The murine norovirus (MNV) core subgenomic RNA promoter consists of a stable stem-loop that can direct accurate initiation of RNA synthesis

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Abstract

All members of the Caliciviridae family of viruses produce a subgenomic RNA during infection. The subgenomic RNA typically encodes only the major and minor capsid proteins, but in murine norovirus (MNV), the subgenomic RNA also encodes the VF1 protein that functions to suppress host innate immune responses. To date, the mechanism of norovirus subgenomic RNA synthesis has not been characterized. We have previously described the presence of an evolutionarily conserved RNA stem-loop structure on the negative-sense RNA, the complementary sequence of which codes for the viral RNA-dependent RNA polymerase (NS7). The conserved stem-loop is positioned 6 nucleotides 3’ of the start site of the subgenomic RNA in all caliciviruses. We demonstrate that the conserved stem-loop is essential for MNV viability. Mutant MNV RNAs with substitutions in the stem-loop replicated poorly until they accumulated mutations that revert to restore the stem-loop sequence and/or structure. The stem-loop sequence functions in a non-coding context as it was possible to restore the replication of a MNV mutant by introducing an additional copy of the stem-loop between the NS7 and VP1 coding regions. Finally, in vitro biochemical data would suggest that stem-loop sequence is sufficient for the initiation of viral RNA synthesis by the recombinant MNV RdRp, confirming that the stem-loop forms the core of the norovirus subgenomic promoter.

Importance
Noroviruses are a significant cause of viral gastroenteritis and it is important to understand the mechanism of norovirus RNA synthesis. Herein we describe the identification of an RNA stem-loop structure that functions as the core of the norovirus subgenomic RNA promoter in cells and in vitro. This work provides new insights into the molecular mechanisms of norovirus RNA synthesis and the sequences that determine the recognition of viral RNA by the RNA dependent RNA polymerase.
Introduction

Noroviruses, members of the *Caliciviridae* family of small positive-sense RNA viruses are a major cause of viral gastroenteritis in the developed world (1, 2). Despite their impact, noroviruses remain poorly characterized as an in-depth understanding of the molecular mechanisms of norovirus genome translation and replication have been hampered by the inability to culture human norovirus (3). Murine norovirus (MNV) is a model system with which to understand the norovirus life cycle (4) as it replicates in cultured cells (5) and a number of tractable reverse genetics systems are available (6-9). Studies of MNV have therefore led to a number of significant advances in the understanding of the molecular mechanism of norovirus genome translation and replication (Reviewed in 10).

The norovirus RNA genome typically encodes three open reading frames (11). MNV also encodes an additional ORF in the region overlapping the VP1 coding region (12, 13) (Fig. 1A). All members of the *Caliciviridae* family of small positive-sense RNA viruses synthesize a shorter than genome length, subgenomic, RNA (sgRNA) which directs the translation of the major and minor structural proteins, VP1 and VP2 respectively (10). The MNV sgRNA also encodes the VF1 protein, an antagonist of the innate immune response (12). Production of a sgRNA during the viral life cycle is a common feature of many positive-sense RNA viruses (14) and it is often used to control the expression of the viral proteins.
The mechanism of calicivirus sgRNA synthesis is not well-understood. Initial in vitro biochemical approaches using rabbit hemorrhagic disease virus (RHDV) suggested that a sequence upstream of the first nucleotide of the subgenomic RNA is required for sgRNA synthesis (15). This sequence acts in the complement of the RHDV genomic RNA. Bioinformatic analysis also confirmed the presence of evolutionarily conserved and stable RNA stem-loop structures upstream of the start of the sgRNA of caliciviruses (16). Within the region coding for the viral RNA-dependent RNA polymerase (NS7, RdRp) in all caliciviruses analyzed, a ~24-50 nt stem-loop structure could be predicted on the negative-sense genomic RNA, precisely 6 nt 3' of the sgRNA start site. This stem-loop structure in the MNV minus-strand RNA will be referred to as Sla5045 (for stem-loop anti-sense 5045). We previously observed that the stable structure of Sla5045 is essential for the recovery of viable MNV in cultured cells (16). In the current study we present results consistent with the hypothesis that Sla5045 forms the core of the norovirus sgRNA promoter.

Materials and Methods

Cell lines and plasmid constructs. The murine leukemia macrophage cell line (RAW264.7) was grown and maintained in Dulbecco modified Eagle medium (DMEM; Gibco) with 10% (v/v) fetal calf serum (FCS), penicillin (100 SI units/ml) and streptomycin (100 µg/ml) and 10 mM HEPES buffer (pH 7.6). Baby hamster kidney cells (BHK-21) engineered to express T7 RNA polymerase (BSR-T7 cells,
obtained from Karl-Klaus Conzelmann, Ludwig Maximilian University, Munich, Germany) were maintained in DMEM containing 10% FCS, penicillin (100 SI units/ml), streptomycin (100 µg/ml) and 1.0 mg/ml geneticin (G418). All cells were maintained at 37°C with 10% CO₂.

The full length MNV-1 cDNA clone, pT7:MNV3’Rz, contains the MNV-1 genome under the control of truncated T7 polymerase promoter (7). Mutant derivatives of pT7:MNV3’RZ were made as previously describe (7, 16). These include: (i) a frameshift in the NS7 region of ORF1 (pT7:MNV 3’Rz F/S), (ii) the m53 mutations to destabilize the Sla5045 (pT7:MNVm53 3’Rz) and (iii) the m53r mutations to restore the Sla5045 (pT7:MNVm53r 3’Rz). Other full length MNV-1 cDNA clones carrying m53 mutant suppressors (m53SupA, m53SupG and m53SupH) in m53 backbone construct were generated by overlapping PCR mutagenesis using pT7:MNVm53 3’Rz as a template. All primer sequences used in this work and protocols will be made available upon request. Mutant suppressors were also engineered into pT7:MNV3’Rz, producing WTSupA, WTSupB and WTSupC cDNA constructs. Additional synonymous mutations at position 4922 in the WT and m53 cDNA constructs with adenyltate and guanylate substitutions were also generated by overlapping mutagenesis PCR. The luciferase reporter-expressing MNV replicons used in this study were as reported previously (17). Briefly, the luciferase gene was fused to VP2 in the WT, POL<sup>FS</sup>, m53 and m53r constructs and separated by the foot and mouth disease virus 2A protease (FMDV 2A). The expression of luciferase reporter gene in these constructs is under the control of the MNV TURBS sequence (18, 19).
Translation of the luciferase reporter protein occurs as a VP2 fusion protein and is co-translationally cleaved at the specific FMDV 2A cleavage site to release both luciferase-2A and VP2.

