production and release of virions into the cell supernatant (Guix et al., 2007). Importantly, this experimental system does not allow multicycle replication as the virus generated is unable to reinfect the neighbouring cells.

A number of significant steps in the understanding of norovirus gene expression and replication have also been made using MNV, a GV norovirus initially identified as a lethal infection in immunocompromised mice (Karst et al., 2003). MNV replicates efficiently in primary or immortalized murine dendritic and macrophage cells (Wobus et al., 2004), and has a number of reverse-genetics systems available (Chaudhry et al., 2007; Ward et al., 2007; Yunus et al., 2010). The development of a Norwalk virus replicon system (HuNV), where cells stably maintain Norwalk virus RNA containing an antibiotic selection marker in the capsid-coding region (Chang et al., 2006), added...
Table 1. Nomenclature for HuNV and MNV proteins and their functions

<table>
<thead>
<tr>
<th>MNV</th>
<th>HuNV*</th>
<th>Function</th>
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<tbody>
<tr>
<td>NS1/2</td>
<td>p48 (N-term)</td>
<td>Replication complex formation†, contributes to persistence in MNV infections</td>
</tr>
<tr>
<td>NS3</td>
<td>NTPase (2C-like)</td>
<td>RNA helicase†/NTPase</td>
</tr>
<tr>
<td>NS4</td>
<td>p22 (3A-like)</td>
<td>Replication complex formation†</td>
</tr>
<tr>
<td>NS5</td>
<td>VPg</td>
<td>Genome-linked protein involved in translation and replication</td>
</tr>
<tr>
<td>NS6</td>
<td>Pro (3C-like)</td>
<td>Protease</td>
</tr>
<tr>
<td>NS7</td>
<td>Pol/3Dpol</td>
<td>RdRp</td>
</tr>
<tr>
<td>VP1</td>
<td>VP1</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>VP2</td>
<td>VP2</td>
<td>Minor capsid protein</td>
</tr>
<tr>
<td>VF1</td>
<td>No equivalent</td>
<td>Virulence factor</td>
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*Alternative names for the HuNV proteins are given in parentheses, and originate through comparison of the norovirus proteins and genome organization with those of poliovirus.
†These functions have been proposed, but have yet to be demonstrated fully.

Norovirus binding and entry mechanisms

The interaction of noroviruses with the cell surface is known to involve carbohydrate structures, which in the case of HuNVs include the histo blood group antigens (HBGAs). A detailed description of the interaction of HuNVs with the cell surface and HBGAs, as well as the role this interaction plays in norovirus entry and susceptibility, has been provided in detail recently (Donaldson et al., 2008; reviewed in Donaldson et al., 2010). MNV, currently the only norovirus that replicates efficiently in cell culture, binds host cells via sialic acid moieties, glycolipids and glycopolymers in a strain-dependent manner (Taube et al., 2009, 2012). The mechanism by which MNV enters cells has yet to be elucidated fully, but it is dependent on dynamin and cholesterol (Gerondopoulos et al., 2010; Perry & Wobus, 2010). As mentioned above, receptor binding and/or the entry process appear to be at least one of the limiting factors for the culture of HuNV in immortalized cells as viral RNA transfected into cells is able to undergo limited replication. Additional work in this area may therefore provide a mechanism with which to further understand the block to HuNV replication in cell culture.

Viral protein translation

Once a norovirus VPg-linked RNA genome is released into the cytoplasm of a permissive cell, it behaves as an mRNA template for the ‘pioneer round’ of viral RNA translation (Fig. 2). For this to occur, the viral RNA is recognized by cellular translation initiation factors and is translated into protein by the cellular translational apparatus. RNA viruses typically utilize novel mechanisms for viral genome translation, not only for the initiation of translation, but also to increase the coding capacity of their relatively short genomes (Firth & Brierley, 2012) and this is also true also for noroviruses. Translation of all calicivirus genomes occurs by a novel mechanism involving VPg that is not found in many other animal RNA viruses (Herbert et al., 1997). However, recent data would indicate that a similar mechanism may be employed by members of the family Astroviridae, for which the linkage of VPg to viral RNA is essential for RNA infectivity (Fuentes et al., 2012), as also described for noroviruses (Chaudhry et al., 2006).

