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Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence *in vitro* attenuate virus replication *in vitro* and *in vivo*

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Abstract

West Nile virus (WNV) infections in vertebrates are generally acute but persistent infections have been observed. To investigate the ability of WNV to produce persistent infections, we forced subgenomic WNV replicons to replicate within a cell without causing cell death. Detailed analyses of these cell-adapted genomes revealed mutations within the nonstructural protein genes NS2A (D73H, M108K), NS3 (117Kins), NS4B (E249G) and NS5 (P528H). WNV replicons and WNVs harboring a subset of NS2A or NS3 mutations showed a reduction in genome replication, a reduction in antigen accumulation, a decrease in cytopathic effect, an increased ability to persist in cell culture and/or attenuation *in vivo*. Taken together, these data indicate that WNV with a defect in replication and an increased potential to persist within the host cell can be generated by point mutations at multiple independent loci, suggesting that persistent viruses could arise in nature.

Keywords: West Nile virus; *Flavivirus*; Replicon; Replication; Attenuation; Nonstructural gene; Interferon; Innate immune response; Persistence

Introduction

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) has caused significant morbidity and/or mortality among avian, equine and human populations in North America since 1999. Although a large number of avian and equine deaths have been attributed to WNV infection, viral infection is typically non-fatal for humans. Approximately 20% of infected individuals show symptoms of infection (the majority of these cases present with a mild febrile illness characterized by malaise, headache, altered mental condition and sometimes rash) but neurological complications (including acute flaccid paralysis, meningitis and encephalitis) can occur less frequently and can result in death (Petersen and Marfin, 2002). Currently, there are no FDA-approved vaccines to prevent disease or antiviral therapies available to treat infection. Most patients mount an antibody response and display a rapid and complete recovery from illness. Thus, WNV infections are generally considered acute.

It has been suggested, however, that WNV could be capable of causing a prolonged infection. Several other members of the genus *Flavivirus* including Japanese encephalitis virus (Ravi et al., 1993), Modoc virus (Johnson, 1970), St. Louis encephalitis virus (Slavin, 1943) and tick-borne encephalitis virus (Pogodina et al., 1981) have been reported to cause long-lived or persistent infections. Hamsters (Tesh et al., 2005; Xiao et al., 2001) and rhesus monkeys (Pogodina et al., 1983) experimentally infected with WNV have evidence of viral replication in their tissues months post infection. Less is known about persistent WNV infections in humans. Anti-WNV IgM has been detected in patients' sera over 500 days post symptom onset (Roehrig et al., 2003) or in the central nervous system over 100 days post symptom onset (Kapoor et al., 2004). Recently, a case of persistent WNV infection in a human patient has been reported (Penn et al., 2006). Despite this, little is known of the factors that influence the duration of infection.

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The WNV genome is a single-stranded, positive-sense RNA molecule that is translated as a single polyprotein, which is co- and post-translationally cleaved into 10 structural (C, prM and E) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2001). The functions of some of these proteins have been described (NS2B/NS3 is the protease, NS3 functions as a helicase and NTPase and NS5 is the RNA-dependent RNA-polymerase and methyltransferase) but the exact functions of NS1, NS2A, NS4A and NS4B have not been determined (Lindenbach and Rice, 2001). Viral replication occurs in a replication complex associated with the endoplasmic reticulum (ER) of infected cells, presumably through the interaction of small hydrophobic nonstructural proteins NS2A, NS2B, NS4A and NS4B with ER membranes, other viral nonstructural proteins and host factors (Lindenbach and Rice, 2001). In addition to a putative role in the assembly of viral replication complexes, studies with WNV and other flaviviruses indicate that one or more of these nonstructural proteins could play a role in altering the cell's response to infection (Jones et al., 2005; Liu et al., 2005, 2006; Munoz-Jordan et al., 2005; Munoz-Jordan et al., 2003; Scholle and Mason, 2005).

WNV replicons (WNR) are genomes that lack the majority of the structural gene sequence and are capable of self-replication. Replicons are unable to produce infectious virions and are therefore useful tools to study some aspects of viral replication. WNR contain the 5′ and 3′ untranslated regions, a cyclization sequence within the C coding sequence required for RNA replication and all nonstructural protein genes. Several laboratories have generated WNV and Kunjin virus (KUNV, a subtype of WNV) replicons and these genomes tolerate the addition of new genetic material thereby allowing reporter genes or antibiotic resistance genes to be inserted (Khromykh and Westaway, 1997; Rossi et al., 2005; Scholle and Mason, 2005; Shi et al., 2002). Individual cells containing replicons harboring antibiotic resistance genes can be selected using the appropriate antibiotic within the media and if the replicon is not cytotoxic, replicon-bearing cells can be cloned and expanded to produce clonally-derived cell lines. Replicon-bearing cell lines can be used to study genome replication (Fayzulin et al., 2006; Liu et al., 2004), the interactions between the replicon and the cell (Fredericksen and Gale, 2006; Guo et al., 2005; Liu et al., 2004; Rossi et al., 2005; Scholle and Mason, 2005), or as a tool to identify anti-WNV compounds (Gu et al., 2006; Lo et al., 2003; Rossi et al., 2005).

We previously reported that cells transfected with a WNR encoding the neomycin phosphotransferase (NPT) gene could form colonies under G418 antibiotic selection (Rossi et al., 2005). In these studies, we demonstrated that three cell types (Vero, BHK and Huh7) could successfully harbor WNRs transiently. However, the percentage of transfected cells that could progress to persistent infection and form colonies under G418 selection (colony-formation efficiency or CFE) was notably different among these cell lines. We were readily able to obtain G418-resistant BHK and Vero cells persistently harboring the WNR but in Huh7 cells, the efficiency of recovering G418-resistant cells was less than 1% (Rossi et al., 2005). Once these G418-resistant colonies were established they could be maintained in the absence of G418 for weeks without loss of the replicon, suggesting that a stable adaptation had occurred. Replicon genomes harvested from one cloned G418-resistant Huh7 cell line (Huh7 cl 1.1) produced a CFE of 100% when introduced into naïve cells, demonstrating the genetic adaptation of WNR to persist in cell culture (Rossi et al., 2005).

Replicon-bearing cell lines can provide data detailing specific virus–host interactions. We showed previously that interferon-stimulated response element (ISRE) activation was decreased in a replicon-bearing Huh7 cell line after type I interferon (IFN) stimulation compared to cells that were cured of the replicon (Rossi et al., 2005). Replicon-bearing Huh7 cells also produced less phosphorylated STAT1 after IFN treatment (Scholle and Mason, 2005). Both Vero and Huh7 replicon-bearing cells, however, could be cured of the replicon by adding IFN to the culture medium (Rossi et al., 2005), indicating that these cell-adapted replicons were not able to completely block the antiviral action of IFN. KUNV replicons, adapted to replicate noncytopathically in BHK cells, contained mutations in NS2A and NS5; cells expressing KUNV replicons encoding one of these mutations (NS2A A30P) induced higher levels of IFNβ transcription than cells transfected with wt KUNV (Liu et al., 2004). Additionally, KUNV NS2A A30P could not replicate in cells capable of mounting an IFNα/β response to infection and were attenuated in mice (Liu et al., 2006).