Sla5045Dup contains a second copy of the WT or m53 stem-loop (Sla2) inserted outside of the NS7 coding region in the intergenic region between Orf1 and Orf2 was used to generate constructs WT/WT, WT/m53, m53/WT and m53/m53 by overlapping PCR mutagenesis. A second panel of Sla5045Dup mutant constructs was also generated based on the m53/WT construct as detailed in the text.

**Reverse genetics and virus yield determination.** Recoveries of full-length infectious MNV-1 cDNA clones were performed using the established reverse genetics system (7). Typically, BSR-T7 cells were infected with fowlpox virus expressing T7 RNA polymerase at a multiplicity of infection (MOI) of 0.5 plaque forming units (PFU) per cell before transfection with 1 µg of cDNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Note that the levels of T7 RNA polymerase normally expressed in BSR-T7 cells are not sufficient to drive MNV recovery. Where indicated, the RNA-mediated reverse genetics system was used. In this case, RNA transcripts were produced using *in vitro* transcription reactions which consisted of 200 mM HEPES pH7.5, 32 mM magnesium acetate, 40 mM DTT, 2 mM spermidine, 7.5 mM of each NTP (ATP, UTP, GTP and CTP), 40 unit of RNase inhibitor (Promega), 250 ng of linearized DNA template and 50 µg/ml of T7 RNA
polymerase. The reactions were incubated at 37°C for 2-7 h, treated with DNase I (New England Biolabs) and the RNA was purified by precipitation using lithium chloride and resuspended in RNA storage solution (Ambion) (9). Prior to transfection into BSR-T7 cells, post-transcriptional enzymatic capping was performed on the purified RNA transcripts using a ScriptCap system (Epicentre), according to manufacturers instructions. Typically one microgram of capped RNA was transfected using Lipofectamine 2000 according to the manufacturers’ instructions. The yield of infectious was determined 24 h post transfection of cDNA or capped RNA, using 50% tissue culture infectious dose (TCID\textsubscript{50}). Note that BSR-T7 cells, a BHK cell derivative can support only a single cycle of virus replication, possible due to the lack of a suitable receptor for virus reinfection.

RNA purification and genome copy determination. All RNA purifications from infected and transfected cells were performed using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) according to the manufacturer’s instructions. RNA was quantified by spectrophotometry and qualitatively inspected by on agarose gels stained with ethidium bromide.

The expression level of genomic RNA for WT and m53SupA MNV were quantified using one step RT-qPCR. Viral genome copies were determined using the MESA Blue qPCR Assay (Eurogentech) performed in parallel with control RNAs of known concentration using the primers 404F (GGAGCCTGTGATCGGCTCTATCTTGGAGCAGG) and 491R
(GCCTGGCAGACCGCAGCAGCAGACTGGGTTGTTGACC) to amplify the viral genomic RNA.

Where described, strand-specific RT-qPCR was performed using tagged RT primers to detect either positive or negative-sense genomic RNA as previously described (20). In addition, a set of primers was designed to amplify nucleotides 5473-5422 to facilitate the simultaneous quantification of both genomic and subgenomic RNAs. Control RNAs for the genomic or subgenomic RNAs, of positive or negative-sense polarity, were generated by in vitro transcription and used as standards to facilitate quantification.

Western blotting. Samples for western blot analysis were obtained by lysing cells in radioimmunoprecipitation assay buffer (RIPA - 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS). Protein concentration in the lysates were quantified using the bicinchoninic acid (BCA) protein assay (Pierce). Equal amounts of protein per samples were separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) Immobilon-P transfer membrane (Millipore) for detection using Enhance Chemiluminescence (ECL) reagents by semi-dry transfer. The blocking buffer (PBST) contained 0.1% Tween 20 in PBS containing 5% w/v milk powder and the rabbit anti-MNV NS7 or VP1 serum. Antibody binding was detected using a secondary, horseradish peroxidase-conjugated secondary antibody (source). The signal was detected by enhanced chemiluminescence kit (ECL; Amersham Biosciences).
Luciferase reporter assay. Cell lysates used to detect luciferase activity were prepared by lysing the transfected cells in 1 X Reporter Lysis Buffer (Promega) according to the manufacturers instructions. The protein concentration in each lysates was standardized and total protein (30 μg) from each cell lysate was analyzed. The luciferase assays used a 1:500 dilution of coelentrazine (Promega) in PBS as a substrate. Luciferase activity was measured in an auto-injector luminometer (FLUOstar Omega, BMG Labtech).

Northern blot assay. Total RNAs were purified from infected cells and denatured by glyoxylation according to the manufacturers instructions (Ambion). Denatured RNAs along with the relevant control RNA generated by in vitro transcription to produce full-length MNV genomic and subgenomic RNAs of both polarities, were separated by agarose electrophoresis. Denatured RNAs were transferred to Hybond N+ nylon membranes using mild alkaline conditions and the viral RNA detected using the NorthernMax northern blotting system (Life Technologies). RNA probes used to detect the positive and negative-sense viral RNAs were generated by incorporating $^{32}$P-UTP or CTP using the Maxiscript in vitro labeling system, according to the manufacturer’s instructions (Ambion). The RNA probe used to detect the negative-sense viral RNA consisted of nts 1-200 of the positive sense sgRNA, whereas the positive sense viral RNA was detected using a probe complementary to nts 7161-7382.
5’ rapid amplification of cDNA ends (RACE). Total RNA preparations were subjected to cDNA synthesis using a reverse primer complementary to sequences ~700 bases downstream (on positive polarity) of Sla5045. The resulting cDNAs were treated with ribonuclease to degrade the viral RNA template prior. The purified cDNAs were then used as a template for RACE assay using 5’ RACE according to the manufacturer’s instructions (Life Technologies). Purified cDNAs were first subjected to the 3’ end poly(C) tailing reaction catalyzed by terminal transferase followed by PCR using an anchor primer that anneals to the poly(C) 3’end of cDNA and the MNV specific reverse primer. Sequences of the PCR products were determined using the BigDye terminator kit and analyzed using the Vector NTI software (Invitrogen).

