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Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line

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Abstract

Norwalk virus (NV) is a prototype strain of the genus *Norovirus* in the family Caliciviridae. The human noroviruses have emerged as major agents of acute gastroenteritis in all age groups, but there are no vaccines or antiviral agents partly due to the absence of a cell culture system. We report the generation of cells expressing self-replicating NV RNA (NV replicon) following transfection of NV RNA bearing an engineered neomycin resistance gene into cell lines of human (Huh-7) or hamster (BHK21) origin. Expression of replicon RNA was significantly reduced in the presence of interferon (IFN)-α in a dose-dependent manner in the NV replicon-bearing cells, suggesting a role for innate immunity in the control of human norovirus replication. This stable NV replicon system should lead to new insights into norovirus replication, virus–host interactions, and approaches for the treatment of norovirus disease.

Keywords: Norwalk virus; Replicon-bearing cells; Huh-7; Interferon; RNA replication

Introduction

Caliciviruses are positive strand RNA viruses in the family Caliciviridae that consists of four genera, *Norovirus*, *Sapovirus*, *Lagovirus*, and *Vesivirus*. Viruses in the genera *Norovirus* and *Sapovirus* cause gastroenteritis in humans and animals. Recent studies estimate that noroviruses are responsible for more than 90% of nonbacterial gastroenteritis outbreaks (Fankhauser et al., 1998) and as many as 23 million cases of gastroenteritis in the United States each year (Mead et al., 1999). Norwalk virus (NV), a prototype strain of the noroviruses, was associated with an outbreak of gastroenteritis in Norwalk, Ohio, in 1968 (Kapikian et al., 1972). The viral genome is ~7.7 kb in length and organized into open reading frames (ORFs) 1, 2, and 3, that encode an ~1800-amino-acid (aa) polyprotein, the major capsid protein (VP1), and a minor capsid protein (VP2), respectively (Jiang et al., 1993; Xi et al., 1990). The NV ORF1 polyprotein is processed by the viral proteinase (Pro) into several nonstructural proteins with the gene order: N-terminal protein (45 kDa); NTPase (40 kDa); "3A-like" protein (22 kDa); VPg (16 kDa); Pro (20 kDa); RNA-dependent RNA polymerase (Pol) (57 kDa) (Blakeney et al., 2003; Hardy et al., 2002). The structural proteins, VP1 and VP2, are synthesized from a subgenomic RNA containing ORFs 2 and 3 (Glass et al., 2000; Jiang et al., 1992). Calicivirus replication occurs in association with intracellular membranes and likely proceeds through a minus strand RNA intermediate that is used as the template for the synthesis of positive-sense full-length genome and subgenomic RNA (Green et al., 2002).

Studies of noroviruses associated with disease in humans have been hampered by the continued absence of a cell culture system (Duizer et al., 2004). Two recent advances in norovirus research include the discovery of a murine norovirus that grows in a murine macrophage-like cell line (Wobus et al., 2004), and the demonstration that transfection of a full-length

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cDNA clone of the NV genome (under control of the T7 promoter) into modified vaccinia Ankara (MVA)-T7 infected cells allowed the expression of viral proteins and subsequent NV RNA replication (Asanaka et al., 2005). Here, we report the generation of a stable RNA replicon system for NV that functions in both human Huh-7 cells and hamster BHK21 cells, and that circumvents the need for a helper virus. The replicon-bearing cells expressed NV proteins and RNA and could be maintained after multiple passages in the presence of G418. Importantly, the replicon-bearing cells could be examined for the effects of potential viral inhibitors, and we provide evidence that NV replication is sensitive to the effects of exogenous interferon (IFN). Virus replicon systems have proved an important tool in the investigation of virus–host interactions (Blight et al., 2000; Foy et al., 2003; Gale and Foy, 2005), and the availability of a NV replicon provides a new system in which such interactions can be assessed for the human noroviruses.

Results

Generation and characterization of NV replicon-bearing cells

Plasmid NV101, which contained a cloned cDNA consensus sequence of the RNA genome of NV (Fernandez-Vega et al., 2004), was engineered to encode the neomycin resistance gene within ORF2 (Fig. 1A). The resulting plasmid was designated pNV-Neo. A second plasmid, designated pNV-NeoΔGDD, was engineered to abolish RdRp activity of the viral Pol by deletion of the GDD motif within the Pol coding sequence. Following the transfection of RNA transcripts derived from pNV-Neo or pNV-NeoΔGDD into BHK2, Vero, 293, 293T LLC-PK and Huh-7 cells, viable cell colonies were subsequently selected in the presence of G418 (0.5 mg/ml) only in BHK21 cells transfected with RNA derived from pNV-Neo. The cells were subjected to cloning by limiting dilution, and two clones (G3 and G6) were chosen for further characterization (data not