In this manuscript, we identify and characterize several mutations that have been selected during the adaptation of WNR to persistently replicate in cell culture. Using reverse genetic techniques, we demonstrated that a subset of these mutations could independently produce replicons that readily persisted in Huh7 cells. These mutations reduced the ability of WNR and WNV genomes to replicate and produce cytopathic effect. Viruses harboring these mutations were highly attenuated in mice and a NS2A mutant virus produced lower viremia than the wt WNV. Furthermore, less IFN was detected in the serum of mice infected with this NS2A mutant than in mice infected with wt WNV. These results demonstrate that multiple individual mutations allow WNR to replicate to levels low enough to persist *in vitro*, and suggest that similar changes could allow WNV to persist *in vivo*.

Results

Replicon genomes from antibiotic-resistant cell clones encode various independent mutations

We have previously shown that WNR NS1-5 ET2AN and WNR NS1-5 EG2AN genomes (Fig. 1) that were adapted to replicate in Huh7 cells have acquired mutation(s) that confer a cell-adapted phenotype (Rossi et al., 2005). To identify these mutations, we sequenced the complete replicon genomes harbored within three independent WNR-bearing G418-resistant Huh7 cell clones. Only a few mutations were detected in these genomes (Fig. 2A). Sequence analysis of the 5′ and 3′ UTRs revealed no mutations in any replicon examined. In the coding region, one synonymous (NS3 G32G), one insertion

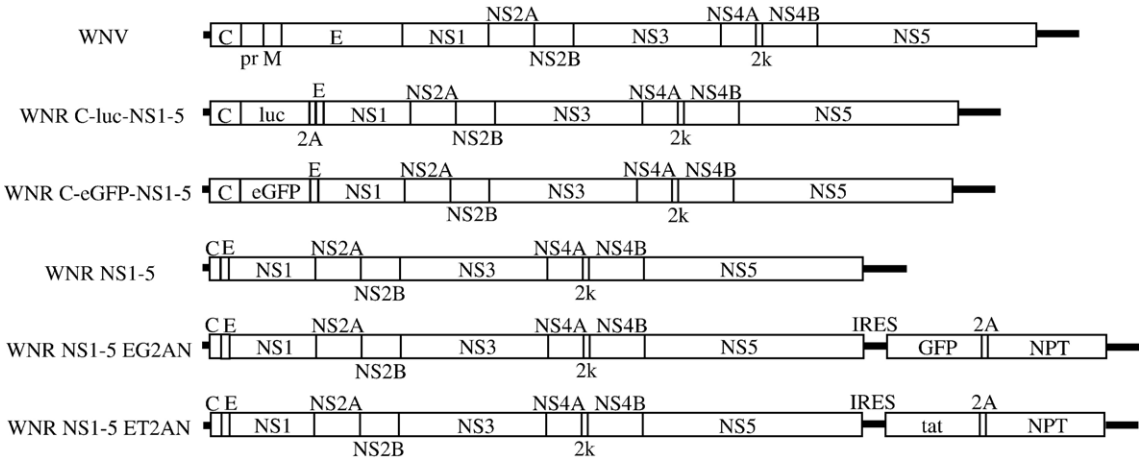

Fig. 1. Schematic diagrams of replicon genomes used in these studies (see Materials and methods). All constructs used in this study are based on the TX02 WNV genome (Rossi et al., 2005).

(NS3 117Kins) and eight non-synonymous mutations were observed. One non-synonymous mutation observed in NS4B (E249E/G) was observed in four clones, but always as a mixed population. Interestingly, this mixed population appeared to be stable since it was observed after up to 41 passages in G418. The small number of synonymous mutations within these three replicon-bearing cultures indicates that there is a selective pressure for specific coding changes within the replicons that successfully adapted to Huh7 cells.

The frequency of mutations observed within the NS2A gene was of particular interest. KUNV replicons adapted to BHK cell cultures also showed NS2A mutations (Liu et al., 2004, 2006), suggesting that this was a potential 'hotspot' for the accumulation of cell-adapted mutations. When additional replicons

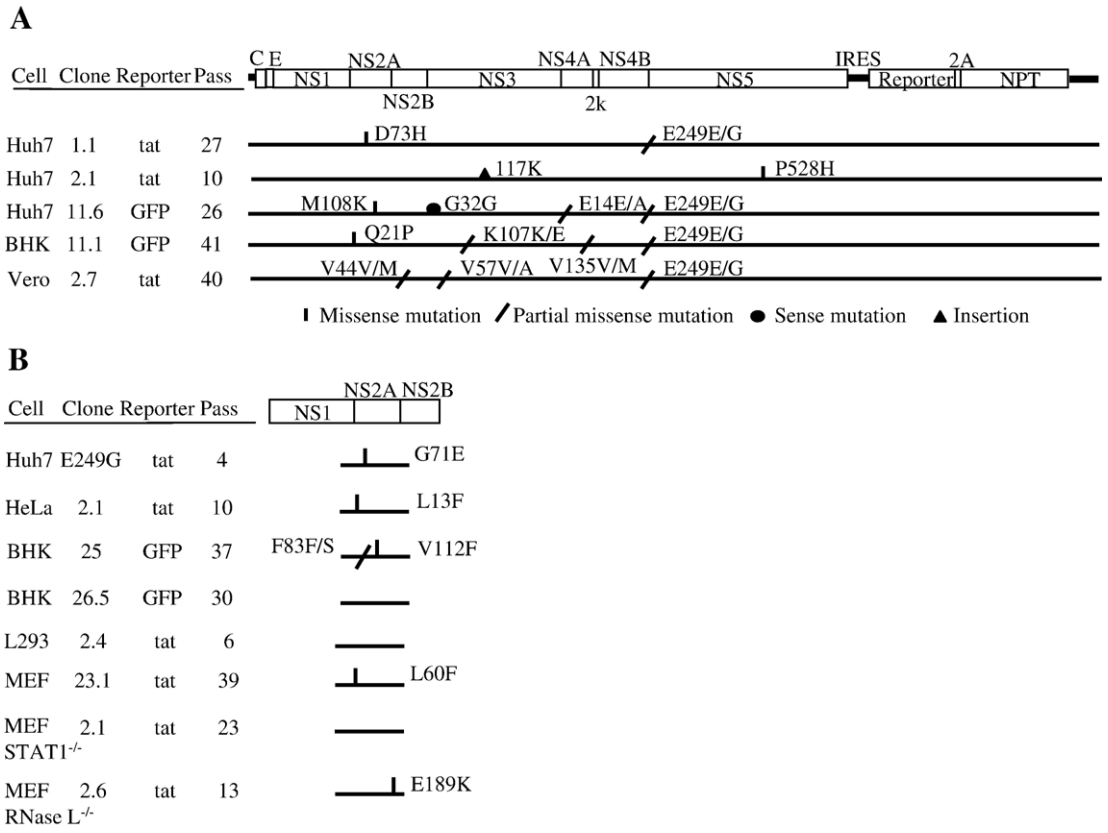

Fig. 2. Position of cell-adapted mutations detected in WNR NS1-5 ET2AN present in replicon-bearing cell lines. The left side of each panel shows parental cell line, clone name, reporter gene and passage number at sequencing. Horizontal lines denote regions where PCR products of harvested replicons were sequenced. (A) Five genomes that were sequenced in their entirety. (B) Eight genomes that were sequenced through the NS2A coding region only. The cell line listed indicates the cell line from which the replicon was harvested. Replicons harvested from MEF cl 23.1 were adapted to MEF STAT1$^{-/-}$ cl2.3 (clone not sequenced) cells prior to sequencing.

contained within other G418-resistant cell lines from multiple cell types were harvested and sequenced in their entirety or across the NS2A gene, more than half of the genomes sequenced had a mutation within the NS2A gene (Fig. 2).