Primer extension analysis. Primer extension analysis was performed on RNA isolated from either infected cells or cells transfected with in vitro transcribed capped RNAs generated from MNV cDNA containing clones. The $^{33}$P $\gamma$-ATP labeled primer 5129R (GGTTGAATGGGGACGGCCTGTTCAACGG) was annealed to 10μg of total RNA was and extended using Superscript III according to the manufacturers’ instructions (Life Technologies). Reactions were stopped by the addition of formamide containing dye followed by separation on 6% urea-PAGE. The extended products were visualized following exposure to autoradiography film and compared to samples isolated form infected cells and control RNA produced by in vitro transcription.
**Recombinant RdRp expression and purification.** The cDNA encoding MNV NS7 (nts 3537 to 5069) was subcloned in pET-15b. The construct contains an N-terminal six-histidine tag to facilitate protein purification. The plasmid expressing MNV NS7 was transformed into *E. coli* Rosetta cells, which were grown at 37°C until the optical density at OD₆₀₀ was between 0.6 and 0.8. Isopropylthiogalactoside was added to a final concentration of 1 mM induce protein expression for 16-18 h at 16°C. The cells were harvested by centrifugation and resuspended in 40 ml lysis buffer (100 mM TrisCl, pH 7.9, 300 mM NaCl, 10% glycerol, 15 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Triton X-100, protease inhibitor cocktail (Sigma)). The recombinant protein was purified by Ni-NTA (Qiagen) according to the manufacturer's instructions. The elute protein was dialyzed in buffer containing 100 mM TrisCl, pH 7.9, 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and stored in aliquots at -80°C.

Recombinant GII.4 NS7 was expressed and purified as previously described (17). Briefly, *E. coli* Top 10 cells transformed with pBAD GII.4NS7 plasmid were cultured at 37°C until an OD₆₀₀ of around 0.6 was reached. L-arabinose was added to a final concentration of 0.02% to induce protein expression. The cells were grown at 37°C for 5 h and then harvested and suspended in lysis buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 10% glycerol, 10 mM Imidazole, proteinase inhibitor cocktail (Sigma)). The protein was purified by Talon metal affinity resin (Clontech Laboratories) and the elute protein dialyzed in buffer containing 100 mM TrisCl, pH 7.9, 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and stored in aliquots at -80°C. All recombinant
proteins were quantified by using the Bradford method followed SDS-PAGE and staining with Coomassie brilliant blue to examine their purity.

**RNA synthesis assay.** RNA synthesis by the recombinant RdRps was assayed as previously described (21) with minor modifications. The 20 μl reaction contained 20 mM sodium glutamate (pH 7.4), 12.5 mM dithiothreitol, 4 mM MgCl₂, 1 mM MnCl₂, 0.5% Triton X-100 (v/v), 0.2 mM GTP, 0.1 mM ATP and UTP, 3.3 nM α⁻³²P-CTP (MP Biomedicals), 50 nM MNV RNA template, 25 nM recombinant RdRp. The reaction was incubated at 30°C for 2 h, and then stopped by the addition of EDTA (pH 8.0) to final concentration of 10 mM. The RdRp products were subjected to electrophoresis in 24% polyacrylamide gel containing 7.5 M urea and 0.5X TBE. The radiolabeled RNA products were visualized and quantified by using a Phospholmager (Typhoon 9210; Amersham Biosciences) and ImageQuant software.

**Results**

The stability of Sla5045 contributes to norovirus infectivity and subgenomic RNA synthesis.

Sequence analyses of published noroviruses genomes indicate that the stem-loop sequence of Sla5045 is highly conserved (data not shown and Fig. 1B). Several positions in the Genogroup II (GII) or V (GV), representing human and murine noroviruses respectively, appear to show some variation in sequence, although in all cases a complex stem-loop structure is retained (Fig.
We initially sought to test the functional significance of the stability of Sla5045. Three nucleotide substitutions were introduced to the MNV genome, resulting in a construct named m53 (Fig. 1C). M53 was previously shown to have a severely reduced recovery of infectious virus (16). However, it was not determined whether the inability to produce detectable virus was due to a defect in RNA encapsidation or sgRNA production, or both. To further validate the role of Sla5045 in the norovirus life cycle and to examine whether the m53 mutations affect viral RNA synthesis, we inserted the previously described m53 substitutions into the MNV luciferase replicon, Mflc-R (17). The replicon consists of a full-length MNV genome expressing a Renilla luciferase-FMDV 2A peptide-VP2 fusion protein in the VP2 coding region. Transfection of BSRT7 cells with enzymatically capped, in vitro transcribed RNA from the Mflc-R cDNA leads to luciferase expression in the absence of infectious virus production. As luciferase is expressed from ORF3 and occurs via a conserved termination-reinitiation mechanism of translation at the translational termination signal of ORF2 (18, 19), it functions as a highly sensitive marker for the production of sgRNA. The introduction of the m53 mutations resulted in luciferase levels similar to a replication-defective replicon containing a frame-shift in the NS7 coding region (Fig. 1D). A construct containing compensatory mutations to m53, named m53r (Fig. 1C), that restored the stability of Sla5045 was found to restore luciferase expression levels to nearly the level of the WT MNV replicon (Fig. 1D). The introduction of m53r mutations also resulted in the recovery of infectious virus (Fig. 1E). These data are consistent with the hypothesis that Sla5045 plays a role
in viral RNA synthesis and does not simply affect a late stage in the viral life cycle such as encapsidation.