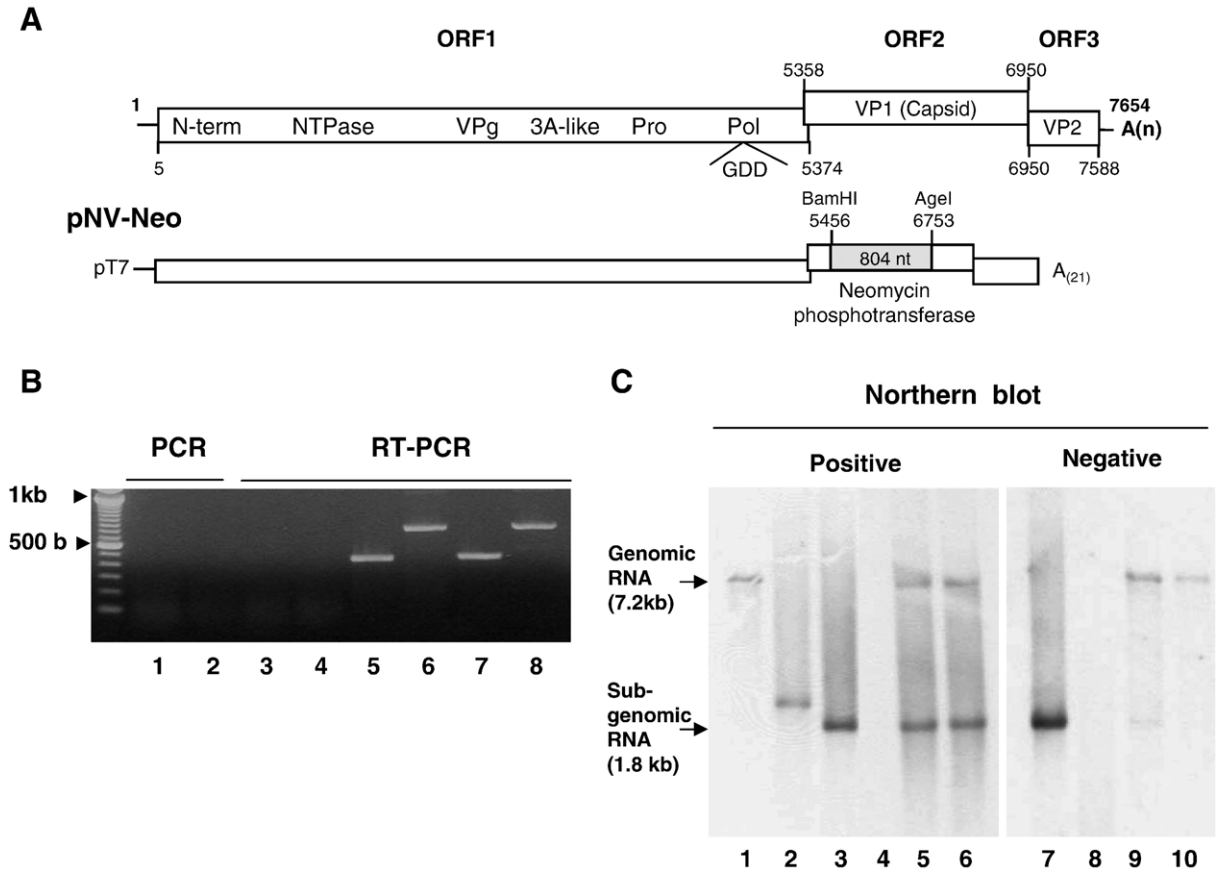

Fig. 1. Generation of construct pNV-Neo and analysis of RNA expression in replicon-bearing cells HG23. (A) Schematic diagram of the genome organization of NV and the NV replicon construct (pNV-Neo). The location of the GDD motif deleted in construct pNV-NeoΔGDD is indicated. (B) Detection of NV-specific RNA in HG23 cells by RT-PCR. Total cellular DNA was isolated from parental Huh-7 (lane 1) or HG23 (lane 2) cells and PCR was performed with primers Neo-F and Neo-R that were designed to amplify the full length neomycin phosphotransferase gene (804 bp). Lanes 3 to 8 contain RT-PCR products obtained by analysis of total RNA extracted from HG23 cells (at two different passages) with NV-specific primers, NVp35 and NVp36 (470 bp) (lanes 5 and 7) or Neo-F and Neo-R (lanes 6 and 8). Controls (lanes 3 and 4) included PCR analysis (in the absence of RT and with primer pair Neo-F and Neo-R) of RNA from HG23 cells. (C) Northern blot analysis of NV positive (lanes 1–6) and negative sense RNA (lanes 7–10). Lanes 1 to 3: positive sense RNA transcript controls representing the full-length RNA encoded in pNV-Neo (lane 1), the subgenomic RNA of parental NV (lane 2) and the subgenomic RNA encoded in pNV-Neo (lane 3). Lanes 4 to 6: RNA purified from Huh-7 (lane 4) and HG23 cells (lanes 5 and 6, two different passages). Lane 7: RNA transcripts corresponding to the negative sense subgenomic RNA encoded in pNV-Neo. Lanes 8 to 10: RNA preparation from Huh-7 (lane 8) and HG23 cells (lanes 9 and 10). For Northern blot analysis, mRNA from Huh-7 or HG23 cells were isolated and enriched with MicroPoly(A) purist kit (Ambion).

shown). Transfection of Huh-7 cells with RNA transcribed from pNV-Neo proved inefficient in the selection of replicon-bearing cells, and a strategy was developed in which replicon RNA purified from G3 cells (passage number 60) was used in the transfection. Viable Huh-7 cell colonies bearing the NV replicon were then selected in the presence of G418, and a cell clone designated HG23 was selected for further study.

A gene-specific PCR assay for the neomycin phosphotransferase gene was used to verify that the neomycin resistance gene had not integrated into the cellular DNA of HG23 cells. Total DNA purified from Huh-7 or HG23 cells was analyzed for the presence of the gene by PCR (in the absence of RT), and no PCR products were observed (Fig. 1B, lanes 1 and 2, respectively). In contrast, analysis of the isolated RNA from HG23 cells (at two different passages) by RT-PCR yielded an 804-bp product consistent with that expected for the neomycin resistance gene (Fig. 1B, lanes 6 and 8). In addition, the presence of NV-specific RNA was confirmed in the two preparations of HG23 cells by RT-PCR with diagnostic primers NVp35 and NVp36 (Atmar et al., 1995), that would produce an expected product of 470 bp (Fig. 1B, lanes 5 and 7). It should also be noted that RNA purified from the parental Huh-7 cells was negative for the presence of the neomycin resistance gene and NV RNA by RT-PCR (data not shown), which was consistent with their inability to survive in the presence of G418.

The RNA species produced in HG23 cells were examined by Northern blot analysis. Sense and anti-sense RNA probes specific for the NV ORF3 region were hybridized with RNA that was extracted from HG23 or parental Huh-7 cells and further enriched with oligo(U) purification. Genomic and subgenomic RNA controls for the Northern blot included a full-length RNA transcript (positive sense) derived from linearized pNV-Neo, and subgenomic-length positive or negative sense RNA molecules (representing wild type or replicon NV) derived by transcription from corresponding PCR products containing an engineered T7 promoter. The full-length RNA transcript obtained from pNV-Neo was detected as a band of ~7.2 kb in size in the Northern blot for positive strand RNA (Fig. 1C, lane 1). The positive sense subgenomic RNA transcripts representing wild type NV and replicon NV were detected as bands sized at ~2.3 kb and ~1.8 kb, respectively (Fig. 1C, lanes 2 and 3). The negative sense subgenomic replicon RNA control was also detected as a band at ~1.8 kb (Fig. 1C, lane 7). The Northern blot analysis for positive strand RNA showed that RNA purified from the two passages of HG23 cells contained both genomic and subgenomic sized RNA species that corresponded in size to the replicon RNA controls (Fig. 1C, lanes 5 and 6). The same RNA samples (loaded at 10 times higher concentration) analyzed in a Northern blot for negative strand RNA showed the presence of a full-length RNA molecule of the expected length and low to undetectable levels of a negative sense subgenomic RNA (Fig. 1C, lanes 9 and 10). Negative sense subgenomic RNA was difficult to detect in BHK21-derived G3 and G6 cells as well, even when total RNA without oligo(U) enrichment was analyzed (data not shown). The lower amounts of negative strand RNA compared to positive strand are an expected feature of positive strand RNA replication (Blight et al., 2000). However, it was of interest that negative strand full-length genomic RNA was present following oligo(U) enrichment. This too might be expected in that a replicative form consisting of a closely associated plus and minus strand has been demonstrated in cells infected with other positive strand RNA viruses (Pogue et al., 1994).