Specific changes in NS2A and NS3 increase the ability of WNR to persist in cell culture

To evaluate the influence of select individual mutations on *in vitro* persistence, several mutations were selected for further analysis (Table 1). The missense mutations from the three replicon-bearing Huh7 cell lines that were sequenced in their entirety were incorporated into WNR NS1-5 ET2AN (Fig. 1) by standard reverse-genetic techniques and these genetically engineered replicons were tested for their CFE. In Huh7 cells, WNR NS1-5 ET2AN harboring NS2A D73H, NS2A M108K or NS3 117Kins displayed CFEs of 100% whereas the wt replicon had a CFE of less than 1%. Interestingly, a NS2A A30P mutation (reported to increase antibiotic-resistant colony-formation in KUNV replicons (Liu et al., 2004)) displayed a CFE of 3.5% in our WNR (Table 1). The NS4B E249G and NS5 P528H mutations produced low CFEs, very similar to wt WNR NS1-5 ET2AN (Table 1).

There was a notable difference in the intensity of antigen staining in cells transfected with some mutant WNR NS1-5 ET2AN. There was no detectable difference in staining intensity among cells containing the wt, NS2A A30P, NS4B E249G or NS5 P528H mutant replicons. However, cells transfected with WNRs with NS2A mutations D73H and M108K or NS3 117Kins stained less intensely than cells expressing wt WNR, suggesting that these mutations reduced WNR replication. Among these mutants, replicons harboring the NS3 117Kins stained most poorly and cells transfected with this WNR required amplification of the antibody signal by biotin–streptavidin for consistent detection.

Cell-adapted mutations reduce genome replication

To quantify the replication of WNRs bearing cell-adapted mutations, we measured enhanced green fluorescent protein (eGFP) fluorescence intensity in cells infected with VLPs containing WNR C-eGFP-NS1-5. Fig. 3A shows histograms depicting eGFP fluorescence intensity at 24 h post infection (hpi) of VLP-infected cells. This figure shows that in cells infected with genomes encoding NS5 P528H (geometric mean or GM = 8561) or NS2A A30P (GM = 8408) the profile of eGFP fluorescence intensity was comparable to wt-infected cells (GM = 8489). EGFP intensity in cells infected with genomes containing the NS4B E249G mutation (GM = 7705) was slightly less than wt. WNR NS2A M108K (GM = 2801) and NS2A D73H (GM = 3317) infected cells showed the weakest expression of eGFP, consistent with the weak antigen staining observed in WNR NS1-5 ET2AN-transfected Huh7 cells. Results from WNR NS3 117Kins were not reported since VLPs encoding this mutation produced a fluorescence intensity similar to wt levels, suggesting that a phenotypic reversion had occurred and that this mutation was unstable under these conditions (see WNV NS3 117Kins characterization below). A reduced level of genome-encoded eGFP fluorescence was previously noted in two WNR NS1-5 EG2AN-bearing cell lines that were passaged 31 times in G418-containing media compared to a cell population that was transiently infected with the same replicon and these data correlated with the RNA levels measured during replication (Rossi et al., 2005).

EGFP VLP infections were also analyzed for changes in eGFP fluorescence over time to observe the rates of WNR C-eGFP-NS1-5 replication. Fig. 3B shows the GM eGFP fluorescence intensity at 12, 16, 20 and 24 hpi. EGFP fluorescence intensity increased over time in all infected samples but more slowly for cultures infected with WNR eGFP-NS1-5 NS2A D73H or NS2A M108K. Genomes expressing the NS2A A30P mutation showed a lag in the establishment of efficient genome replication but at 24 hpi, eGFP expression were similar to wt levels (Fig. 3B).

Table 1
Summary of CFE for individual WNR NS1-5 ET2AN engineered to contain specific mutations

Gene	Mutation	CFE[a] (%)	Increase in CFE over wt
wt	NONE	0.75	n/a[b]
NS2A	A30P	3.5	4.7×
NS2A	D73H	100	133×
NS2A	M108K	100	133×
NS3	117Kins	100	133×
NS4B	E249G	1.5	2×
NS5	P528H	1.3	1.7×

[a] CFE = colony-formation efficiency.
[b] Not applicable.

Replicons bearing cell-adapted mutations are less cytopathic

To quantify the cytopathology produced by the WNR NS1-5 encapsidated into VLPs, Huh7 monolayers were infected with VLPs harboring selected NS2A mutations at a multiplicity of infection (MOI) of 5 and monitored for cell number using an MTT assay (Fig. 4A). The number of cells was measured at each day for 5 days and normalized to an uninfected control. Although no differences in cell number were observed between the different VLP-infected cultures for the first 4 days, cultures containing the wt and NS2A A30P mutant showed a precipitous drop in cell number at day 5. Moreover, cells infected with the VLPs encoding either cell-adapted mutation NS2A D73H or NS2A M108K appeared similar in morphology to mock-infected cells at this time point (results not shown). When these cultures were immunostained at day 6, nearly all of the cells were antigen-positive (Fig. 4B), independent of the VLP they were infected with, confirming that most of the cells were infected, an important control since uninfected cells could confound the results obtained within this experiment. Most uninfected and NS2A D73H-infected cells had cellular morphology consistent with healthy and dividing cells whereas wt or NS2A A30P infected cells were in the process of dying (condensed and rounded; Fig. 4B).