We analyzed the production of sgRNA in cells transfected with capped RNAs of m53 and m53r using a primer extension assay. A radiolabelled primer complementary to nucleotides 5121-5129 was annealed to RNA isolated from transfected cells and extended using reverse transcriptase, generating a product of 105 nts corresponding to the start of the sgRNA. Whilst sgRNA was detected in infected or cells transfected with WT RNA, it was absent in m53 but restored to almost wild-type levels in the m53r (Fig. 1F). Taken together, these data confirm that the stability of Sla5045 contributes to MNV sgRNA synthesis, as well as that the production of luciferase and the presence of infectious virus correlate with sgRNA synthesis.

Rapid reversion and suppression of Sla5045 disruption occur in cell culture.

The replication cycle of many positive-sense RNA viruses are error-prone and the reversion or phenotypic suppression of the introduced mutations can occur even during low levels of viral replication. Such reversion or suppression can become apparent after repeated passage of samples obtained by reverse genetics recovery. To examine whether it was possible to isolate revertants or suppressors of MNV containing the m53 mutations, named MNVm53, clarified tissue culture supernatants from three independent reverse genetics recoveries
were serially passaged in RAW264.7 cells until cytopathic effect (CPE) was observed. In all cases CPE was observed after 3 passages in permissive cells. Passages performed with similar samples obtained using the POL$^{FS}$ construct failed to result in CPE. Individual viral isolates were isolated by limiting dilution and the sequence of the NS7 coding region was determined. In all cases we observed additional changes either in Sla5045 or sequences elsewhere in the viral genome (Fig. 2). In total we identified 12 different independently-derived viable viruses that could be divided into two groups; phenotypic revertant mutants (Rev 1-9) that contained mutations that phenotypically restored the base-pairing of Sla5045 and suppressor mutants (Sup A-C) that retained the m53 mutations but had additional changes elsewhere. All Rev isolates contained mutations that appeared to restore the stability of Sla5045 by either reintroducing the WT sequence (Rev 2-6 and 8), or by introducing a stabilizing mutation on the opposite side of the stem (Fig. 2B). In contrast the suppressor mutants retained the m53 mutations but had additional changes in the NS7-coding region. Of the three Sup mutations identified, SupA and Sup C had synonymous mutations that did not alter the corresponding amino acid (data not shown), while SupB resulted in a threonine to alanine substitution at residue 439 of NS7.

**Both coding and non-coding suppressor mutations in the NS7 coding region complement the defect in MNVm53**

To confirm that SupA-C can suppress the defect in MNVm53 replication, individual mutations were introduced into full-length cDNA clones from WT MNV
or m53 and the effect on virus recovery examined in BSRT7 cells. The BHK-
derived BSRT7 cells are permissive for viral replication but not viral infection,
with the viral yields being indicative of a single cycle of viral replication (7). All
three suppressor mutations were viable in the m53 background, confirming their
ability to restore the replication defect of MNVm53 (Fig. 3A). SupA, with an
A4922G substitution on the negative-sense RNA, was consistently better at
restoring the replication defect of MNVm53 than the SupB or C. improving viral
yields by more than 10-fold when compared to the virus with both the m53 and
either the SupB or SupC mutations. All three Sup mutations were also viable in
the WT cDNA background, with SupA producing viral yields that were
comparable to WT MNV by 48 h after transfection (Fig. 3A and 3B). Sequence
analysis indicated that all viruses were stable for at least three passages in cell
culture (data not shown). Given that Sup A and C had synonymous mutations,
these results strongly suggest that the suppression of the m53 defect acted on
the viral RNA.

The replication of MNVm53SupA was further characterized by one-step
growth curve analysis (Fig. 3B-D). The production of infectious virus (Fig. 3B),
viral protein (Fig. 3C) and viral RNA (Fig. 3D) were all reduced in MNVm53SupA
when compared to WT MNV. The mutation introduced in SupA results in a silent
U4922C mutation on the positive-sense RNA (Fig. 4A). This position
corresponded to an alanine at codon 461 in NS7 and enables additional silent
mutations to be introduced. We examined whether substitutions to the other
three nucleotides at this position would also suppress the m53 defect as well as affect WT MNV. All 4 mutations were well tolerated in a WT MNV background whereas only the substitutions equivalent to SupA, A4922G on the minus strand, or A4922U on the minus-strand RNA, restored replication in the MNVm53 backbone (Fig. 4B). All viable viruses could be repeatedly passaged in cell culture and the sequences introduced remained stable (data not shown). These data suggest that there is a sequence specific requirement for the ability of position 4922 to suppress the defect in MNVm53.

**Sla5045 function at a different location in the MNV genome**

Due to the location of Sla5045 in the NS7 coding region, mutational analysis was limited to synonymous changes that do not alter the NS7 protein sequence. To facilitate additional mutational analysis, we added a second copy of Sla5045, named Sla2, after the ORF1 termination codon. Sla2 is located within a noncoding intragenic region introduced between the coding sequence of NS7 and VP1 in construct Sla5054Dup (Fig. 5A). The cDNA of Sla5045Dup was engineered to contain either a WT or m53 version of Sla5045 or in the region containing Sla2. *In vitro* transcribed and capped RNA was then transfected into cells and the effect on virus yield examined 24 hours post transfection. RNA-mediated reverse genetics recovery was used to significantly improve the yield of recovered viruses and to enhance our ability to detect viruses that replicate poorly. As expected, RNA generated from the WT MNV cDNA clone resulted in \(~10^5\) infectious units per ml, whereas m53 and a polymerase mutant failed to
produce any detectable virus (Fig. 5B). Sla5045Dup containing Sla5045 WT/Sla2 WT (WT/WT) reproducibly yielded viable virus whereas the WT/m53 construct, containing a mutated Sla2 did not (Fig. 5B). In constructs where Sla5045 contained the m53 mutations, a WT Sla2 restored replication whereas a m53 Sla2 did not (Fig. 5B). These results demonstrate that only stem-loop sequences positioned at Sla2 function as a sgRNA promoter in the context of the Sla5045Dup cDNA constructs. Importantly however, given that the yield of virus obtained from the Sla5045Dup constructs WT/WT and m53/WT was significantly lower than that of the WT virus (Fig. 5B), it appears that the sgRNA promoter functions less well in the Sla2 position.