Some aspects of the mechanism of calicivirus RNA genome translation are similar to that used by the plant viruses in the family Potyviridae (Lénard et al., 2006). VPg attached covalently to the 5′ end of the genome mediates translation of viral RNA, acting as a cap substitute, and recruits host cell translation initiation factors (Fig. 3). The structure of the MNV VPg protein has recently been described as containing a compact helical core, flanked by more flexible intrinsically disordered N- and C-terminal regions (Leen et al., 2013). This high degree of flexibility is likely to be due to the numerous roles that VPg plays in the norovirus life cycle (Goodfellow, 2011). The VPg proteins of HuNV and MNV have both been shown to interact with components of the eIF4F translation initiation factor complex, in particular eIF4E (Chaudhry et al., 2006; Goodfellow et al., 2005), the cap-binding protein, and eIF3 (Daughenbaugh et al., 2003), which is recruited to the complex and in turn helps to recruit the 43S ribosomal pre-initiation complex (Fig. 3). Functional studies have to date only been possible with MNV as it is not possible to obtain a sufficient quantity of authentic VPg-linked HuNV RNA due to the inability to culture HuNV in immortalized cells. These studies confirmed that translation of MNV RNA requires the eukaryotic initiation factor eIF4A, the
RNA helicase component of eIF4F, potentially for unwinding RNA structures present in the 5’ end of the genome, as both dominant-negative forms of eIF4A and small-molecule inhibitors of eIF4A inhibited MNV RNA translation in vitro (Chaudhry et al., 2006). To date only direct interactions with the norovirus VPG have been demonstrated for eIF3 and eIF4E, but functions for these interactions have not been described. Despite the direct interaction, in vitro studies have indicated that eIF4E is not required for the translation of MNV RNA in a rabbit reticulocyte lysate (Chaudhry et al., 2006). Therefore, it is likely that VPG forms multiple contacts with components of the translation initiation factor complex, not all of which are essential for viral RNA translation (Fig. 3). A direct, high-affinity, functional interaction between the norovirus VPG and a component of the translation apparatus has yet to be described.

The extremities of calicivirus genomes contain evolutionarily conserved RNA structures that are known to interact with host cell factors to promote viral replication and translation (Simmonds et al., 2008; Vashist et al., 2012). For example, structures in the Norwalk virus 5’ and 3’ ends are thought to interact with the cellular proteins La, polypyrimidine tract-binding protein (PTB) and poly(A)-binding protein (PABP), and these proteins have also been identified as binding RNA structures in the MNV genome (Gutiérrez-Escolano et al., 2000b, 2003; Vashist et al., 2012). The function of these interactions has yet to be elucidated fully, but studies with MNV have confirmed that they play a role in virus replication, as is well established for other RNA viruses (Bailey et al., 2010b; Simmonds et al., 2008). Many of the host proteins found to interact with norovirus RNA structures have previously been identified as regulators of the translation of other RNA viruses, e.g. La and PTB stimulate picornavirus internal ribosome entry site (IRES)-mediated translation (reviewed by Fitzgerald & Semler, 2009). Therefore, binding of these and other factors may serve to enhance viral protein translation, perhaps through stabilization of long-range RNA interactions that promote the circularization of the norovirus genome, as a process of RNA circularization is known to stimulate host cell mRNA translation (Wells et al., 1998). In both Norwalk virus and MNV, the interaction of complementary sequences in the 5’ and 3’ extremities of the viral genome is stimulated by cellular proteins (López-Manriquez et al., 2013; Sandoval-Jaime & Gutiérrez-Escolano, 2009). Two cellular proteins in particular, the poly(C)-binding protein PCBP2 and the heterogeneous nuclear ribonucleoprotein hnRNPA1, are involved in the circularization of the MNV genome (López-Manriquez et al., 2013). A number of host factors have also been identified as binding to the extremities of the MNV genome using a proteomics-based approach and summary tables of all these interactions can be found in Vashist et al. (2012). As many of these factors form direct protein–protein interactions with each other, it is possible that numerous cellular RNA-binding proteins contribute to the circularization of the viral genome during translation and/or replication. In addition to PCBP2 and hnRNPA1, RNA interference (RNAi)-mediated reduction of three of the proteins identified in the proteomics screen, i.e. PTB, DDX3 and La, significantly reduced MNV replication in cell culture (Vashist et al., 2012). Importantly, the role of the cellular RNA-binding proteins in norovirus translation is unknown as currently no experimental system exists that allows viral RNA translation in cells to be decoupled from viral RNA replication; therefore, effects on either process have a negative impact on viral replication. It is possible that these types of RNA–protein interactions play both positive and negative regulatory roles in the life cycle of noroviruses as data from a related calicivirus indicated that binding of PTB to the viral 5’ end impacts negatively virus translation and may contribute to the switch from RNA translation to replication (Karakasiliotis et al., 2010).