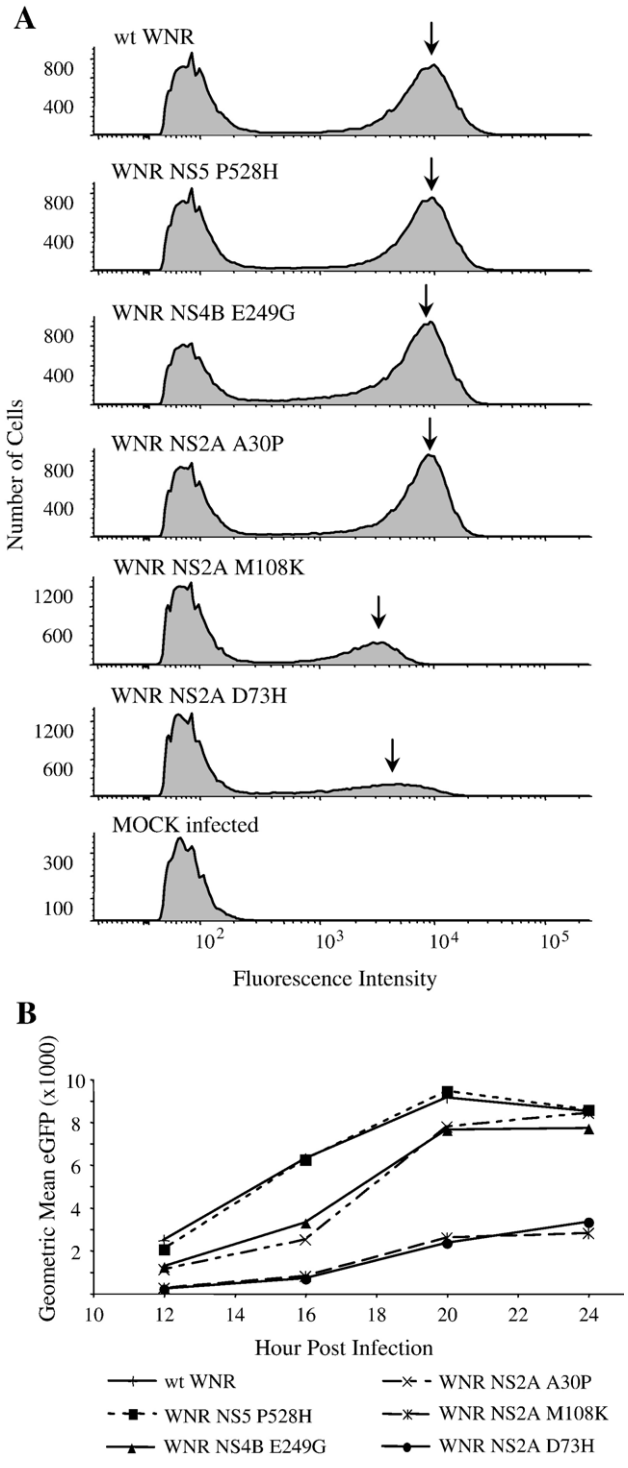

Fig. 3. Levels of eGFP fluorescence in cultures infected with VLPs containing WNR C-eGFP-NS1-5 with selected cell-adapted mutations. (A) Histograms showing eGFP fluorescence in cell populations at 24 hpi with WNR C-eGFP-NS1-5 VLPs containing the indicated mutations. In this experiment, uninfected cells showed a peak fluorescence intensity of approximately 66. The arrow above each peak of eGFP-expressing cells indicates the mean geometric fluorescence intensity of eGFP expression. (B) Time course of eGFP fluorescence intensity at different times post infection. Both panels show data from one of three experiments that showed similar trends.

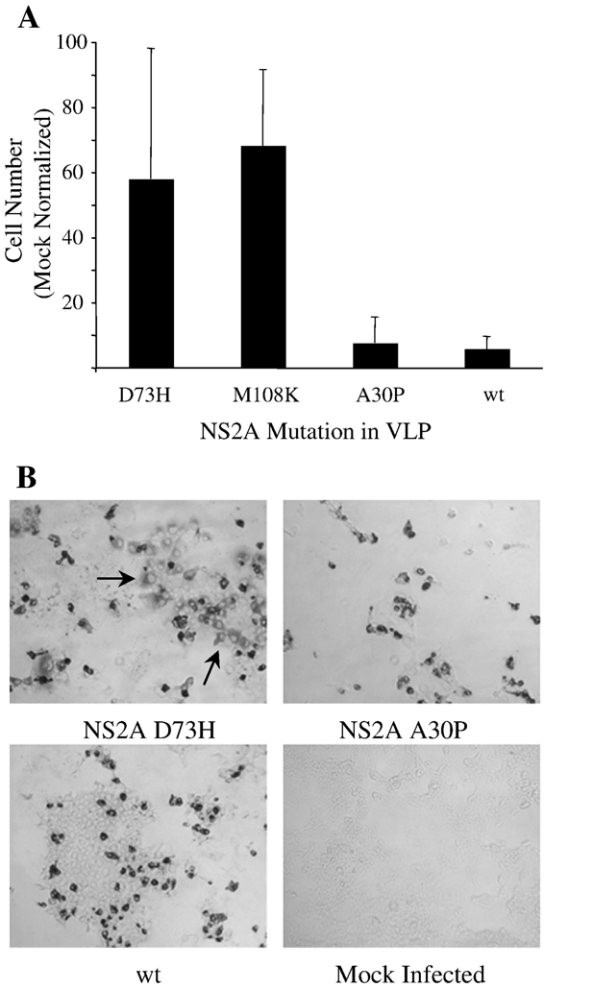

Fig. 4. Survival of Huh7 cells infected with VLPs encoding selected NS2A cell-adapted mutations. Huh7 cells were infected with VLPs (harboring WNR NS1-5) at an MOI of 5. (A) Cell number determined by MTT staining at 5 days post infection (dpi); values are shown normalized to MTT data from mock-infected cells. Error bars denote standard deviation. (B) Micrographs (20× magnification) of cell monolayers from parallel cultures that were fixed 6 dpi and immunostained for WNV proteins. Cultures infected with WNR NS1-5 NS2A M108K were not fixed and stained for analysis. Arrows denote viable cells positive for WNR antigen.

Viruses encoding cell-adapted mutations replicate poorly in vitro

To evaluate the effect of cell-adapted mutations on WNV, full-length genomes were engineered to contain these mutations using standard molecular biology techniques. The sizes of infectious foci produced from these viruses were dramatically different between the wt virus and viruses harboring cell-adapted mutations or a 3′ UTR deletion (Fig. 5A). Specifically, viruses harboring cell-adapted mutations NS2A D73H, NS2A M108K, 3′ UTRΔ produced much smaller antigen-positive foci than the wt virus in Vero cells at 48 hpi, whereas the NS2A A30P and NS4B E249G produced foci in Vero cells that were slightly smaller than the foci produced by wt virus, and the NS5 P528H virus displayed foci indistinguishable from those produced by the wt virus (Fig. 5A). Interesting, when a subset of these viruses

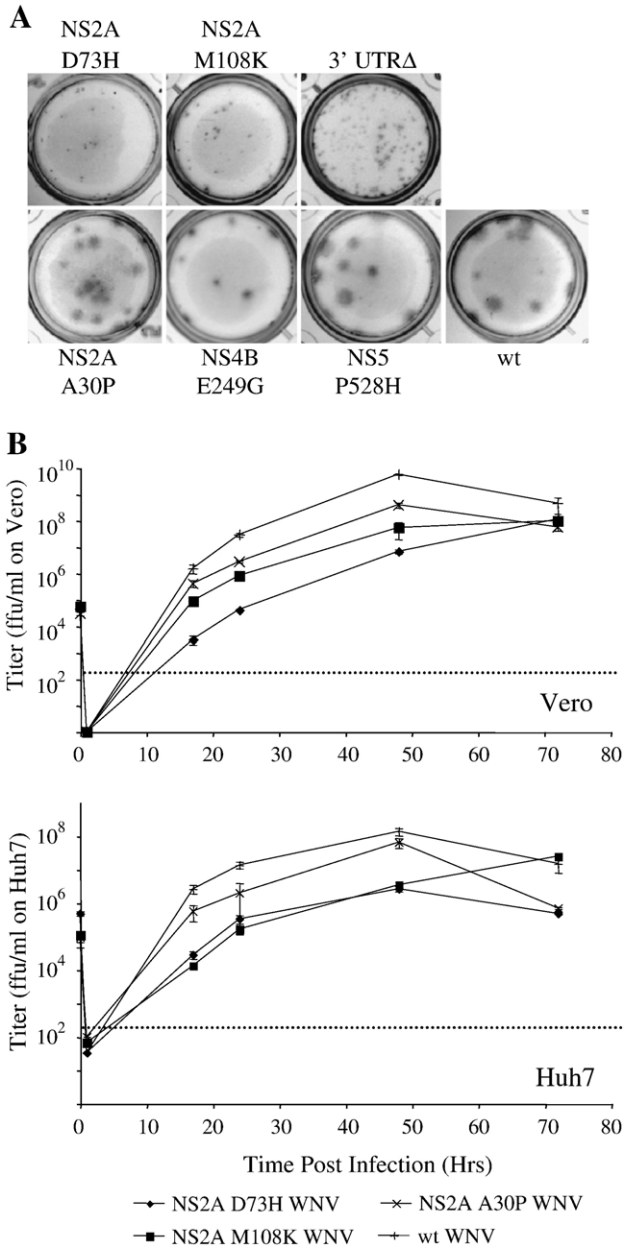

Fig. 5. Comparison of focus formation and growth of mutant and wt WNV. (A) Vero cell monolayers infected with viruses and immunostained 2 dpi to detect viral antigen-containing foci (see in Materials and methods). (B) Viral growth curve of Vero and Huh7 cells infected at a MOI of 0.1 with the indicated viruses (see Materials and methods). Samples were titrated on either Vero or Huh7 monolayers as indicated in the figure. Error bars denote the standard deviation of the titration. The limit of detection for this assay (shown by the dotted line) was 3.3×10^2 ffu/ml.