Expression of NV proteins in HG23 cells was examined by immunofluorescence assay (IFA) and Western blot analysis (Fig. 2). Controls for these experiments included the analysis of NV proteins expressed in Huh-7 cells infected with MVA-T7 and transfected with the NV full-length clone NV101. Hyperimmune serum raised in a guinea pig against recombinant NV ProPol showed a positive signal in the IFA when reacted with HG23 cells (shown in Fig. 2A, panels 1 and 2 at high (200×) and low (100×) magnification and with Huh-7 cells infected with MVA/T7 and transfected with NV101 (Fig. 2A, panel 5) (100×). Of interest, discrete areas of localized antigen expression were observed in the cytoplasm of HG23 cells near and around the nucleus reminiscent of the vesiculated areas observed in murine norovirus-infected cells (Wobus et al., 2004) and the perinuclear localization of the NV N-terminal protein expressed alone in transfected cells (Fernandez-Vega et al., 2004). The NV ProPol hyperimmune serum showed no reactivity in the IFA with the parental Huh-7 cells (Fig. 2B, panel 4) or with Huh-7 cells infected with MVA/T7 (Fig. 2A, panel 6). In addition, preimmune serum from the same guinea pig did not react with the HG23 cells (Fig. 2A, panel 3). Hyperimmune sera raised against the NV ProPol or NTPase detected proteins by Western blot analysis consistent in observed mass with the mature Pol (57 kDa) and Pro (19 kDa) or NTPase (40 kDa), respectively, in NV101-transfected cells infected with MVA/T7 virus (Fig. 2B, lane 2 and Fig. 2C, lane 6). A protein profile similar to that of the NV101 expression experiment was observed in the Western blot analysis of three different passages of HG23 cells probed with the NV-specific ProPol (Fig. 2B, lanes 4–6) or NTPase-specific sera (Fig. 2C, lanes 2–4). No evidence for NV-specific proteins were detected in Huh-7 cells (Fig. 2B, lane 3 and Fig. 2C, lane 1) or in Huh-7 cells infected with MVA-T7 (Fig. 2B, lane 1 and Fig. 2C, lane 5). These data show that the nonstructural proteins are expressed in the replicon-bearing cells, and that authentic proteolytic processing of the nonstructural polyprotein encoded in ORF1 likely occurs. Sequence analysis of the replicon RNA purified from G3 and G6 cells soon after selection in G418 (less than passage number [P] 10) found no sequence differences compared to that of the NV replicon encoded in plasmid NV101. At passage 60, the RNA from G3 cells was purified and examined again by sequence analysis. At this later passage, two nucleotide changes were detected that resulted in two amino acids changes: one in ORF1 (N-terminal protein) and one in ORF3 (VP2) (Table 1). Following transfection of the P60 RNA from G3 cells into Huh-7 cells, additional mutations were detected in the analyzed P8 RNA that included four HG23 cell-specific mutations in ORF1 (one amino acid each

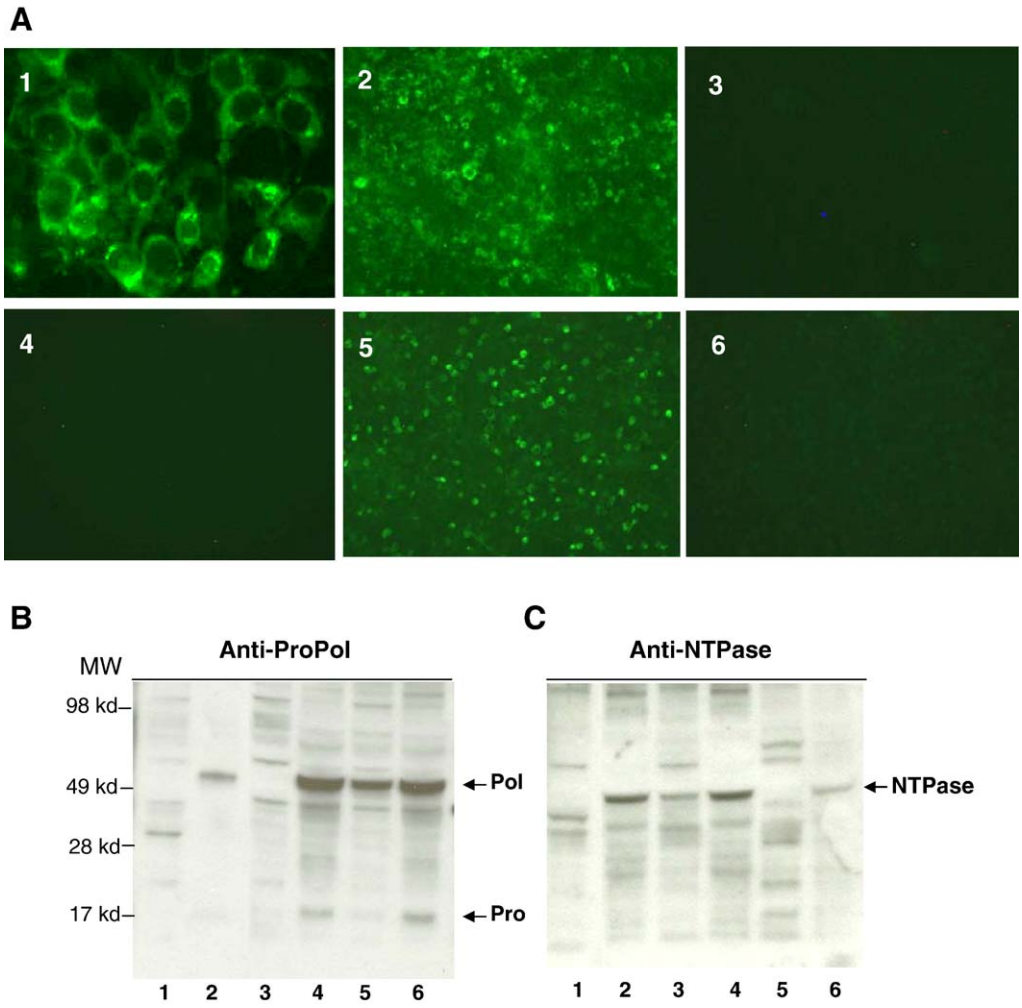

Fig. 2. Detection of Norwalk virus protein expression in replicon-bearing cells (HG23) by IFA and Western blot analysis. (A) IFA staining was performed with preimmunization or post-immunization serum obtained from a guinea pig hyperimmunized with NV recombinant ProPol protein. Panels 1 and 2: HG23 cells reacted with ProPol post-immunization serum and shown at high (200×) or low (100×) magnification, respectively. Panel 3: HG23 cells reacted with the preimmunization serum from the same guinea pig. Panel 4: parental Huh-7 cells reacted with the ProPol hyperimmune serum. Panels 5 and 6: Huh-7 cells that were infected with MVA/T7 and then transfected with pNV101 (Panel 5) or mock-transfected with PBS (Panel 6). (B) Western blot analysis with antibodies specific for the NV ProPol. Lanes 1 and 2: total lysates of Huh-7 cells that were infected with MVA/T7 and then either mock transfected with PBS (Lane 1) or transfected with pNV101 (Lane 2). Lane 3: a lysate prepared from Huh-7 cells without treatment. Lanes 4–6: lysates prepared from three different passages of HG23 cells. C. Lane 1: a lysate prepared from Huh-7 cells without treatment. Lanes 2–4: lysates prepared from three different passages of HG23 cells (the same samples analyzed above in panel B). Lanes 5 and 6: lysates from Huh-7 cells infected with MVA/T7 and then either mock transfected with mock PBS and pNV101 after MVA-T7 infection, respectively.

in the N-terminal protein, NTPase, Pro and Pol) and three additional mutations (two amino acids and one silent mutation) in the ORF3 region (Table 1). No mutations were observed in the 5′ and 3′ nontranslated regions of HG23 replicon RNA as determined by 5′ and 3′ RACE, respectively. Both G3 and HG23 cell lines were stable and NV replicon positive without significant changes in the expression of NV proteins at least 100 passages (the maximum number we passaged them so far) in the presence of G418.