Translation of the viral proteins VP1 and VP2 (and VF1 in MNV) occurs primarily from the subgenomic RNA (Fig. 1). This is most likely a strategy to produce higher levels of the major capsid protein for virus assembly as the subgenomic RNA is present at higher levels in infected cells than the viral genomic RNA and each icosahedral capsid contains 180 copies of VP1 arranged in 90 dimers (Prasad et al., 1994). Translation of VP2 occurs by a termination–reinitiation mechanism as the norovirus subgenomic RNA is polycistronic (Naphine et al., 2009). Upon termination of ORF2 translation (VP1), ribosomes are thought to remain associated with the RNA to reinitiate at the start of ORF3 (VP2). This is facilitated by overlapping stop and start codons of ORF2 and ORF3, respectively (e.g. UAAUG in MNV). An upstream RNA motif known as the termination upstream ribosomal-binding sequence (TURBS) is also required, which is in part complementary to 18S rRNA and is thought to tether the ribosome through direct RNA–RNA base pair interactions (Meyers, 2007; Naphine et al., 2009). There is also evidence from transfection of bovine norovirus RNA that translation of VP1 occurs by a similar termination–reinitiation between ORF1 and ORF2 on the genomic RNA (McCormick et al., 2008). This was proposed as a unique translation mechanism for GI3 viruses and although it is feasible that it could contribute to VP1 translation in other genogroups, the mechanism has yet to be demonstrated within the context of viral infection for any norovirus. The start codon for translation of ORF4 in MNV is positioned 13 bases downstream of the start of ORF2 and may be initiated by leaky scanning and a −2 slip in the ribosome (McFadden et al., 2011).

**Replication complex formation**

Translation of the ORF1 polyprotein is followed by co- and post-translational processing by the viral NS6 protease, and results in the release of the viral NS proteins ready for replication complex formation and their precursors, some
Fig. 2. Outline of the norovirus life cycle. (1) HuNV and MNV are thought to attach to the cell surface using various carbohydrate attachment factors. This is not sufficient to mediate entry and binding to an unidentified protein receptor is thought to be required (2). Entry (3) and uncoating (4) proceed through as-yet-undefined pathways. (5) The incoming viral genome is translated, through interactions with VPg at the 5’ end of the genome (red triangle) and the cellular translation machinery. (6) The ORF1 polyprotein is co- and post-translationally cleaved by the viral protease NS6. (7) The replication complex is formed by recruitment of cellular membranes to the perinuclear region of the cell (not shown), through interactions in part with NS1/2 and NS4. (8) Genome replication occurs via a negative-strand intermediate, and genomic and subgenomic RNA are generated by the viral RdRp (NS7), using both de novo and VPg-dependent mechanisms of RNA synthesis. (9) The replicated genomes are translated (within the replication complex) or packaged into the capsid, VP1, for virion assembly and exit (10).
of which are thought to be functionally active in replication (Belliot et al., 2005). The structure and function of the HuNV protease (Pro, Fig. 1) have been characterized extensively, and the active site is thought to be a catalytic triad of H30, E54 and C139, although E54 is thought largely to determine substrate specificity rather than being essential for catalysis (Someya et al., 2002, 2008; Zeitler et al., 2006). HuNV Pro and MNV NS6 share >60% amino acid identity (Leen et al., 2012), and sequence alignments showed that H30 and C139 are conserved, whereas position 54 is aspartic acid rather than glutamic acid in MNV NS6, although this implies it has a similar function. Taken together with structural and mutagenic studies it is therefore thought that the catalytic triad and mechanism of catalysis may be conserved between HuNV 3CL<sup>Pro</sup> and MNV NS6 (Leen et al., 2012). The structure of MNV NS6 has recently been determined to high resolution and was adventitiously solved with the C terminus of an adjacent NS6 molecule bound in the active site as a peptide substrate, which facilitated identification of the key residues involved in peptide binding and specificity (Leen et al., 2012).

Similar to other positive-strand RNA viruses, norovirus replication occurs in close association with host-derived membrane complexes in the cytoplasm (Belov & van Kuppeveld, 2012; Wobus et al., 2004) (Fig. 2). Evidence for the replication complex comes from studies with MNV, as to date the mechanism of replication complex formation has not been investigated in cells replicating HuNV RNA. MNV infection induces the formation of membranous vesicle clusters in infected cells, and proliferation of the viral RNA polymerase (NS7) and dsRNA, a viral RNA replication intermediate, occurs at punctate foci in the perinuclear region (Hyde et al., 2009; Wobus et al., 2004). The NS proteins, as well as the major and minor capsid proteins co-localize with NS7 and dsRNA at this location, forming the replication complex (Hyde & Mackenzie, 2010; Thorne et al., 2012). More recently the MNV replication complex was shown to be juxtaposed with the microtubule organizing centre (MTOC) within the perinuclear region, suggesting that MNV may use the cytoskeletal network early in infection to position the complex (Hyde et al., 2012). This process is mediated potentially by an interaction between VP1 and acetylated tubulin, although a direct interaction in infected cells has yet to be demonstrated.