were used to infect MEF cells, NS4B E249G and NS5 P528H viruses formed foci identical in size to wt virus, whereas NS2A D73H, NS2A M108K, and 3′ UTRΔ formed substantially smaller foci (Supplemental Fig. 1). Viruses encoding the NS3 117Kins also produced large foci that were indistinguishable from wt WNV, indicating that a phenotypic reversion had occurred (results not shown). Sequence analyses of the recovered WNV 117Kins revealed a missense mutation in the inserted residue, which changed the K to an M. Viruses that produced small foci also produced little CPE in cell culture. For viruses encoding NS2A D73H, NS2A M108K or 3′ UTRΔ, little CPE was noted in infected cultures at 72 hpi. In contrast, CPE was readily evident in cultures infected with WNV NS2A A30P, WNV NS4B E249G, WNV NS5 P528H and wt WNV at the 72 hpi.

Vero and Huh7 cells were used to produce growth curves for selected viruses. Cell monolayers were infected with an MOI of 0.1 for 1 h, then washed and refed with minimal growth media (DMEM with 20 mM HEPES 1% FBS and 1% antibiotics). Samples of the supernatant were taken at indicated time points and titrated on Vero or Huh7 cell monolayers (Fig. 5B). At 48 hpi, peak titers were reached for wt WNV and WNV NS2A A30P at which point WNV NS2A A30P produced a log fewer infectious particles than wt WNV. At 48 hpi, titers of WNV NS2A M108K and WNV NS2A D73H were 2–3 logs lower than wt in both cell types. By 72 hpi, peak titers had not been attained for WNV harboring either NS2A D73H or M108K mutations in Vero cells or for NS2A M108K infection of Huh7 cells. These findings suggest that viral replication level per se (rather than particular mutations that alter the interaction of mutant viruses with particular host factors) is the most important aspect determining *in vitro* persistence since the growth trends of these NS2A D73H and M108K viruses were similar between two cell lines, even thought differences in *in vitro* persistence are only observed in the Huh7 cells (see above). As mentioned above, at 72 hpi, CPE was only evident in cells infected with wt WNV or WNV NS2A A30P and was not present in mock-infected cultures or cultures infected with WNV NS2A D73H and WNV NS2A M108K.

Viruses that replicate poorly in vitro are attenuated in vivo

The virulence of mutant and wt WNV were determined using Swiss-Webster mice. The survival data and 50% lethal dose (LD_{50}) values determined for these viruses are shown in Table 2. Viruses that showed no (or very little) reduction in replication and CPE *in vitro* (harboring mutations NS2A A30P, NS4B E249G or NS5 P528H) compared to the wt WNV were virulent *in vivo* with similar LD_{50} values. On the other hand, viruses that replicated poorly and caused little CPE (harboring mutations NS2A D73H, WNV NS2A M108K or WNV 3′ UTRΔ) were not virulent in mice (Table 2). These viruses did not cause death in any infected mouse except for WNV NS2A D73H, which killed 1 out of 5 mice given 100 ffu. No mice infected with 1000 ffu of WNV NS2A D73H, WNV NS2A M108K and WNV 3′ UTRΔ died so LD_{50} values for these viruses could not be accurately calculated from this experiment and were recorded at above 1000 ffu (Table 2).

To determine if the differences in LD_{50} were due to lack of productive infection, surviving animals were tested for the presence of antibody to WNV by neutralization assay. All mice that survived infection with 100 ffu of the inoculated viruses seroconverted, but those animals inoculated with the poorest replicating virus, 3′ UTRΔ, showed lower convalescent antibody titers (Table 2). These results indicate that these mice were

Table 2
Summary of phenotypes and genotypes of WNV *in vivo*

Virus	LD_{50} [a]	Detailed data from 100 ffu inoculation dose			
		MDD [b]	% Survival	Seroconversion [c]	Genotype [d]
NS2A A30P	4.2	10(1.41)	20	+++	P,P
NS2A D73H	>1000 [e]	13(0)	80 [f]	++,++,+++,+++	D
NS2A M108K	>1000 [e]	n/a [g]	100 [h]	+++,+++,+++,+++,+++	n/a
NS4B E249G	6.9	10.3(1.5)	20	+++	G
NS5 P528H	4.2	9.25(1.5)	20	+++	H
3′ UTRΔ	>1000 [e]	n/a	100 [h]	+,+,++,+++,+++	n/a
wt	3.2	8.8(1.3)	0	n/a	n/a

[a] LD_{50} values calculated by the Reed and Muench method for calculating 50% endpoint dilutions (Reed and Meunch, 1938) using 14-day survival data for groups inoculated with 0.1–100 ffu, except where noted.
[b] Mean day of death, number in parentheses denotes standard deviation.
[c] Neutralization antibody titer from mice collected 1 month post inoculation (see Materials and methods). Titers of 1/320, 1/1280 and >1/2560 are denoted by +, ++ and +++, respectively.
[d] Amino acid sequence detected in viruses recovered from brains collected at death from one or more individuals (see Materials and methods).
[e] Less than 50% death in each group inoculated with 0.1–1000 ffu.
[f] $p<0.05$ compared to wt WNV by Fisher's exact test.
[g] Not applicable.
[h] $p<0.01$ compared to wt WNV by Fisher's exact test.

able to recognize infection even with a poorly replicating virus and mount an immune antibody response.

Mutant WNV lethality was associated, in some cases, with reversion

Mortality detected in mice infected with viruses showing a cell-adapted phenotype was the result of phenotypic reversion. As indicated above, no mice infected with any administered dose of WNV NS2A M108K or WNV 3′ UTRΔ died. One mouse died from infection with 100 ffu of WNV NS2A D73H and, in this case, the virus reverted back to the wt genotype at the mutated locus (Table 2), indicating that WNV NS2A D73H has an attenuated phenotype and that reversion to the wt residue at this position was responsible for the lethal phenotype observed. Virus recovered from moribund mice infected with viruses harboring NS2A A30P, NS4B E249G and NS5 P528H showed no reversion to the wt amino acid at the mutated codon, retaining their respective mutations (Table 2).

Mice infected with WNV NS2A D73H produce a reduced viremia and IFN response to infection

We next sought to assess the relative levels of viral load in the serum of mice infected with either WNV NS2A D73H or wt WNV. Mice were infected intraperitoneally (i.p.) with 1×10^5 ffu of virus and serum was harvested from three animals each day for 6 days. Mice infected with the WNV NS2A D73H produced a lower viremia than mice infected with wt WNV (Fig. 6A). The mice infected with WNV NS2A D73H had a peak titer on day 1-post infection that was 100× lower than wt levels, a difference trending toward significance (*t*-test; $p<0.06$). After day 3, no virus was detectable in mice infected with WNV NS2A D73H. The wt-infected mice had a peak viremia at day 2 with an average of 1×10^5 ffu/ml serum and lasted for 4 days. Virus was undetectable in all mice on days 5 and 6 post infection.