Infectivity of RNA isolated from replicon-bearing cells

A colony-forming assay was developed in order to assess the infectivity of RNA purified from replicon-bearing cells. Transfection of RNA replicon extracted from HG23 cells into fresh BHK21 cells produced viable cell colonies in the presence of G418 (Fig. 3B, panel 2), and an enzyme-based immunostaining technique that used NV ProPol-specific antiserum confirmed the expression of NV proteins (Fig. 3, panel 5). Controls for the immunostaining experiments included an G3 cell monolayer that showed positive reactivity with the ProPol serum (Fig. 3, panel 6), and an BHK21 monolayer that showed no reactivity with the same serum (Fig. 3, panel 8). The number of colonies selected after transfection of BHK21 cells with replicon RNA isolated from HG23 cells was higher than that after transfection of BHK21 cells with RNA transcripts (pNV-Neo) (compare Fig 3B, panels 1 and 2). The copy number (genome equivalents) of the RNA transcripts and RNA replicon (from HG23 cells) transfected into BHK21 cells in Fig 3B was calculated as

Table 1
Sequence analysis of NV replicon RNA from G3 and HG23 cells

Open reading frame (ORF)	Genomic position of nucleotide [a]	Position of amino acid (viral protein) [b]	NV101	G3 (P60)	HG23 (P8)
ORF1	311	103 (N-term)	TGG (Trp)	CGG (Arg)	CGG (Arg)
	792	263 (N-term)	GAA (Glu)	GAA	GGA (Gly)
	2061	686 (NTPase)	GAA (Glu)	GAA	GGA (Gly)
	3515	1171 (Pro)	ATG (Met)	ATG	GTG (Val)
	4079	1359 (Pol)	GAG (Glu)	GAG	AAG (Lys)
ORF3	7052	35 (VP2)	TAT (Tyr)	TAT	CAT (His)
	7196	83 (VP2)	GCT (Ala)	GCT	ACT (Thr)
	7372	142 (VP2)	AAT (Pro)	AAT	AAC (Pro)
	7577	210 (VP2)	AAT (Asn)	GAT (Asp)	GAT (Asp)

[a] Nucleotide are numbered according to sequence of NV virus genome as follows: ORF1 (nt 5–5374) and ORF3 (nt 6950–7588), encoding a large polyprotein (viral nonstructural proteins) and VP2, respectively.

[b] Amino acid residues are numbered according to location in the corresponding ORF. The nucleotide of interest is underlined and its location in the codon of the translated ORF is shown. N-term: N-terminal protein. Pro: proteinase. Pol: polymerase.

2.6×10^{11} (1 μg of RNA) and 8.0×10^{8}, respectively by qRT-PCR. Because the infectivity of calicivirus RNA purified from virions (or infected cells) is severely reduced by the treatment of RNA with proteinase K (Black et al., 1978; Chang et al., 2002; Herbert et al., 1997), we examined whether treatment of the replicon RNA with this enzyme decreased its infectivity in the colony-forming assay. Treatment of the replicon RNA with proteinase K (100 μg/ml) at 37 C for 4 h prior to transfection abolished the selection of cell colonies in the presence of G418 (Fig. 3B, panel 4). This result suggests that the infectivity of replicon RNA purified from cells is enhanced by the presence of the VPg protein.

Effect of IFN-α on the NV replicon

Recent studies have reported that innate immunity plays an important role in the control of murine norovirus infection (Karst et al., 2003; Wobus et al., 2004), so we examined whether NV replication in Huh-7 cells was sensitive to the effects of IFN-α. The HG23 cells were treated with increasing concentrations of human IFN-α (up to 20 U/ml), and its effect on protein expression was monitored by IFA (Fig. 4A) and Western blot analysis (Fig. 4B). In addition, a qRT-PCR assay developed by Kageyama et al. (2003) was used for a comparative analysis of RNA levels present in mock-treated or IFN-α-treated replicon-bearing cells. The addition of IFN-α to HG23 cells inhibited NV protein expression in a dose-dependent manner (Figs. 4A and B), while the cells themselves showed no toxic effects of the treatment (data not shown). Additional controls in the Western blot analysis showed that the expression of STAT1 increased proportionately to the concentrations of IFN-α, while the β-actin loading control remained constant (Fig. 4B, lanes 2–7). The levels of NV-specific RNA as measured by qRT-PCR decreased also in a dose-dependent manner (Fig. 4C). The presence of 20 U/ml of IFN-α for 72 h resulted in nearly complete clearance of the replicon proteins and RNA, and the effective dose of IFN-α for reducing NV protein

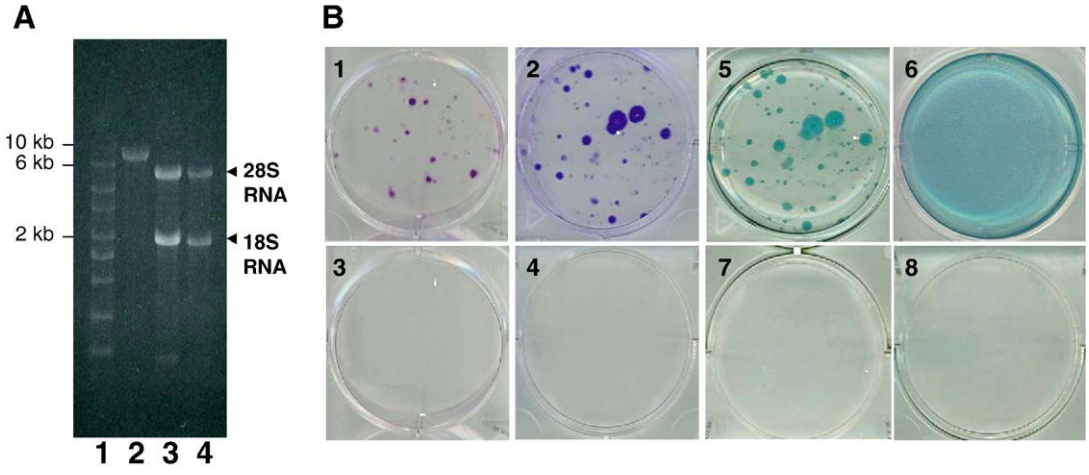

Fig. 3. Colony formation assay in BHK21 cells. BHK21 cells were transfected with RNA derived either by in vitro transcription from pNV-Neo or by isolation from replicon-bearing HG23 cells. G418-resistant cell colonies were then selected in the presence of the antibiotic. (A) RNA preparations used in the transfection. Lane 2: RNA transcripts from pNV-Neo, and lanes 3 and 4: total RNA from HG23 cells after treatment with proteinase K (lane 3) or PBS (lane 4). (B) Colony formation after RNA transfection and incubation with G418. Colonies were visualized by crystal violet staining (panels 1–4) or by an enzyme-based immunostaining technique that used ProPol antibody (panels 5–8). Panels 1 and 3: BHK21 cells at 2 weeks following transfection with RNA transcripts derived from pNV-Neo and or mock transfection with PBS, respectively. Panels 2 and 4: BHK21 cells at 2 weeks following transfection with RNA extracted from HG23 cells that was treated with PBS (mock control) or proteinase K, respectively. Panels 5 and 7: the same cells as panels 2 and 4 (cells were stained with crystal violet after the immunostaining procedure). Panels 6 and 8: immunostaining assays on confluent G3 and BHK cells, respectively.