The host membranes that comprise the MNV replication complex are derived from components of the secretory pathway, notably the endoplasmic reticulum (ER), trans-Golgi network and endosomes, which together serve as a platform for replication (Hyde et al., 2009). The mechanisms by which noroviruses recruit cellular membranes to establish the replication complex in the host cell remain unclear. For MNV, NS1/2 and NS4 have been implicated in driving complex formation by recruiting cellular membranes, based on their localization to components of the endocytic and secretory pathways (Bailey et al., 2010a; Hyde & Mackenzie, 2010). Furthermore, the MNV NS4 protein induces Golgi disassembly and mildly inhibits protein secretion, demonstrating that it is capable of driving the rearrangement of cellular membranes (Sharp et al., 2012). The HuNV NS4 equivalent, p22, inhibits cellular protein secretion and has a more potent inhibitory effect on this pathway than MNV NS4 (Sharp et al., 2010, 2012). HuNV p22 contains an ER export signal that is thought to promote its uptake into COP II vesicles involved in transport from the ER to the Golgi apparatus, although a direct interaction between p22 and COP II has not yet been demonstrated. Nevertheless, uptake of p22 has been proposed to result in mislocalization of COP II vesicles, thereby inhibiting proper trafficking to the Golgi, and in turn leading to Golgi disassembly and loss of protein secretion (Sharp et al., 2010). MNV NS4 does not contain the same ER export signal, and the possible involvement of
COPI and COPII vesicles in MNV replication complex formation has been excluded (Hyde et al., 2009), suggesting NS4 may act by an alternative mechanism. The HuNV p48 protein (equivalent to the MNV NS1/2 protein) promotes Golgi disassembly, and disrupts expression and trafficking of cell surface proteins in transfected cells (Ettayebi & Hardy, 2003; Fernandez-Vega et al., 2004). Disruption by p48 is thought to be mediated through a direct interaction with VAP-A (SNARE regulator vesicle-associated membrane protein-associated protein A), which is involved in regulating cellular vesicle transport (Ettayebi & Hardy, 2003). Studies on HuNV proteins have focused largely on the disruption to protein secretion; however, it is plausible that the observed membrane rearrangements either contribute to or are a direct consequence of the recruitment of cellular membranes for the replication complex.

**Genome replication**

As for all positive-sense RNA viruses, genome replication occurs via a negative-sense RNA intermediate and is performed by the viral RdRp. The norovirus RdRps, generally referred to as NS7, contain active-site residues conserved among other positive-strand RNA virus RdRps, and are conserved structurally and functionally (Högboom et al., 2009). The replicative properties of the MNV RdRp (NS7) are comparable *in vitro* with those of the HuNV RdRp, supporting the use of MNV for studies of replication (Bull et al., 2011). Subsequent to the ‘pioneer round’ of translation of the incoming parental viral RNA genome, the mRNA template then functions as a template for the formation of a double-stranded replicative form (RF). The process of initiation of negative-sense RNA synthesis on the incoming parental viral RNA is not understood fully, but the norovirus RdRp has two mechanisms of initiation that have been demonstrated both *in vitro* and in cells: *de novo* and VPg-dependent (Rohayem et al., 2006). Unlike for picornavirus replication where the negative-sense RNA is VPg-linked (Pettersson et al., 1978), there is no evidence as yet to suggest whether or not norovirus negative-sense RNA in the double-stranded RF is linked to VPg. However, a recently proposed model has suggested that *de novo* initiation is used for the synthesis of negative-sense genomic and subgenomic RNAs (Subba-Reddy et al., 2012), both of which can be detected in calicivirus-infected cells (Green et al., 2002). The *de novo* initiation of norovirus RdRps is enhanced through direct interactions with the shell domain of VP1, in a species-specific and concentration-dependent manner (Subba-Reddy et al., 2012). This observation was made using cell-based assays that rely on the co-expression of norovirus proteins, including the NS7 RdRp in cells. *De novo* RNA synthesis is then measured indirectly via the RIG-I-mediated sensing of 5′-triphosphorylated RNA produced by the RdRp using host cell RNAs as a template, which subsequently leads to the activation of the IFN-β promoter linked to luciferase (Subba-Reddy et al., 2011).