Serum samples were also assayed for the presence of IFN by a biological assay. In this assay, 2–4 units (U)/ml of murine IFNβ yielded a 50% reduction in VLP infection, but sera has an effect on the sensitivity of this assay so the calibration provided in Fig. 6B consists of normal mouse serum (NMS) containing murine IFNβ added to a final concentration of 0, 50 or 250 U/ml. IFN activity was measured in serum samples from all mice harvested on the first 4 days of infection. The number of mice that produced detectable levels of IFN in response to WNV NS2A D73H infection (3 out of 12) was statistically different from the number of mice (12 out of 12) that produced IFN in response to wt WNV infection (Fisher's exact test; $p<0.0005$, Fig. 6B). There was also a statistically significant difference between the level of VLP neutralization detected in sera in mice sacrificed 2 days post infection with WNV NS2A D73H compared to wt WNV (*t*-test; $p<0.001$) and a trend towards significance (*t*-test; $p<0.06$) for these same values obtained with sera collected on day 4 post infection (Fig. 6B). Taken together, these data indicate that a virus with a reduced ability to replicate elicits a lower level of IFN *in vivo*.

Discussion

Eukaryotic cells employ several mechanisms to detect and eliminate viral infection whereas viruses have evolved ways to avoid, alter, delay or redirect the cell's innate immune responses. Studying the interactions between WNRs and cells can reveal information related to this complex interaction, which can result either in cytopathology, cell death, or a persistent infection. Here, we describe mutations observed after WNR genomes were forced to adapt to cell culture and the phenotypes of replicon and viral genomes harboring some of these cell-adapted mutations.

Mice infected with WNV NS2A D73H produce a delayed and decreased IFN response to infection compared to wt-infected mice. This could be due to individual cells producing little IFN in response to infection or be due to there being fewer

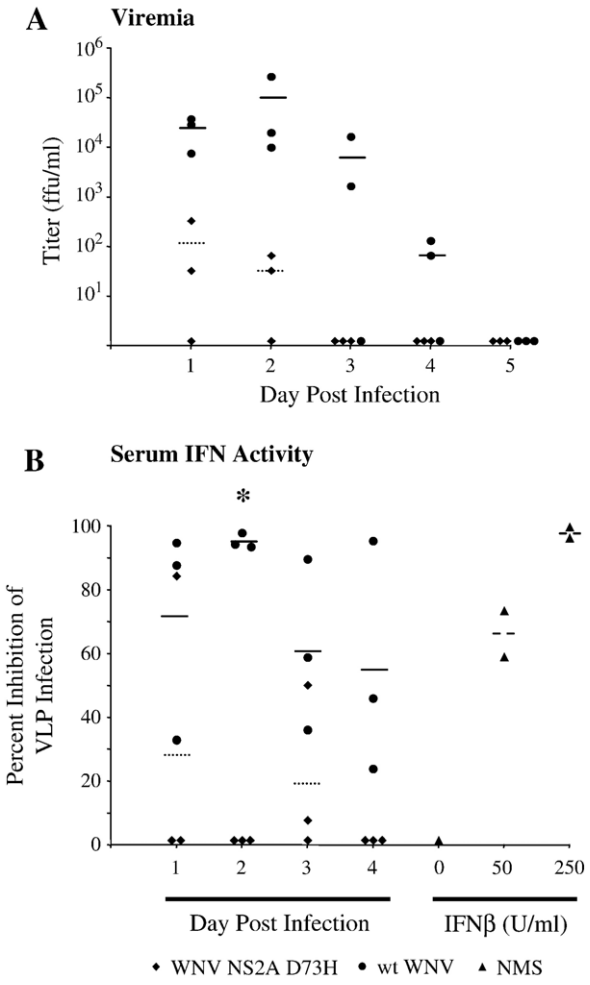

Fig. 6. Viremia and serum IFN levels in mice infected with 1×10^5 ffu of wt WNV or WNV NS2A D73H. (A) Viremia in mice infected with either wt or NS2A D73H WNV. Each plot point represents the data collected from one mouse on the indicated day. The limit of detection for this assay is 33 ffu/ml. Solid and dotted horizontal lines show the average titer for samples at that time point for wt WNV and WNV NS2A D73H viruses, respectively. (B) IFN activity detected in serum of mice infected with either wt or NS2A D73H WNV. Values are expressed as percent inhibition of luciferase-VLP infection relative to wells infected with these VLPs in the presence of NMS. Each plot point represents the data collected from one mouse at the indicated day or treatment. Solid and dotted horizontal lines show the mean titer for samples at that time point for wt and WNV NS2A D73H viruses, respectively. The right side of this figure shows the effect of murine IFNβ added to NMS at a final concentration of 50 U/ml or 250 U/ml within sera. The average of these two points is shown as a dashed horizontal line. Pretreatment of selected samples showing a high inhibition of VLP infection with anti-IFN blocked their activity, indicating that the inhibition detected in this assay was due to IFN (results not shown). Significance for difference between titers, as shown by the asterisk, was determined by two-tailed, unpaired Student's *t*-test (*$p<0.001$).

infected cells capable of producing large amounts of IFN. It is also unknown if cells infected with genomes harboring the cell-adapted mutations described in this manuscript are more sensitive to exogenous IFN treatment than wt-infected cells. The ability of WNV NS2A D73H to either induce less IFN production and/or cause cells to respond differently to exogenous IFN compared to wt WNV is of interest and is under further investigation. The attenuated phenotypes of WNV NS2A D73H and WNV NS2A M108K are similar to WNV-MAD78 and therefore it is possible that our mutated lineage I viruses would have a similar sensitivity to IFN as lineage II viruses. On the other hand, it is possible that viruses that replicate poorly would not produce viral products at levels high enough to block STAT1 phosphorylation and all downstream functions of ISGF3 signaling. If this were true, then cells infected with these viruses would still be responsive to IFN, which could lead to viral clearance from the cell and the animal host. Consistent with these findings, we have shown that cell-adapted WNR, which display low levels of replication, are readily cleared by IFN treatment (Rossi et al., 2005), but the individual cell line in which replicons persist can also influence this interaction (Scholle and Mason, 2005).

The data presented in this manuscript support our hypothesis that reduced replication is associated with a lack of replicon cytopathogenicity. Here we show that WNRs and WNVs harboring cell adapted mutations within the NS2A and NS3 genes are capable of reducing genome replication, reducing cytopathogenicity *in vitro* and allowing establishment of persistent replication in cell culture compared to wt genomes.

In this study, more than half of the WNR genomes harvested from replicon-bearing cell lines contained missense mutations in the NS2A gene. Interestingly, others have reported that mutations within the NS2A gene of flavivirus replicons appear to be important for the establishment of noncytopathic persistent replication in mammalian cells (Liu et al., 2004; Rossi et al., 2005). Genomes harboring either NS2A D73H or NS2A M108K mutations have high CFE values, replicate poorly and fail to cause mortality in mice. In our hands, WNV and WNR harboring the NS2A A30P mutation showed phenotypes similar to wt WNV in most experiments. Although the NS2A A30P mutation did not affect the ability of KUNV replicons to replicate in cell culture, this mutation allowed replicons to readily form antibiotic-resistant cell colonies: 17-fold greater than wt replicons in BHK cells and approximately 50-fold greater than wt replicons in HepG2 and HEK293 cells (Liu et al., 2004). When the NS2A A30P mutation was introduced into WNR NS1-5 ET2AN, however, the CFE in Huh7 cells was only 4.7-fold higher than wt replicons. *In vitro*, WNR C-eGFP-NS1-5 harboring NS2A A30P was reduced slightly in genome replication early during infection but replicated similarly to wt replicons after 24 hpi. WNV NS2A A30P also grew to a slightly lower titer but produced large antigen-positive foci in Vero cells. Additionally, WNR NS1-5 NS2A A30P VLPs were able to induce CPE in tissue culture. WNV NS2A A30P is lethal with a similar LD_{50} to wt in 6-week-old outbred Swiss-Webster mice. Conversely, KUNV NS2A A30P is highly attenuated and reported to be similar to in mice only 3 weeks old (Liu et al., 2006). These results indicate that the phenotype of the NS2A A30P mutation within KUNV is not the same as within a North American WNV. This disparity is likely due to other attenuating mutations in KUNV and argues that the A30P mutation may be unsuitable for use in generating an attenuated vaccine strain.