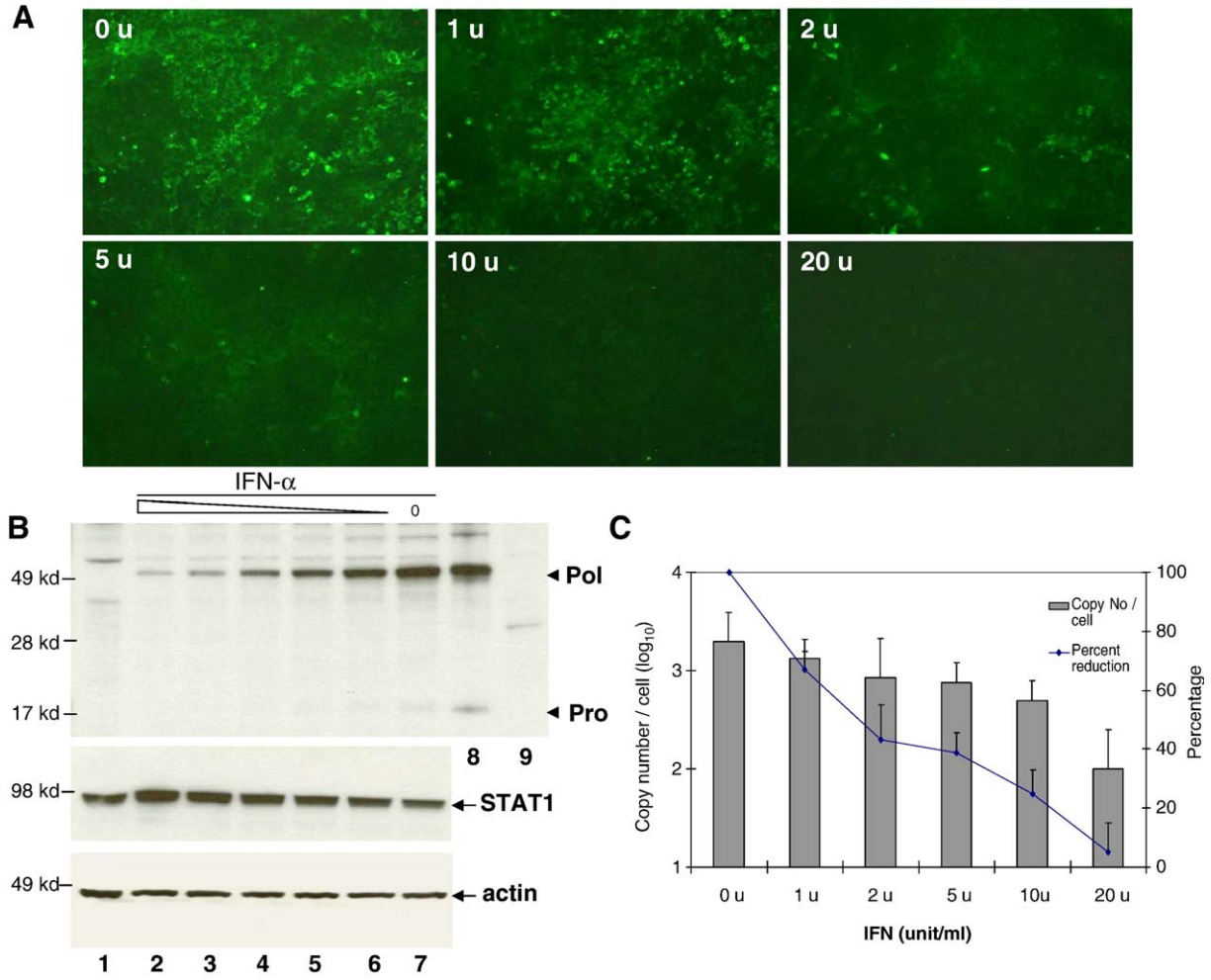

Fig. 4. Effect of IFN-α treatment on the NV replicon in HG23 cells. HG23 cells were treated with increasing concentrations of IFN-α (0 to 20 U/ml) and examined after 72 h. (A) IFA staining (using the ProPol antibody) of HG23 cells incubated with the indicated concentrations of IFN-α. (B) Western blot analysis of HG23 cell lysates incubated with varying concentrations of IFN-α and analyzed with antibodies specific for NV ProPol protein (upper panel), STAT1 (middle panel) and β-actin (lower panel). In all panels, lane 1: lysates from nontreated Huh-7 cell lysates, and lanes 2 to 7: lysates from HG23 cells treated with 20, 10, 5, 2, 1 and 0 U/ml of IFN-α, respectively. Lanes 8 and 9: lysates from Huh-7 cells infected with MVA/T7, and then transfected with pNV101 or PBS, respectively. (C) Copy numbers of NV genome per cell as determined by qRT-PCR. Bars represent the copy number/cell (log10) of NV genome and symbols ♦ represent the percent reduction (compared to cells without treatment, 0 IFN-α) of NV genome in HG23 cells treated with varying concentrations of IFN-α, respectively. A comparison of the total replicon RNA purified from nontreated HG23 cells with a standard curve (generated by the dilution of RNA transcripts derived from pNV-Neo) showed that total RNA replicon was usually present in a calculated range of 1000–3000 copies per cell.

(ProPol) and genome copies in HG23 cells to 50% (ED_{50}) of that observed in the nontreated (mock) control at 72 h was calculated to be approximately 2 U/ml (Figs. 5B and C). The sensitivity of the NV replicon to human IFN-α was observed also in the BHK21-based G3 cells, but the ED_{50} was ten times higher (20 U/ml) (data not shown).