Western blot analysis was used as an indirect measure of viral RNA transfection efficiency as we have previously observed that viral antigen expression after transfection of RNA is due to translation of the capped RNA only and not due to viral replication (9). Similar levels of the NS7 protein were detectable in all cases, although the Sla5045Dup construct that contained Sla5045 WT/Sla2 WT produced a protein detected by the anti-NS7 antisera with higher molecular weight (identified by an asterisk in Fig. 5B). We suspect that this was due to the insertion of an additional stable stem-loop structure in close proximity to the NS7 stop codon resulting in suppression of translational termination as this has been widely documented in the literature (Reviewed in 22). This hypothesis is also supported by the absence of the extended product when the Sla5045 was disrupted by the introduction of the m53 mutations. The
lack of NS7 expression in the POL\textsuperscript{FS} polymerase mutant was due to the introduction of a frame-shift mutation leading to the production of a truncated NS7 protein that is rapidly degraded (9).

Although the titers of the Sla5045Dup viruses recovered were low, some of the introduced mutations were stable and could be passaged in cell culture, indicating that they are viable (Fig. 5C). Sequence analysis and 5' RACE confirmed that the introduced mutations were stable for up to 3 passages in cell culture and that the start of the sgRNA produced during authentic norovirus replication in cell culture is position 5052, as has been predicted previously (Fig. 5D) (4).

**The sequence, positioning and stability of Sla5045 are essential for norovirus replication.**

Our data indicates that the Sla5045 is essential for norovirus replication and that its function can be complemented by an additional copy in *cis* in a non-coding region upstream of the capsid-coding sequence. To examine the sequence requirement further we made constructs that contained a mutated m53 within the NS7 coding region along with various mutant forms in the second copy present in the non-coding region. The construct Sla5045Dup m53/Sla2 m53r contained the m53r derivative of Sla5045 that is predicted to compensate for the disrupted base pairs in m53 (Fig. 6A). As expected, the Sla5045Dup m53/Sla2 m53r produced viable virus after reverse genetics recovery (Fig. 6B).
resultant virus could be passaged in cell culture (Fig. 6C) and that stably maintain both copies of Sla5045 (data not shown). In contrast Sla5045Dup m53/Sla2 WT+8, where the spacing between the stem-loop and the predicted initiation site of the sgRNA was increased from 6 to 8 nts was not viable. Significant alterations to the base stem sequences (Sla5045Dup m53/Sla2 SL+ or SL-) or disruption of the top stem sequence (Sla5045Dup m53/Sla2 S2) also prevented replication (Fig. 6A-C). In contrast, the introduction of two nucleotide changes in the terminal loop region of Sla5045 in the construct Sla5045Dup m53/Sla2 TL-Dis had no effect on the ability of Sla5045 to complement the defect in the m53 mutant backbone did not produce viable virus (Fig. 6A-C).

**Sla5045 functions as a template for the norovirus RNA polymerase NS7 in vitro.**

Results from characterization of Sla5045 are all consistent with the hypothesis that Sla5045 forms the core of the promoter for MNV sgRNA synthesis and is used to direct sgRNA synthesis. However, it is unclear whether it functions directly in RNA synthesis. To determine whether this is the case, we examined whether RNAs that contain Sla5054 can direct RNA-dependent RNA synthesis by the recombinant MNV NS7 protein *in vitro* (Fig. 7A). An RNA containing Sla5045 with a 5' overhang that contains the initiation nucleotide 5052 was chemically synthesized in a RNA we termed the MNV proscript, MNVps. The term proscript is used to denote that the RNA contains both a putative promoter and the template sequence (23) (Fig. 7B). Should the MNV proscript direct
accurate initiation of RNA synthesis from nt 5052, an 11-nt product should result. Based on comparison with RNAs of known lengths, the products of MNVps were 11-nt in length. We also observed a 10-nt RNA that is presumed to be a premature termination product that has been previously observed with other recombinant RdRps. (Fig. 7C; 24). To ascertain that the RNA was initiated from the expected cytidylate at nt 5052, we tested an RNA that is identical to MNVps except that nt 5052 was substituted with an adenylate named IM. IM failed to direct the synthesis of either the 10- and 11-nt RNA products (Fig. 7C). These data confirm that Sla5045 can accurately direct de novo initiation of the norovirus sgRNA synthesis.

In the in vitro RNA synthesis reactions we also detected an RdRp product of ca. 60-nt, longer in length than the input proscript. This RNA is likely formed by viral RdRp using the 3’ terminal nucleotide of the template RNA to form a primer-extended product (25). This activity is common to numerous viral RdRps (26, 27). Notably, IM generated a higher abundance of the primer-extended RNA products than did MNVps. Since de novo initiated RNA product generated from MNVps was more abundant that those from primer extension, we conclude that the recombinant MNV RdRp has a propensity for de novo initiated RNA synthesis from the MNV sgRNA.

To examine further whether the MNV Sla5045 can direct a genotype-specific RNA synthesis by the MNV RdRp, we performed in vitro RNA synthesis reaction with proscript containing the comparable sequence from a human GII.4 norovirus named GII.4Ps (Fig. 7B). Accurate de novo initiated RNA synthesis
from GII.4Ps should give rise to an 8-nt product (Fig. 7D). The recombinant MNV RdRp did produce RNAs of 8-nt and a 7-nt from GII.4Ps (Fig. 7D). However, in more than six independent assays, the amount of the RNA product was 27% of the amount produced from the MNV RdRp. In contrast, the recombinant GII.4 RdRp produced more than 30-fold the amount of products from the GII.4Ps than from the MNVps (Fig. 7D). The MNV RdRp reproducibly produced a reduced amount of the primer-extension product from the GII.4ps (13%, Fig. 7D).