In NS3, we detected one sense mutation (G32G), one insertion of three nucleotides (117K) and two mixed populations of missense mutations and wt sequences (V57V/A and K107K/E).

The mutations V57A and K107E mutations were not examined but since they occurred as mixed missense mutations within the persistent WNR population and thus it seemed unlikely that their phenotype would have a strong advantage to the establishment of persistence *in vitro* (see discussion of NS4B E249G below). WNR NS1-5 ET2AN 117Kins showed a noncytopathic phenotype and had a high CFE, but reverted to a wt phenotype quickly when introduced into WNR C-eGFP-NS1-5 or WNV genomes in cell culture. Replicon genomes with this mutation had a selective advantage during the establishment of replicon-bearing cell lines (under G418 selection) but were at a strong disadvantage during genome replication in non-selective media and were therefore not analyzed further in this study.

Surprisingly, no mutations were observed in the 5′ and 3′ UTRs, NS1, NS2B or NS5 that specifically altered genome replication and yielded a cell-adapted phenotype. Genomes harboring the NS4B E249G and NS5 P528H mutations had wt phenotypes. Interestingly, the NS4B E249G mutation has been observed in several natural WNV isolates (Davis et al., 2004; Lanciotti et al., 2002). Naturally occurring viruses harboring the NS4B E249G mutation produced small foci, were temperature sensitive and not lethal in mice (Davis et al., 2004). However, a NY99 WNV genetically engineered to only contain the NS4B E249G mutation produced large antigen-positive foci, showed no difference in growth kinetics in Vero cells to wt NY99 WNV and were lethal for mice (C. T. Davis and A.D.T. Barrett, personal communication), consistent with the highly virulent wt phenotype of WNV NS4B E249G described here. Recently, Shi and coworkers reported the characterization of a C3H/He mouse cell-adapted replicon based on a NY00 strain of WNV. During adaptation, this WNR acquired the same NS4B E249G mutation that we observed, and genetically engineered WNR with this mutation were shown to be twice as likely as the parental replicon to produce antibiotic-resistant colonies in C3H/He and BHK21 cells (Puig-Basagoiti et al., in press). Additionally, this group reported that WNR NS4B E249G replicated more poorly compared to wt replicons in C3H/He and BHK21 but not Vero cells (Puig-Basagoiti et al., in press). Finally, Puig-Basagoiti et al. showed that NY00 WNV NS4B E249G was slightly less virulent than NY00 WNV by footpad inoculation of 1000 plaque-forming units in 6-week-old C3H/HeN mice (Puig-Basagoiti et al., in press). These *in vitro* data correlate well with our data showing that TX02 WNR with this same mutation produced a two-fold higher CFE relative to the wt TX02 WNR. However, the *in vivo* attenuating properties of the NS4B E249G mutation reported by Shi and coworkers, differ from our data with a TX02 WNV NS4B E249G mutant (Table 2) or work of Davis et al. with a NY99 WNV NS4B E249G mutant (C.T. Davis and A.D.T. Barrett, personal communication), which failed to demonstrate an affect of this NS4B E249G mutation on LD_{50} values in i.p.-inoculated outbred mice. The discrepancy among these *in vivo* results might reflect the differences in WNV strains, mouse strains, and/or inoculation routes employed in these studies.

It is unclear why the NS2A gene appears to be a hotspot for cell-adapted mutations. If a reduction in replication alone were important for establishing persistence, then it would be expected that changes in the polymerase (NS5) or 3′ and 5′ UTRs could have produced a similar adapted phenotype. NS2A is one of several flaviviral proteins that are believed to intercalate into the ER membrane anchoring the RNA replication complex, facilitating efficient genome replication (Lindenbach and Rice, 2001). Although the possibility that NS2A could coordinate virion assembly (Kummerer and Rice, 2002; Liu et al., 2003) and genome replication is of great interest, this activity is unlikely to be relevant to the adaptation of WNR to persist in the cell. However, NS2A's ability to cause changes in RNA replication levels or changes in NS2A's ability to block IFN signaling could directly influence WNR adaptation to persist *in vitro*. Point mutations detected within NS2A reported here and observed by others [in the laboratory propagated (Liu et al., 2003, 2004) and natural WNV isolates (Beasley et al., 2005; Lanciotti et al., 2002)] occur in linear amino acid sequences within hydrophilic domains interspersed between long hydrophobic domains which are likely buried in the ER membrane. These mutations could induce subtle changes in RNA replication activity that might be harder to induce by point mutations in soluble globular proteins such as helicases, proteases and polymerases, helping to explain why NS2A appears to be a hotspot for adaptive mutations. Changes in the UTRs that produce an adapted phenotype might require large-scale alterations, which might not be readily achievable by point mutations.

Materials and methods

Cells, plasmids, viruses and VLPs

Vero, BHK, Huh7, Huh7 cells harboring WNRs, and BHK packaging cells were maintained as previously described (Fayzulin et al., 2006; Rossi et al., 2005). HeLa cells were maintained in DMEM with 10% FBS, 20 μg/ml gentamycin and 1% antibiotics. MEF, MEF RNase $L^{-/-}$ (Zhou et al., 1999) and MEF $STAT1^{-/-}$ (Durbin et al., 1996) cells were grown in DMEM with 5% FBS 20 μg/ml gentamycin and 1% antibiotics, non-essential amino acids and sodium pyruvate.

Generation, characterization and manipulation of a WNV infectious clone (in pACNR plasmid) and multiple WNRs clones have been previously described (Fayzulin et al., 2006; Rossi et al., 2005). One additional clone designated WNR C-eGFP-NS1-5 was constructed to include the entire coding sequence of C and eGFP between the structural and nonstructural genes. For increased stability, the WNV cDNA was also transferred to a bacterial artificial chromosome plasmid (pBAC, described in Suzuki et al., in press). All viruses used in this study were recovered from BHK cells electroporated with *in vitro* transcribed RNA [T7 MEGAscript (Ambion) supplemented with 7-MeG cap analog (New England Biolabs)] obtained from these pACNR-propagated or pBAC-propagated cDNA templates. Virus-like particles (VLPs) were produced as previously described (Fayzulin et al., 2006). Supernatants containing VLPs were harvested from electroporated packaging cells 2–7 days post transfection with *in vitro* transcribed replicon RNA. Schematic diagrams of all WNV or WNR constructs are shown in Fig. 1.