The NV replicon does not interfere with the induction of an IFN response in HG23 cells

We next examined whether the presence of the NV replicon in Huh-7 cells affected the ability of the cells to respond to the induction of IFN by Sendai virus (SeV). Similar studies of the HCV replicon system in Huh-7 cells had led to the identification of strategies utilized by HCV to subvert the innate immune response (Foy et al., 2003). Reporter plasmids for luciferase expression under control of the DNA promoters ISRE (pISRE-TA-luc), NF-kB (pNF-kB-TA-luc), or IFN-β (pIFNβ-TA-luc) were transfected into Huh-7 cells or HG23 cells and expression of luciferase was measured in the presence or absence of SeV infection. The pRL-CMV (for renillar luciferase under CMV promoter) was co-transfected in all experiments to control the efficiency of the transfection and standardize luciferase expression levels. In some experiments, the reporter plasmid was co-transfected with pCI-NVcap, which would express the NV major capsid protein VP1. Analysis of the effect of SeV infection on cells transfected with the reporter plasmids showed a marked similarity between the Huh-7 and HG23 cells in their overall response to the induction of IFN by SeV. The luciferase expression increased up to 30-fold in both parental Huh-7 and HG23 cells transfected with pISRE-TA-luc and infected with

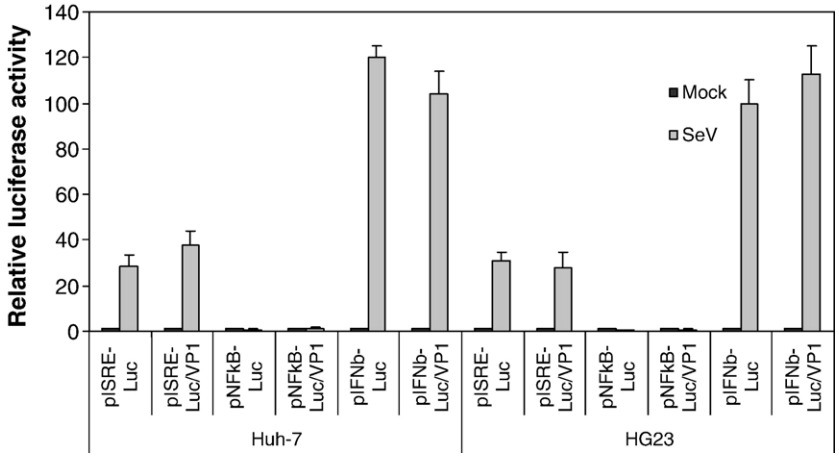

Fig. 5. Luciferase expression under the control of DNA elements of ISRE, NF-kB or IFN-β in Huh-7 and HG23 cells with or without Sendai virus (SeV) infection. One-day-old (~90% confluent) Huh-7 or HG23 cells in 12-well plates were transfected with each reporter plasmid (pISRE-TA-Luc, pNF-kB-TA-Luc or pIFNb-TA-Luc) and pRL-CMV with or without pCI-NVCap (VP1). After 24 h of the transfection, the cells were infected with SeV or mock infected with medium. Luciferase expression was measured at 18 h after SeV infection, and data presented as fold increase of luciferase expression by SeV infection. The luciferase expression (firefly luciferase) was normalized against the expression level of the renilla luciferase. Bars represent standard deviations of at least 3 independent experiments.

SeV (Fig. 5). Similarly, luciferase expression under control of the IFN-β promoter was induced up to 100-fold by SeV infection regardless of the presence of NV replicon (Fig. 5). There were minimum responses of luciferase expression under control of the NF-kB element in both Huh-7 and HG23 cells (Fig. 5). Co-transfection of pCI-NVCap (which provided the NV VP1 capsid protein not present in the replicon) with the reporter plasmids did not result in significant differences in luciferase expression (Fig. 5).

Discussion

In the absence of a permissive cell culture system for the human noroviruses, the availability of a stable cell line expressing NV RNA would allow the study of virus and host interactions and provide a platform for screening anti-viral compounds. This report describes the generation of a NV RNA replicon system in BHK21 and Huh-7 cells. A neomycin resistance gene was engineered into the VP1-encoding region of ORF2, disrupting expression of an intact VP1, but preserving the likely subgenomic promoter, an intact ORF3, and the genomic 3′-end. The presence of NV-specific RNA was demonstrated in the replicon-bearing cells by Northern blot analysis and RT-PCR. Analysis of NV-specific proteins in the replicon-bearing cells showed expression of NV antigen in the cytoplasm and provided evidence for authentic proteolytic processing of the NV nonstructural polyprotein in cells. The NV replicon-bearing cells (G3 and HG23) showed growth kinetics similar to those of the parental BHK21 and Huh-7 cells, with no apparent cytotoxic effects associated with endogenous expression of the NV genome. Sequence analysis of the replicon RNA of G3 and G6 BHK21-based cells showed that no adaptive mutations had occurred immediately after selection and in the early passages. In contrast, the replicon RNA of HG23 Huh-7-based cells accumulated several mutations in ORF1 and ORF3 soon after selection (P8). It remains important to elucidate whether the mutations facilitate efficient replication of replicon RNA in Huh-7 cells (HG23).

The replicon RNA was infectious in that it allowed the selection of new antibiotic-resistant cells when isolated and transfected back into fresh cells. The “infectivity” of the replicon RNA was abolished by treatment with proteinase K. Furthermore, the efficiency of generating antibiotic-resistant cell colonies by replicon RNA purified from the replicon-bearing cells was higher than that of RNA transcripts derived from pNV-Neo. A likely explanation for this latter observation was that the presence of VPg enhanced the infectivity of the replicon RNA, as shown for the viral RNA of other caliciviruses (Black et al., 1978; Chang et al., 2002; Herbert et al., 1997). Of interest, uncapped RNA transcripts derived from pNV-Neo did not produce viable cell colonies following transfection and incubation of cells in the presence of G418 (data not shown). This observation was consistent with studies of RNA transcripts generated in the FCV and PEC calicivirus reverse genetics systems from cDNA clones, in which uncapped RNA was not infectious (Chang et al., 2005; Sosnovtsev and Green, 1995). The NV VPg interacts with host translation initiation factor eIF3, and thus, may play a role in translation of the viral RNA (Daughenbaugh et al., 2003). The infectivity of the NV replicon RNA in both hamster and human cells suggests that the NV VPg can interact with the translation machinery of both species.

The stable NV replicon system allowed the initiation of studies to investigate NV and host interactions relating to innate immunity. A similar cell-based RNA replicon system for HCV allowed the identification of certain evasion mechanisms of intracellular host defense likely used by the virus during infection (Blight et al., 2000; Foy et al., 2003; Gale and Foy, 2005). The NS3/4 protein of HCV was shown to block IRF-3 activation, thereby inhibiting the production of IFN-β in response to SeV infection (Foy et al., 2003). The NS5A protein of HCV, an inhibitor of PKR, was an important site where