This assay demonstrated that specific loop sequences in the shell domain of VP1 interact with the RdRp to specifically stimulate *de novo* initiation, without affecting VPg-dependent initiation. These observations have resulted in a model for the initial rounds of viral RNA synthesis, whereby the shell domain from the incoming parental viral capsid, or from the early rounds of viral RNA translation, binds to the RdRp and stimulates negative-sense RNA synthesis. The model would propose that as the levels of VP1 increase due to the production of viral positive-sense RNA, VP1 forms multimeric complexes, preventing the interaction with the RdRp and leading to capsid assembly and the formation of new infectious viral particles. This model would also explain the observation that bovine noroviruses have a termination–reinitiation sequence (TURBS) upstream of VP1, similar to that used to produce VP2 (McCormick et al., 2008). The effect of this strategy is that a small percentage of ribosomes translating ORF1 will reinitiate on ORF2 to produce VP1, leading to low levels of VP1 during the ‘pioneer’ rounds of viral translation. Whether other noroviruses utilize a similar mechanism for the synthesis of VP1 during the early stages of the viral life cycle is unknown currently. Species-specific interactions with NS1/2 also promote norovirus RdRp activity in this assay, whereas interactions with VP2 are inhibitory (Subba-Reddy et al., 2011). Taken together, this suggests that the viral proteins may play alternative regulatory roles, perhaps at different stages in the norovirus life cycle. Recent work has highlighted that the HuNV RdRp may be regulated by phosphorylation by the cellular survival kinase Akt (Eden et al., 2011). Akt phosphorylates HuNV NS7 RdRp on residue T33, which is conserved among pandemic HuNV GIL4 strains, but absent in non-pandemic strains. However, the implications of Akt regulation of RdRp activity on infection have not been investigated fully.

Following the generation of a double-stranded RF, the synthesis of positive-sense genomic and subgenomic RNA occurs (Fig. 2). It is accepted generally that this process occurs in a VPg-dependent manner as positive-sense infectious norovirus RNA isolated from MNV-infected cells is linked to VPg (Chaudhry et al., 2006). For this process to occur, the NS7 RdRp uses VPg as a protein primer for RNA synthesis, initiating at the 3′ end of the negative-sense RNA. This mechanism is also employed by other caliciviruses and members of the family *Picornaviridae* of positive-sense RNA viruses (Liu et al., 2009; Rohayem et al., 2006). In the case of noroviruses, the NS7 RdRp initiates RNA synthesis by covalently attaching VPg via a phosphodiester bond to the initiating nucleotide, which is invariably guanine in all members of the family *Caliciviridae*. This process is referred to as VPg nucleotidylation or guanylation. The linkage is formed between the guanine at the 5′ end of the genome and a conserved tyrosine residue in VPg (Y26 in MNV and Y27 in HuNV) (Subba-Reddy et al., 2011). This linkage is essential for infectivity as enzymic removal of VPg from purified norovirus RNA significantly reduces infectivity (Chaudhry et al., 2006; Guix et al., 2007). Biochemical and
structural studies have indicated that the conformation of VPg and its helical core is central in facilitating the VPg–RdRp interaction, but have also highlighted that VPg is likely to unfold during the process of guanylation to enable the positioning of the tyrosine in the active site of the RdRp (Leen et al., 2013). This process of VPg nucleotidylation has been studied widely in the picornaviruses where a small RNA structure, first identified in the 2C-coding region of poliovirus (Goodfellow et al., 2000, 2003) was used as a template to produce VPg-pUpU that was then used to initiate viral RNA synthesis at the termini of the template RNAs (Liu et al., 2009). A similar structure has been proposed for noroviruses, located in the NS7-coding region (Victoria et al., 2009); however, this structure is not required for in vitro nucleotidylation of the norovirus VPg. There is in fact evidence that an element at the 3′ end of the genome enhances the efficiency of nucleotidylation, at least in vitro (Belliot et al., 2005).