Construction of mutant WNR and full-length infectious clones

Total RNA was extracted from G418-resistant clones of Huh7 cells bearing replicons by using either the Trizol LS Reagent (Life Technologies) or RNaqueous kit (Ambion). This RNA was used as a template for reverse transcription (ImProm II, Promega) reactions using either random hexamers and/or specific antisense primers that anneal at the end of the 3′ UTR of WNV (5′ AGATCCTGTGTTCTCGCACC 3′). Fragments of replicon cDNA amplified from these reactions by PCR using WNV-specific primer pairs were gel purified and sequenced by standard techniques (Protein Chemistry Core Laboratory, UTMB). Mutations of interest were recreated by overlap PCR mutagenesis (Higuchi et al., 1988) or obtained from the PCR amplified cDNA fragments collected from Huh7 replicon-bearing cells and were cloned into replicon-encoding plasmids using standard recombinant DNA techniques. All mutant cDNA plasmid clones were sequenced through the PCR-amplified region to ensure no other missense mutations were present and *in vitro* transcribed RNA from these constructs was transfected into cells to ensure their ability to replicate. A full-length clone with a large deletion in the 3′ UTR of the virus (between nucleotides 43 and 388 of the 3′ UTR) was also generated using similar methods. In selected cases, fragments of cDNA were transferred to pBAC encoding the full-length infectious clone cDNA to create mutant viruses.

Immunohistochemical (IHC) staining of monolayers to detect WNV antigen

Viral antigen was detected by immunohistochemistry using a polyclonal mouse hyperimmune ascites fluid from WNV-infected mice as previously described (Rossi et al., 2005). In the cases where antigen staining was weak, the signal was enhanced using a biotin-conjugated secondary antibody, followed by streptavidin amplification (Vectastain ABC kit, Vector Technologies).

Determination of CFE and expansion of G418-resistance colonies

Colony-formation efficiency CFE was determined by electroporating replicon RNA harboring the NPT gene (WNR NS1-5 ET2AN; see Fig. 2) into Huh7 cells. An aliquot of these cells was seeded in a 24-well plate, fixed and immunostained 48 h post electroporation to determine the number of productively transfected cells. The remaining electroporated cells were serially diluted into 6-well plates and 48 h after electroporation, these cells were fed new growth media supplemented with 33% conditioned media and 400 μg/ml G418. This selective media was changed every 3 days for 2 weeks. Two weeks after electroporation, the cell monolayers were stained with crystal violet and the antibiotic-resistant colonies were counted. No colonies were detected in Huh7 cultures that were electroporated with no RNA and maintained under the same conditions. CFE was calculated as the ratio of G418-resistant colonies to the number of positively transfected cells.

Focus-formation assays and virus/VLP titration

All viruses were titrated on Vero cells except for samples taken for the Huh7 growth curve (see Fig. 5, Panel B), which were titrated on Huh7 cells. Monolayers in 96-well plates were infected with virus dilutions, overlaid with 0.6% tragacanth-containing media, incubated for 24–36 h and processed by IHC. VLPs were titrated in a similar manner except these cultures were not overlaid with tragacanth. Virus titration of mouse sera was performed as described above except in 24-well plates and incubated for 2–3 days prior to fixation and IHC staining. The numbers of infectious foci (or cells in the case of VLPs) were then used to determine the titer in focus forming units (ffu; virus) or in infectious units (iu; VLPs) per milliliter.

Flow cytometry

Vero cells were infected with VLPs bearing eGFP (WNR C-eGFP-NS1-5; see Fig. 2) at a MOI of 1, incubated for 1 h to allow for VLP adsorption, washed, refed with growth media and incubated for the indicated times. At harvest, cells were trypsinized, resuspended in PBS supplemented with 1% fetal calf serum and analyzed by flow cytometry (Flow Cytometry and Cell Sorting Core Laboratory, UTMB). Histograms of eGFP fluorescence at emission 530 nm of the viable cell population was determined by standard techniques and FloJo software (version 8.1) was used to calculate GM eGFP fluorescence for each sample.

MTT cell viability assays

Cell viability was measured by growing cells for 3 h in medium containing 1 mg/ml of 3,[4.5-dimethyltriazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and measuring the amount of MTT converted to an insoluble product as previously described (Rossi et al., 2005).

In vivo assessment of attenuation

Groups of 5 four-week-old female Swiss-Webster mice (Harlan) were inoculated i.p. with virus diluted in PBS supplemented with 10% FBS. Mice were observed daily for 15 days for signs of illness or death. Moribund mice were humanely euthanized to reduce their suffering and the next day was recorded as the day of death in compliance with UTMB Animal Care and Use requirements. LD_{50} values were calculated for each virus (Reed and Meunch, 1938).

Serum and brain tissue were collected from all euthanized animals and stored frozen (−80 °C). One of the frozen brain hemispheres was thawed in 1 ml L15 media and physically disrupted using a homogenizer (4 min at 30 cycles/s) and a steel ball (GlenMills Inc.). Supernatant was collected after clarification by centrifugation of homogenized brain tissue and stored at −80 °C. For analysis of progeny viral genomes in infected mice, Vero cells were infected with 1/10th the volume of the brain

homogenate supernatant. The cells were harvested 24 h later, RNA was isolated, viral genomes were amplified by RT-PCR and sequenced as described above.

Neutralization assay

Serum was collected from all surviving mice 1 month after challenge and tested for the presence of neutralizing WNV antibodies. Sera were heat inactivated at 56 °C for 30 min, diluted two-fold, incubated at 37 °C for 2 h with approximately 100 ffu of WNV VLPs containing a replicon encoding the full C gene and NS1-5 (R. Fayzulin and P.W. Mason, unpublished data), and then plated on Vero cells seeded in 96-well clear-wall plates. Twenty-four hours later, cells were fixed and immunostained, and antigen-positive cells were counted and used to determine the serum dilution that produced an 80% reduction in VLP-positive cells. In this assay, NMS collected from age-matched outbred mice displayed an 80% reduction in antigen-positive cells at a dilution of 1/80.

Detecting virus and IFN produced in mice

Four-week-old female Swiss-Webster mice were inoculated i.p. with 1×10^5 ffu of NS2A D73H WNV or wt WNV. Three mice/virus were humanely euthanized days 1–6 post inoculation and serum was collected as described above. Virus was detected by titration on Vero cells as described above.

IFN levels were measured using a bioassay. Serum samples were diluted with three volumes of 1× MEM without serum or phenol red and treated with UV light (254 nm, 4W, 10 cm) for 10 min to inactivate infectious virus. The UV-inactivated samples were then further diluted four-fold and used to inoculate MEF monolayers in 96-well, black-wall plates. Plates were incubated overnight (14–18 h), the serum was then aspirated and the monolayers were infected with VLPs containing WNR C-luc-NS1-5 (see Fig. 2). Twenty-four hours after VLP infection, cells were lysed by the addition of lysis buffer containing D-luciferin and ATP (SteadyGlo, Promega) and after a 5-min incubation, the plates were read on a luminometer. Percent inhibition was determined by normalizing photon/s of each sample to a sample that contained the same concentration of NMS alone. NMS containing a known amount of murine IFNβ (NIH standard; 0, 50 or 250 U/ml final concentration) were analyzed in parallel to show the percent inhibition of infection produced in mouse sera containing known IFN concentrations. The NIH standard murine IFNβ produced from L-cells and antibody to type I IFN were obtained from the NIAID Reference Reagent Repository, which is operated by KamTek, Inc. or Braton Biotech, Inc., respectively. Anti-type I IFN was also used to determine the specificity of our inhibition by preincubating serum with this antibody prior to analysis.

Statistical analyses

Comparisons among samples within a group were made using a two-tailed, unpaired Student's *t*-test. Incidence data were compared by Fisher's exact test.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.02.009.

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