Furthermore, the GII.4 RdRp also produced a reduced level of primer-extension products from the MNVps (Fig. 7D). These results suggest that MNV RdRp can specifically recognize the MNVps.

Negative-sense subgenomic RNA is produced during MNV replication

The presence of Sla5045 on the negative-sense genomic RNA and the ability of Sla5045 to function in vitro as a template for priming by the RdRp would fit with the hypothesis that MNV uses a process of internal initiation for the generation of sgRNA. However, negative-sense sgRNA is readily detected in cells infected with feline calicivirus (28) and in Norwalk replicon containing cells (29). Northern blot and strand-specific RT-qPCR analysis of RNA isolated from MNV infected cells also confirmed the presence of a negative-sense sgRNA intermediate (Fig. 8A). Whilst we observed similar levels of negative-sense genomic and sgRNA produced in infected cells, the levels of sgRNA were typically >26 fold higher than the genomic RNA levels (Fig. 8B), fitting with previous observation on FCV and Norwalk virus (28, 29). These data are
consistent with the hypothesis that newly synthesized MNV sgRNA may function as a template for further rounds of replication via a negative-sense sgRNA intermediate.

Discussion

In this study we used a combination of genetic and biochemical approaches to characterize a stem-loop RNA sequence that we predicted to be the MNV sgRNA promoter. A mutant MNV that has substitutions that disrupted the predicted secondary structure of the stem-loop was poorly infectious while nucleotide substitutions that restored the stem-loop structure allowed the virus to regain infectivity. Suppressor mutations found after repeated passages in cell culture also had restored Sla5045 structure, although additional suppressor mutants were also isolated outside of the Sla5045. Examination of a second copy of the sla5045 in a non-coding region of the MNV genome revealed that the stem-loop structure functions in cis. Using purified recombinant viral RdRp, we demonstrate an RNA containing Sla5054 can direct the genotype-specific initiate RNA synthesis from Sla5045.

A change in the codon of viral RdRp NS7 was found in SupB, which suppressed the changes in m53. Further analysis of this nucleotide position indicated that the effect was mediated in a non-coding context as changing this codon (ACU) to GUU to code for valine, or to GAU to code for aspartic acid, also restored replication to the m53 (data not shown). There are several explanations
for our ability to isolate second site suppressor mutations outside of Sla5045; the first is that Sla5045 may form higher order RNA structures with other regions of the viral genome, including the positions identified as second site suppressors, and that the second site suppressors stabilize these interactions. Our bioinformatic analysis has failed to identify any obvious interactions between the regions (data not shown). However, accurate predictions of long- or medium-range RNA-RNA interactions are not currently available and we cannot at this point rule out that long-range RNA-RNA interaction contribute to Sla5045 function. In addition, the introduction of the m53 mutations could result in promiscuous base-pairing of Sla5045 with other sequences flanking the start of the sgRNA. The suppressor mutations then function to disrupt the ‘off-target’ RNA-RNA interactions, favoring the restoration of the Sla5045 structure. Distinguishing between these two possibilities will be challenging, however our observation that the addition of Sla5045 to the 3’ end of mini-genome RNA encoding a reporter gene in the antisense orientation is not sufficient to drive RNA synthesis and reporter gene expression in infected cells (data not shown), suggests that additional RNA sequences may contribute to RNA synthesis directed by core promoter that consists of Sla5045.

The production of a sgRNA is conserved in all members of the Caliciviridae yet the mechanism of its synthesis is poorly understood. In contrast, the synthesis of sgRNA is well characterized in numerous other RNA viruses (Reviewed in 14). For viruses that possess only a single genomic RNA and
generate a single sgRNA, one commonly used mechanism for sgRNA synthesis is the premature termination of negative-strand RNA synthesis that is used by the viral replicase as a template to produce the positive-sense sgRNA. A hallmark of the premature termination mechanism is the production of a truncated negative-sense sgRNA during virus replication. Low levels of negative-sense sgRNA have been identified in cells stably replicating the Norwalk virus replicon (29), in purified replication complex from feline calicivirus infected cells (28) and as now in MNV infected cells (Fig 8). However, our data and those from previous publications (15) would suggest an alternative model for MNV sgRNA synthesis. This mechanism is used by plant viruses (23, 30, 31) and alphaviruses (32, 33) and involves the binding of the viral replicase complex to a specific sequence and/or structure present in negative-sense genomic RNA. Our data is consistent with Sla5045 being required as an RNA structure needed for MNV infectivity and to function as a template for RNA synthesis by the RdRp. It is also important to note that in the plant viruses, it is not clear which subunit(s) of the viral replicase can specifically recognized the sgRNA promoter and our results demonstrate that the norovirus RdRp is sufficient for recognition of the sgRNA core promoter. In addition we observed that a genotype-specific interaction between the RdRp and the subgenomic proscript affected the amount of RNA synthesis (Fig. 7D). Our data would however indicate that as negative-sense sgRNA is present in MNV infected cells, the newly synthesized MNV sgRNA may function as a template for additional rounds of RNA replication by the viral RdRp via a dsRNA intermediate.
The precise initiation site of the calicivirus sgRNA has been identified previously in a number of studies. The human norovirus sgRNA initiation site has been previously confirmed using a helper virus system to drive the production of viral genomic RNA in cells (34). In the related vesivirus FCV, 5'RACE was also used to identify the specific sgRNA initiation site (35). To our knowledge, our work provides the first confirmation of the start site of norovirus sgRNA during an authentic infectious norovirus replication cycle.

Our data would indicate that the positioning of the core sgRNA promoter is critical for its function as in the Sla5045Dup constructs, only the sgRNA promoter sequences in Sla2 region functioned (Fig. 5). In addition, increasing the distance between the stem-loop structure and the sgRNA initiation site from 6 to 8 nts, also debilitated virus replication (Fig. 6). These data are in agreement with our previous observations using bioinformatics analysis of all calicivirus genomes that indicate an absolute conservation of the spacing i.e. invariably 6 nts (16).