adaptive mutations occurred that allowed higher levels of replication by the HCV replicon (Blight et al., 2000; Gale and Foy, 2005). Although evasion mechanisms have been documented for HCV, the HCV replicon was shown to be susceptible to the effects of exogenous IFN in vitro (Blight et al., 2000; Guo et al., 2003). Furthermore, IFN has been a major component in treatment strategies for HCV infection (Feld and Hoofnagle, 2005). In contrast, the West Nile virus (WNV) replicon was resistant to exogenous IFN in vitro (Guo et al., 2003, 2005), and a effective mechanism to disrupt the STAT1 pathway was linked to the nonstructural proteins (Guo et al., 2003, 2005). In this study, we showed that IFN-α efficiently cleared the NV replicon in vitro. The ED_{50} of IFN-α for the NV replicon in HG23 cells (2 U/ml) was comparable to that for HCV in its Huh-7-based replicon system (Blight et al., 2000; Guo et al., 2003). Furthermore, the incubation of IFN-α with HG23 cells induced the expression of STAT1, which suggested a role for the STAT1 pathway in the inhibitory effects of IFN-α on the RNA replicon. It was noteworthy that STAT-1 increased in IFN-α-treated HG23 cells, which would argue against an NV mechanism to degrade the STAT1 protein as documented for certain other viruses (Samuel, 2001). The presence of the NV replicon in Huh-7 cells failed also to inhibit the ability of the cell to mount an innate immune response upon infection with SeV as measured with reporter plasmids pISRE-TA-luc and pIFNβ-TA-luc. Taken together, these data suggest that NV is sensitive to exogenous IFN and, moreover, may lack an active evasion strategy to counteract host cellular defenses involving STAT1-mediated pathways. There have been no reports of a direct mechanism used by other caliciviruses for the control of the innate immune response in cells. Viruses known to lack anti-innate immunity mechanisms (such as IFN-sensitive viruses) often show severe growth restriction in target cells (Durbin et al., 1996; Garcia-Sastre et al., 1998), and it is possible that the inability of NV to exert such mechanisms might be related to its fastidious characteristics in cell culture. An understanding of these virus–host factors involving cellular antiviral defense may facilitate the development of a fully permissive cell culture system for the human noroviruses. In addition, it remains important to elucidate whether interactions occur between the NV replicon and other cellular defense pathways that do not involve IFN.

Although norovirus infection is generally considered self-limiting and a short-term illness, recent findings showed that the infection could last longer than several days or even several months in immunocompromised patients (Green et al., 2001; Nilsson et al., 2003). The treatment options for norovirus infection are limited partly due to the absence of screening systems for antiviral drugs. Interferons are well studied and widely used to treat many viral infections including HCV (Feld and Hoofnagle, 2005). Our data suggest a potential therapeutic application of IFN-α to prolonged norovirus infection, but additional studies will be needed to clarify this activity in vivo. The availability of the first helper virus-free NV replicon system should lead to new insights into norovirus replication, the host cell response to infection, and approaches for the treatment of norovirus disease.

Materials and methods

Cells and reagents

The BHK2, Vero, 293, 293T and Huh-7 cells were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics [chlortetracycline (25 μg/ml), penicillin (250 U/ml), and streptomycin (250 μg/ml)] (DMEM-C). LLC-PK cells (ATCC) were maintained in Eagle's minimal essential medium (EMEM) containing 5% fetal bovine serum and the antibiotics. Recombinant IFN type 1 (human IFN-αA + IFN-αD fusion protein) was purchased from Serotec Inc. (Raleigh, NC). SeV was purchased from Charles Liver Laboratories (Wilmington, MA). Antibodies specific for STAT1 (nonphosphorylated) or β-actin were obtained from Cell Signaling Tech (Danvers, MA).

Plasmid construction and generation of region-specific antisera

Standard recombinant DNA methods were used for construction of plasmids. The consensus full-length clone of NV, NV101, has been described previously (Fernandez-Vega et al., 2004). For the construction of pNV-Neo, the neomycin phosphotransferase (neo) gene was amplified by PCR from the pIRESNeo vector (Clontech, Mountain View CA) with primers 5′-ataattggatccTATGGGATCGGCCATTGAAC-3′ (Neo-NV-F) and 5′-ttaattaccggtTCAGAAGAACTCGTCAAG-3′ (Neo-NV-R). The PCR product was digested with *Bam*HI and *Age*I (underlined), and cloned into the corresponding sites of the NV101 plasmid. The resulting construct contained the neomycin resistance gene engineered into the 5′-end region of the ORF2 so that the expressed product would contain the first 33 aa of the NV VP1 fused in frame with neomycin phosphotransferase (with an engineered termination codon) (Fig. 1A). The viral Pol active site GDD in pNV-Neo was deleted by site-directed mutagenesis (Promega, Madison, WI) using primers, CATGTCATATTTCTCATTTTATGA-GATTGTGTCAACTGACATAG (N-ΔGDD-F) and CTATGT-CAGTTGACACAATCTCATAAAATGAGAAATATGA-CATG (N-ΔGDD-R), generating the pNV-NeoΔGDD plasmid.

Sense and anti-sense VP2 gene (ORF3)-specific probes were designed to detect either positive or negative strand NV genomic RNA. The VP2 gene was PCR amplified from NV101 using primers ATGGCCCAAGCCATAATTGG (N-VP2-F) and TCATCGCCTATTATTTGC (N-VP2-R), and cloned to pCR2.1 downstream of T7 promoter using a TA cloning kit (Invitrogen, Calrsbad, CA). We chose two recombinant plasmids with opposite orientations to the T7 promoter.

Nucleotides (nts) 1712–2287 and 3000–4000 of the Norwalk virus genome were each cloned into pET-28a(+) (Novagen, Madison WI) that would express partial NV protein (NTPase and ProPol) sequences fused to a His_6-tag at the N-terminus. After purification of each protein, antisera specific for the recombinant proteins were raised in guinea pigs as described previously (Sosnovtsev et al., 1998). The ORF2 of the NV genome was amplified from NV101 with forward

primer: 5′-AGGCCTACGCGTCTCGAGGAAATGATGATGGCGTCTAAGGACGCTACATCAAGC-3′ (with an incorporated XhoI site underlined) and reverse primer 5′-GCTCGAGACGCGTTTATCGGCGCAGACCAAGCCTACCTCTTGCC-3′ (with an incorporated MluI site underlined). The resulting PCR fragment was digested with XhoI and MluI and cloned into the corresponding restriction enzyme sites of the pCI (Promega) eukaryotic expression vector. The resulting clone was designated pCI-NVCap.

Transfection of RNA transcripts and selection of neomycin-resistant cells

RNA transcripts were synthesized with the mMessenger mMessage in vitro transcription kit (Ambion, Austin, TX) using restriction enzyme (MluI)-linearized plasmids (pNV-Neo or pNV-NeoΔGDD) as templates. All transfections were performed with Lipofectamine 2000 (Invitrogen) in 6-well tissue culture plates (containing BHK2, Vero, 293, 293T, LLC-PK, and Huh-7 cells) with 1 μg of RNA per well. After the transfection, neomycin, G418 (Cellgro, Herndon, VA), was added at concentrations of 0.5–1 mg/ml to DMEM-C. Medium was removed and replaced with fresh DMEM-C containing the antibiotic every 3 days.