Based on the observation that both positive- and negative-sense forms of the viral genomic and subgenomic RNAs have been detected in calicivirus-infected cells (Green et al., 2002), two models have been put forward for the mechanism of norovirus subgenomic RNA synthesis (Fig. 4). The first involves premature termination during

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**Fig. 4.** Mechanisms of norovirus subgenomic RNA synthesis. Schematic representation of the two proposed models for norovirus subgenomic RNA synthesis. (a) The presence of a termination signal upstream of the VP1-coding region (termination signal) results in premature termination during negative-sense RNA synthesis by the viral RNA polymerase (NS7/Pol). The VPg-linked positive-sense RNA template is drawn in black and initiation occurs de novo. The resulting negative-sense subgenomic RNA (blue) is then used for VPg-dependent RNA synthesis to produce a subgenomic dsRNA. The newly synthesized positive-sense ‘daughter’ subgenomic RNA (green) can then be used as a template for the synthesis of negative-sense subgenomic RNA. These in turn function as templates for additional rounds of ‘daughter’ subgenomic RNA synthesis. (b) The internal initiation model relies on the VPg-dependent subgenomic RNA synthesis occurring on a promoter sequence present downstream of the VP1-coding region on the negative-sense RNA (blue, SG promoter). The viral RNA polymerase initiates RNA synthesis, presumably in a VPg-dependent manner, to produce new ‘daughter’ VPg-linked subgenomic RNA (green), which may in turn function as a template for additional rounds of subgenomic RNA synthesis as shown in the premature termination model.
synthesis of the negative-sense genomic RNA, arising from a termination signal. The resulting negative-sense subgenomic RNA would then serve as a template for the production of positive-sense subgenomic RNA. The second model is that an RNA secondary structure upstream of the ORF2 in the negative-sense genomic RNA acts as a promoter for synthesis of positive-sense subgenomic RNA. In agreement with this model, bioinformatic analysis has indicated the presence of a highly conserved RNA stem–loop structure downstream of the VP1-coding region on the negative-sense RNA, 6 nt from the start of the subgenomic RNA in all members of the family Caliciviridae (Simmonds et al., 2008). Mutational analysis of this structure confirmed its essential role in the norovirus life cycle, but whether it plays a direct role in viral subgenomic RNA synthesis is unknown. In vitro biochemical studies with a related member of the genus Lagovirus, rabbit haemorrhagic disease virus, suggests that the calicivirus RdRp can utilize antisense genomic RNA as a template for the production of subgenomic-like RNA in vitro, at least (Morales et al., 2004). Further studies in this area are warranted to determine if this model is correct, but it is worth noting that the models are not mutually exclusive as positive-sense subgenomic RNA produced in the internal initiation model could be used as a template for replication by NS5. Once the synthesis of positive-sense viral genomic and subgenomic RNAs is initiated, multiple rounds of translation of the newly synthesized RNAs ensues, followed by additional rounds of RNA synthesis.

Interactions with host cell factors and pathways

Interactions with host cell proteins and cellular pathways are essential for achieving the productive replication of all positive-strand RNA viruses (Nagy & Pogany, 2011). Knowledge of host cell interactions involved in the norovirus life cycle currently lags behind that of other RNA viruses. As mentioned above, several studies have demonstrated interactions between host cell proteins and structures in the viral RNA, which play a role in the viral life cycle (Bailey et al., 2010b; Gutiérrez-Éscolano et al., 2000b; Sandoval-Jaime & Gutiérrez-Éscolano, 2009; Vashist et al., 2012). The host cell proteins DDX3, La and PTB were all found subsequently to at least partially localize to the viral replication complex during infection, and RNAi-mediated knockdown of these factors reduced MNV replication in cell culture, indicating that these interactions play a role in replication, although their specific functions are not known (Vashist et al., 2012). Another study identified an interaction between PTB and the polypyrimidine tract in a stem–loop structure at the 3′ end of the MNV genome (Bailey et al., 2010b). Mutation of the polypyrimidine tract reduced PTB binding and had no effect on replication in cell culture, but was sufficient to attenuate MNV in vivo, demonstrating a role of RNA–protein interactions in norovirus replication and as possible determinants of pathogenesis (Bailey et al., 2010b).

To create a beneficial cellular environment for replication, noroviruses are thought to disrupt a number of host cell pathways. As described above, the host cell protein secretion pathway is one such target that is disrupted during replication of both HuNV and MNV to modify and recruit membranes for replication (Sharp et al., 2010, 2012; Wobus et al., 2004). It is possible, however, that disruption of this pathway and abrogation of cell surface protein expression confers additional advantages during norovirus infections, such as interfering with antigen presentation or cytokine secretion, as has been reported for other RNA viruses that disrupt the host cell secretion pathway (Dodd et al., 2001). The HuNV replicon system has facilitated a number of studies into HuNV replication in the absence of an efficient cell culture system. It consists of a human hepatoma cell line (Huh-7) expressing self-replicating Norwalk virus genomic RNA and has been used to investigate cellular pathways that are altered by HuNV replication (Chang, 2009; Chang et al., 2006). In this way, both cholesterol and carbohydrate biosynthesis pathways were found to be altered in replicon-bearing cells by DNA microarray analysis. Cholesterol biosynthesis was downregulated and the use of statins to lower cholesterol was found to promote replication (Chang, 2009). Likewise, simvastatin has been found to increase replication in gnotobiotic pigs infected with HuNV (Jung et al., 2012) and epidemiological data has since raised concerns regarding the use of statins as an increased risk factor in the elderly (Rondy et al., 2011). The enhanced replication resulting from treatment with statins in vivo is thought in part to be mediated through suppression of innate immunity (Jung et al., 2012); however, the direct molecular basis for the interaction between norovirus and cholesterol synthesis has yet to be determined.