Overall our data demonstrate that norovirus sgRNA synthesis relies on a sequence and genotype-specific interaction of the viral RdRp with a stem-loop sequence on the minus-strand RNA. These observations add to our growing understanding of the norovirus life cycle and the molecular mechanisms used by caliciviruses to control viral genome translation and replication.

Acknowledgements
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References


Figure legends:

Fig. 1. The stability of Sla5045 is required for norovirus infectivity, replication and sgRNA synthesis.

A) A schematic of the murine norovirus (MNV) genome, highlighting the four open reading frames and Sla5045. B) Genetic conservation of the Sla5045 sequences on the norovirus negative-sense viral RNA. The position of the potential norovirus subgenomic RNA initiation site is indicated with an arrow. The position of the VP1 initiation codon on the positive-sense RNA is highlighted in bold and sequence variation observed in different isolates is shown as bold italic text. Genogroup II noroviruses (GII); the sequence of the GII isolate Hu/GII.4/MD-2004/2004/US is shown (DQ658413) with variation between the following isolates shown: FJ595907.1, AB447425.1, HM635151, HM635164, JF262610.1 and JF262592. Genogroup V (GV) murine norovirus; the sequence of the CW1 isolate (DQ285629) is shown with variation between the following isolates displayed; JN975491 and JN975492. Note that for simplicity, only sequences showing variation in the stem-loop sequence were used in the analysis and shown in the figure. C) The structure of Sla5045 and the mutations introduced to generate the mutant m53 and m53r as described previously (16). In the case of m53r, the mutations shown are in addition to those present in m53. D) Luciferase replicon based analysis of the effect of Sla5045 disruption. Luciferase levels represent the mean of triplicate independent samples. E) Virus yield after reverse genetics recovery of full-length cDNA constructs containing
either MNV wild type (WT), a polymerase frame shift in the NS7 (POL\textsuperscript{FS}), m53 or m53r. The virus titres represent the mean values obtained 24 hours post transfection of viral cDNA expression constructs. The detection limit of the assay is indicated with a dotted line. In all cases where shown, error bars represent the SEM. F) Primer extension-mediated detection of sgRNA synthesis in MNV infected cells (Inf) or cells transfected with capped RNAs of WT, POL\textsuperscript{FS}, m53 or m53r clones. \textit{In vitro} transcribed sgRNA is was used as a positive control (sgRNA) and produces a product 3 nts longer due to the addition of three 5’ G nts by T7 RNA polymerase. Note that 1000 fold less RNA was used for the reaction using RNA from infected cells.

\textbf{Fig. 2: Sequence analysis of m53 phenotypic revertants and second site suppressor mutants.}

A) Positions mutated in the m53 mutant are shown in bold, with the any changes observed in the phenotypic revertants highlighted in grey. The nucleotide sequences shown are for the negative-sense RNA genome. An asterisk indicates that the identified sequence change results in a coding change in the viral NS7 protein (T439A). Revertant mutants are defined as those that phenotypically restore the base pairing of Sla5045. Suppressors are those that retain the mutations in Sla5045 but have additional mutations outside of the region. B) Location of reversion mutations isolated after repeated passage of m53 in cell
culture. Note that the suppressor mutations are not shown as they lie outside of the region containing Sla5045.

**Fig. 3: Characterization of m53 suppressor mutations.**

A) Virus yield following reverse genetics rescue of cDNA constructs containing with WT or m53 MNV cDNA with or without the additional second site mutations as detailed in Table 1. Reverse genetics recovery was performed as described in the materials and methods then the virus yield at 24 hours post transfection determined by TCID50. The dotted line represents the detection limit of the assay with error bars representing the SEM. Single step growth curve analysis of m53SupA replication in comparison to the WT MNV. RAW264.7 cells were infected at a multiplicity of infection of 4 TCID50 per cell and samples harvest at the indicated time post infection. Infectious virus was released by freeze-thaw and titrated. Infectious virus titres are shown in panel B, protein expression levels of NS7 and VP1 in Panel C and viral genomic RNA levels in panel D. In panel D only samples from the exponential growth phase are shown. Error bars represent the SEM.

**Fig. 4: Sequence changes at position 4922 can compensate a defect in Sla5045.**

A) Schematic illustration of the region of the MNV genome mutated in m53 and m53SupA. The sequence of the region shown is represented in the positive polarity to highlight the NS7 reading frame with the negative polarity sequence
shown below. The amino acids encoded by the region are highlighted, as well as the position of the SupA mutation (in italics) and the mutations introduced in m53.

B) Virus yield following reverse genetics rescue of cDNA constructs containing with wild-type (WT) or m53 MNV cDNA with various changes at position 4922. Note that the nucleotide sequences shown represent the sequence of the anti-sense RNA genome with G representing the previously isolated SupA suppressor mutation and A representing the nucleotide present in the wild-type cDNA construct. Reverse genetics recovery was performed as described in the materials and methods then the virus yield at 24 hours post transfection determined by TCID50. The dotted line represents the detection limit of the assay with error bars representing the SEM.

**Fig. 5: Duplication of Sla5045 enables mutagenesis in a non-coding context**

A) Schematic illustration of the reconstruction of Sla5045Dup stem-loop duplication constructs. The position of the authentic Sla5045 on the anti-sense RNA genome is shown and the introduced additional Sla5045 shown as Sla2. For simplicity, only wild-type copies of the stem-loop are shown. The positions of the NS7 stop and VP1 start codons on the corresponding positive sense viral RNA are also shown for reference.