Detection of Norwalk virus RNA and proteins

RT-PCR. RT-PCR for detecting the neomycin resistant gene or NV genome was performed using primers Neo-F (5′-ATGGGATCGGCCATTGAAC-3′) and Neo-R (5′-TCAGAAGAACTCGTCAAG-3′) or NVp35 and NVp36 (Atmar et al., 1995), respectively. *Northern blot analysis.* Northern blot analysis was conducted with the reagents and conditions specified in the NorthernMax-Gly kit (Ambion) and biotinylated RNA probes. The RNA transcripts of NV ORF3 were prepared as anti-sense and sense probes designed to detect either positive or negative strand NV genomic RNA, respectively. For the generation of RNA controls in the Northern blot analysis, RNA was produced by in vitro transcription (Ambion) from the following DNA templates: pNV-Neo linearized with Mlu1; PCR products corresponding to the pNV-Neo subgenomic region; and PCR products corresponding to the NV101 subgenomic region. The PCR products contained an engineered T7 promoter in either the forward or reverse orientation to produce positive or negative sense subgenomic RNA, respectively. *Immunofluorescence assay (IFA)*. The ProPol serum was added to methanol-fixed monolayers of cells, and the binding of antibodies was detected with fluorescein isothiocyanate (FITC)-conjugated, affinity-purified goat antibodies to guinea pig immunoglobulin G (IgG) (ICN Biomedicals, Aurora, OH) as described previously (Chang et al., 2002). *Western blot analysis.* Protein samples of Huh-7 and HG23 cells were prepared in SDS-PAGE sample buffer containing 2% 2-mercaptoethanol, and sonicated for 20 s. The proteins were resolved in a 10% Novex Tris–Bis gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with guinea pig antibodies specific for the ProPol and NTPase proteins, and the binding of the antibodies was detected with peroxidase-conjugated, goat anti-guinea pig IgG (Sigma, St. Louis, MO). Following incubation with a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce biotechnology, Rockford, IL), the signals were detected with X-ray film. Controls for IFA and Western blot analysis included Huh-7 cells transfected with NV101 following MVA-T7 infection (Wyatt et al., 1995). The MVA-T7 virus was a gift from Dr. Bernard Moss, NIAID, NIH. *Sequence analysis.* The templates for sequence analysis included the RNA replicon of early passage number of G3 and G6 cells (both lower than passage number [P] 10), P60 of G3 cells (which was used in the transfection of Huh-7 cells for selection of the HG23 cells) and P8 of HG23 cells. The RNA was amplified by RT-PCR and the PCR products were gel-purified and sequenced directly. Primers used in the RT-PCR and sequence analysis are available upon request. The 5′ and 3′ ends of the RNA replicon of HG23 cells (P8) were determined by the 5′ and 3′ RACE system (Invitrogen), respectively. *Real-time qRT-PCR.* The quantity of NV genome in the replicon-bearing cells was measured by real-time qRT-PCR with the One-step Platinum qRT-PCR kit (Invitrogen), following an established protocol with and GI-specific primers and FAM-labeled G1 probes (Kageyama et al., 2003) as described previously. For quantity control of cellular RNA, qRT-PCR for the β-actin was performed as described previously (Spann et al., 2004). For qRT-PCR, the total RNA in cells (in 6-well plates) was extracted with the RNeasy kit (Qiagen, Valencia, CA). Each RNA sample was prepared from a standardized the same number of cells ($\sim 3 \times 10^6$) to calculate NV genomic RNA level per cell. A standard concentration curve was generated with serial dilutions of RNA transcripts derived from pNV-Neo in each experiment to calculate the total number of genome copies present. The relative genome copy number per cell (RNA level) was calculated by dividing the total number of genome copies present by the number of cells in the experiment.

Colony formation assay in BHK21 cells

The replicon RNA was purified from HG23 cells as described above and transfected into 1-day-old BHK21 cells with Lipofectamine 2000. Controls included transfection of RNA transcripts derived from pNVneo as well as the addition of replicon RNA to BHK21 cells in the absence of lipofectamine. The transfection mixture was removed after 24 h and DMEM-C containing G418 (0.5 μg/ml) was added. In some experiments, the replicon RNA was treated with proteinase K (100 μg/ml) or PBS only at 37 C for 4 h and purified with the RNeasy kit prior to transfection into BHK21 cells. The DMEM-C containing G418 was replaced every 2 or 3 days up to 3 weeks. When colonies became visible (usually at 2 weeks following transfection), the cells were fixed with 4% formalin and stained with crystal violet. In some experiments, the cells were fixed with 4% formalin, permeabilized with 0.5% Triton X-100, and analyzed in an immunostaining procedure that employed ProPol antiserum and peroxidase-conjugated, goat anti-guinea pig IgG (Sigma). Insoluble TMB (Invitrogen) was used as a substrate to

visualize antibody binding. Following immunostaining, cells were then stained with crystal violet to further facilitate the visualization of colonies.

Treatment of NV-harboring cells with IFN-α

The effect of IFN-α on the NV replicon was examined at concentrations ranging from 1 to 20 U/ml (for HG23 cells) or 1 to 100 U/ml (for G3 cells). Varying concentrations of IFN-α were added to 1 day old, 80–90% confluent HG23 or G3 cells, and the cells were analyzed for viral protein and genome expression at 24, 48, 72 or 96 h after treatment. The NV protein and genome expression levels were examined by IFA and Western blot analysis and qRT-PCR, respectively, as described above. Western blot analysis included the detection of STAT1 and β-actin using antibodies to STAT1 and β-actin, respectively, as described above except that a peroxidase-conjugated, goat anti-rabbit IgG (Sigma) was used. The inhibitory effect of IFN-α on the NV replicon was calculated as the concentration of IFN-α that resulted in 50% reduction of NV genome (ED_{50}) as detected by qRT-PCR.

Promoter-luciferase assay for IFN synthesis and response elements

Plasmids, pISRE-TA-Luc and pNF-kB-TA-Luc were purchased from Clontech. The pIFNβ-TA-Luc was engineered by replacing the ISRE in pISRE-TA-Luc with the IFN-β promoter (−183 to +2) using restrictions sites, KpnI and BglII. The IFN-β promoter was amplified with RT-PCR reaction using primers IFNB-F, AATTAAGGTACCAAAATGTAAATGACATAGGAAAAC (with an incorporated KpnI site underlined) and IFNB-R AATTAAAGATCTATGTTGACAACACGAACAGTGTC (with an incorporated BlgII site underlined), and total RNA extracted from Huh-7 cells as a template. The induction of firefly luciferase gene expression was achieved by infecting Huh-7 parental or HG23 cells with SeV. Because the majority of the NV capsid gene was deleted in pNV-Neo, the plasmid, pCI-NVCap was co-transfected with the reporter plasmids in some experiments to provide the NV VP1 capsid protein in *trans* to examine whether VP1 (present in authentic viral infection) might affect interferon induction. One-day-old (~90% confluent) Huh-7 or HG23 cells in 12-well plates were transfected with each reporter plasmid (pISRE-TA-Luc, pNF-kB-TA-Luc or pIFNb-TA-Luc) and pRL-CMV with or without pCI-NVCap. The pRL-CMV (for renillar luciferase under CMV promoter, Promega) served as a control for the efficiency of the transfection and for standardization of luciferase expression levels. Cells were incubated for 24 h before SeV (200 HA U/ml) or mock medium was added. After an additional 18 h, cells were harvested for analysis of luciferase expression. The luciferase assay was carried out with the Dual Glo luciferase assay system (Promega) in a luminometer (Promega). The luciferase expression (firefly luciferase) from each reporter plasmid was normalized against the expression level of the renilla luciferase encoded in pRL-CMV.

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