Studies on related positive-sense RNA viruses suggest an extensive modification of the cellular transcriptional and translational apparatus occurs with infection, which is beneficial to virus replication. In addition to the modification of the cholesterol pathway, in vitro biochemical studies have indicated that the norovirus NS6 protease possess the ability to cleave components of the cellular translation apparatus, namely PABP (Kuyumcu-Martinez et al., 2004). Cleavage of PABP, as well as other components of the elf4F complex, has been observed during FCV infection (Willcocks et al., 2004), but whether or not this occurs during authentic norovirus replication has yet to be determined.

Modification of the nuclear–cytoplasmic shuttling pathways has also been reported in other positive-sense RNA viruses and several observations would suggest that this may also occur during norovirus infection of permissive cells. (i) Infection with FCV results in the movement of the PTB protein from the nucleus to the cytoplasm where it negatively regulates FCV translation (Karakasiliotis et al., 2010). Importantly, this appears to occur prior to a global effect on nuclear export. (ii) During MNV infection of cells the nuclear–cytoplasmic shuttling protein hRNPA1, predominantly nuclear in uninfected macrophage cells,
Antagonistic interactions with the innate immune system are a well-known feature of RNA virus infections. Given the short time course of HuNV, innate immunity has been implicated highly in the control of infection, which has been supported by studies with HuNV and MNV. Treatment with IFN-α/β reduced HuNV RNA replication in the replicon system in gnotobiotic pigs and decreased replication of MNV in vitro (Chang & George, 2007; Jung et al., 2012). The role of the signal transducer and activator of transcription STAT1 signalling in the IFN-mediated innate immune response has also been well documented to control the appearance of clinical disease and viral dissemination during MNV infection in vivo (Karst et al., 2003; Mumphrey et al., 2007; Wobus et al., 2004). The initial detection of norovirus RNA is thought to occur primarily by the cellular helicase MDA-5, which senses dsRNA, rather than the RIG-I RNA sensor, which did not confer resistance to HuNV replication in transfected Huh-7 cells (Guix et al., 2007; McCartney et al., 2008). Accordingly, MNV replicates to higher titres in MDA-5−/− mice and also, to a smaller extent, higher in TLR3−/− mice, suggesting it may also contribute to detection of dsRNA (McCartney et al., 2008). The signalling cascade initiated by MDA-5 is mediated by the mitochondrial antiviral signalling (MAVS) protein, which results in activation of the transcription factors IFN-regulatory factor (IRF)-3, IRF-7 and NF-κB. This in turn results in the production of type I IFNs and the upregulation of IFN-stimulated genes (ISGs) (Kawai et al., 2005; Seth et al., 2005). Both IRF-3 and IRF-7 are required for IFN-mediated control of MNV (Thackray et al., 2012). MNV is able to interfere with innate immune signalling at the cellular level at least partially through the actions of VF1, the product of ORF4 (McFadden et al., 2011). In vitro, VF1 was found recently to delay the upregulation of IFN-β and other ISGs, and loss of VF1 resulted in a fitness cost in vitro and attenuation in vivo (McFadden et al., 2011). Interestingly, attenuation was observed in a STAT1−/− model of infection, suggesting that STAT1-independent signalling pathways may also be involved in, but are not sufficient to control, MNV infection alone. Given its mitochondrial localization, VF1 is expected to interfere with signalling through the MAVS complex or a downstream component affecting IRF-3 and IRF-7 activation, although further studies are required to elucidate the direct target. As HuNV does not express a homologue to VF1, it is unknown at present if it has the same capacity as MNV to antagonize the innate immune response; however, functional duplication of VF1 remains a possibility.