B) Virus yield following reverse genetics rescue of capped *in vitro* transcribed RNA of wild type (WT), polymerase frameshift (POL<sup>FS</sup>), m53 or Sla5045 stem-loop duplication (Sla5045Dup) constructs. The Sla5045Dup constructs are shown using the nomenclature copy1/copy2 as described in the text. Reverse genetics recovery was performed as described in
the materials and methods using in vitro transcribed, enzymatically capped RNA and the virus yield at 24 hours post transfection determined by TCID50. A western blot analysis for NS7 expression is shown below the virus yield data. The asterisk indicates a greater than full length NS7 product observed in cells transfected with SLa5045Dup WT/WT. C) Virus yield assay following a single passage of virus recovered following RNA transfection of wild type MNV (WT) or various Sla5045 stem-loop duplications. The dotted line represents the detection limit of the assay with error bars representing the SEM. D) 5'RACE analysis of WT MNV or Sla5045 stem-loop duplications. Viral RNA was isolated from infected cells and subjected to 5' RACE analysis. Samples were prepared with or without reverse transcription (RT) and then subjected to PCR as described in the materials and methods. The wild-type MNV virus was included as a control. Samples were then sequenced and aligned as shown. The lower case polyG tract is introduced as a result of the 5' RACE methodology.

Fig. 6. Mutational analysis of Sla5045 in a non-coding context.
A) Schematic illustration of Sla5045 mutants characterized in the Sla5045Dup cDNA backbone. The Sla5045 is shown in the 3'-5' direction with the site of subgenomic RNA initiation shown in italics. Mutations introduced are highlighted in bold. B) Virus yield following reverse genetics rescue of capped in vitro transcribed RNA of WT, polymerase frame-shift (POL<sup>FS</sup>), m53 or Sla5045 stem-loop duplication (Sla5045Dup) constructs. The Sla5045Dup constructs are shown using the nomenclature copy1/copy2 as described in the text. Reverse
genetics recovery was performed as described in the materials and methods using *in vitro* transcribed, enzymatically capped RNA and the virus yield at 24 hours post transfection determined by TCID$_{50}$. C) Virus yield assay following a single passage of virus recovered following RNA transfection the various Sla5045 stem-loop duplications. The dotted line represents the detection limit of the assay with error bars representing the SEM.

**Fig. 7: Sla5045 can direct for the initiation of RNA synthesis by the MNV RdRp *in vitro***.

A) Recombinant RdRps from MNV and the human GII.4 norovirus used in this study. The RdRps was expressed and purified as described in the materials and methods. The gel images were from SDS-PAGE stained with Coomassie Blue.

B) Schematics of the synthetic MNV proscript, MNVps, and the GII.4 RNA proscript, GII.4Ps, used for the *in vitro* RNA synthesis assays. The initiation nucleotide used to initiate MNV subgenomic RNA synthesis is in bold and denoted with a bent arrow. A proscript containing a mutated initiation site named IM is used to control for specificity. C) Denaturing PAGE analysis of *in vitro* RNA synthesis assays containing the MNV NS7 with either MNVps or the initiation mutant (IM). The positions of primer extension (PE) and *de novo* (DN) initiation products are highlighted with arrows. The lengths of the RNAs were assigned by comparison to RNAs of 8 to 51-nt that were labeled at the 5′ terminus with a $^{32}$P.

D) Virus genotype-specific RdRp-proscript interaction results in higher level of
RNA-dependent RNA synthesis. The results are representative of six independent experiments.

**Fig. 8: Negative-sense subgenomic RNA is produced during MNV replication.** A) Northern blot analysis of RNA isolated from either mock infected (mock) or MNV infected (Inf) cells. 1.5μg of RNA harvested 10 hours post infection of cells with a multiplicity of infection of 5 TCID50 per cell, was denatured by glyoxylation prior to agarose gel electrophoresis. Positive and negative-sense RNA was detected using strand specific RNA probes as described in materials and methods. Control *in vitro* transcribed RNAs were generated representing the positive- (+) or negative-sense (-) viral RNAs were used as a control. B). Quantitative real-time PCR analysis of the RNA sample shown in panel A. The RNA was analyzed using a strand-specific RT-qPCR assay designed to detect only the genomic RNA or both genomic and subgenomic RNAs simultaneously. The genome copy number is shown as genome equivalent per 25ng of total RNA and was determined by comparison to *in vitro* transcribed control RNAs. The data show was represent the mean and standard deviation of 4 independent repeats.
### Virus Nucleotide position within Sla5045 Changes outside of Sla5045

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**Diagram**

![Diagram showing nucleotide positions and mutations](attachment:image.png)
A

NS7

VF1

Sla5045

WT A(SupA) G

U C

WT A(SupA) G

U C

1 2 3 4

Position 4922 Sequence

TCID50/mL (Log_{10})

WT back bone

m53 backbone

B

WT

m53 SupA

Positive sense

Negative sense

UGA CAC CGA AGG-90 nts-CUA CGA CUC UGG

UGA CAC CGG AGG-90 nts-CUG CGG CUU UGG

5'
A

![Diagram of NS7 and VF1 genes with sgRNA Start 1 and sgRNA Start 2](image)

B

![Graph showing TCID50/mL (Log10) for WT, POL, FS, m33, WT/m33, m33/WT, m33/m33](image)

C

![Bar chart showing TCID50/mL (Log10) for WT, WT/WT, WT/m33, m33/WT, m33/m33](image)

D

![Image of gel electrophoresis with arrow indicating 60kDa and NS7](image)

![Image of gel electrophoresis with bands labeled Sla5045Dup WT, Sla5045Dup WT/WT, Sla5045Dup m33/WT](image)
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### Graph B

- **Y-axis**: TCID50/mL (Log_{10})
- **X-axis**: Sample Types
- **Legend**: WT, POLfs, m53/m53r, m53/m53 WT+8, m53/TL-dis, m53/SL+, m53/SL-, m53/S2+

### Graph C

- **Y-axis**: TCID50/mL (Log_{10})
- **X-axis**: m53/m53, m53/m53 WT+8, m53/TL-dis, m53/SL+, m53/SL-, m53/S2+
In vitro transcribed control RNA

B

Genome Eq/25ng RNA (Log10)

Positive-Sense RNA  Negative-Sense RNA

gRNA  gRNA + sgRNA  gRNA  gRNA + sgRNA