Assembly and exit

The processes behind viral assembly, encapsidation and the exit of noroviruses are largely unknown. The ability of VP1 to self-assemble into virus-like particles (VLPs) indistinguishable morphologically and antigenically from native virions suggests that it may be sufficient to drive capsid assembly during virus replication (Bertolotti-Ciarlet et al., 2002), although the involvement of cellular proteins cannot be excluded. This self-assembly property of the norovirus VP1 protein has been utilized to generate a recombinant VLP-based vaccine that has proven efficacious in human volunteer studies (Atmar et al., 2011). Whilst VP2 is not required for VLP assembly, it is thought to promote the stability of VP1 and is essential for the production of infectious virions (Bertolotti-Ciarlet et al., 2002; Sosnovtsev et al., 2005). The highly basic nature of VP2 has formed the basis of a long-held theory that it may be involved in encapsidation via an interaction with the acidic viral RNA (Sosnovtsev et al., 2005). In support of this, VP1 and VP2 have been shown to interact, and the binding of VP2 was mapped to a conserved motif in the shell domain of VP1 (Vongphansawad et al., 2013). This places VP2 in the interior of the capsid, consistent with a role in encapsidation. However, to date, a direct interaction between VP2 and viral genomic RNA has not been demonstrated. A possible interaction between the VPg protein linked covalently to the viral RNA and the capsid protein VP1 has also been observed in yeast two-hybrid studies with the related FCV (Kaiser et al., 2006). Such an interaction would provide a mechanism for the specific encapsidation of replicated viral RNA in preference to cellular mRNAs, although further studies are required to validate such a model.

There have been no direct studies on the exit and release of the assembled norovirus virion to complete the viral life cycle. One proposed exit strategy for other calciviruses involves the induction of apoptosis (Alonso et al., 1998; Bok et al., 2009; Sosnovtsev et al., 2003). Whether noroviruses employ a similar strategy is unknown; however, accumulation of apoptotic epithelial cells has been observed in intestinal biopsies from infected patients (Bok et al., 2009; Furman et al., 2009; Troeger et al., 2009). Furthermore, as highlighted above, apoptosis is induced with active MNV replication and plays a role in the processing of NS1/2 during the latter stages of the viral life cycle. The induction is associated with downregulation of survivin, a pro-survival factor, followed by caspase and cathepsin B activation and cytochrome c release (Bok et al., 2009; Furman et al., 2009). Whether the downregulation of survivin occurs by a direct interaction with a viral protein or as a response to indirect effects of MNV on the host cell is as yet unknown. Instead, VF1 has been shown to delay the onset of apoptosis through its localization at the mitochondria (McFadden et al., 2011), although it is possible that this may serve to prolong the window for replication prior to the final stages of exit and release. Inhibition of apoptosis results in reduced MNV production, either way demonstrating a clear requirement for apoptosis for an aspect of the norovirus life cycle (Furman et al., 2009).

Concluding remarks

Although our understanding of the norovirus life cycle is far from complete, the past decade has seen a dramatic increase in publications relating to various aspects of
norovirus biology, including the molecular mechanisms of norovirus genome translation and replication. This is due to increased awareness of the prevalence and clinical importance of noroviruses, but also due to the significant efforts in the development of tools and resources for the study of norovirus biology. A lack of an efficient culture system for HuNVs is a major hurdle that researchers in the field have yet to overcome, but steps towards this are already under way (Asanaka et al., 2005; Guix et al., 2007). Work with related viruses has provided novel insights into the biology of members of the family *Caliciviridae*, but mechanistic differences between the various members of the family are apparent. For example, whilst further processing of the MNV NS1/2 protein occurs during virus replication, whether the HuNV p48 (N-term) protein is further processed in not known. Also, whilst FCV translation initiation is clearly dependent on eIF4E, the only study to date on norovirus translation initiation found that eIF4E is in fact dispensable (Chaudhry et al., 2006). With this in mind, continued efforts and the use of novel approaches to norovirus culture will be required to provide the breakthrough needed. One such approach has found recently that mice deficient in aspects of the immune response can be infected by intraperitoneal injection of HuNV (Taube et al., 2013). This experimental system, which does not recapitulate norovirus gastrointestinal disease, provides a useful tool for the study of aspects of norovirus pathology. More importantly, this system, combined with the advances in other areas, provides a starting block from which to move forward to the ‘Holy Grail’ of norovirus biology, i.e. a cell culture system that enables HuNVs to undergo a full replication cycle. Such a system, when combined with a reverse-genetics system to enable viral genome manipulation and a small, preferably inexpensive and genetically defined, animal model, would provide the complete toolset with which to fill in the remaining gaps in our understanding of norovirus biology.

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**References**


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