Evaluation of replicative capacity and genetic stability of West Nile virus replicons using highly efficient packaging cell lines

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Received 6 February 2006; returned to author for revision 23 February 2006; accepted 25 February 2006
Available online 2 May 2006

Abstract

A stable cell system for high-efficiency packaging of West Nile virus (WNV) subgenomic replicons into virus-like particles (VLPs) was developed. VLPs could be propagated on these packaging cells and produced infectious foci similar to foci produced by WNV. Focus size correlated with the replicative capacity of WNV replicons, indicating that genome copy number, rather than amount of trans-complementing structural proteins, was rate-limiting in packaging of VLPs. Comparison of VLP production from replicon genomes encoding partial or complete C genes indicated that portions of C downstream of the cyclization sequence could improve genome replication or that cis expression of C could enhance packaging. Interestingly, a rapid loss of replicon-encoded reporter gene activity was detected within two serial passages of reporter gene-containing VLPs. The loss of reporter activity correlated with gene deletion and better VLP growth, indicating a powerful selection pressure for WNV genomes lacking reporter genes.

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Keywords: Flavivirus; Replicon; Packaging; Virus-like particle; Noncytopathic replicon

Introduction

West Nile virus (WNV) has widely spread in the USA since its introduction in 1999 and poses a significant threat to public health by producing large numbers of neuroinvasive infections that can cause death or serious sequelae. Like other flaviviruses, WNV has a single-stranded, positive-sense RNA of about 11 kb in length that encodes a single polyprotein that is co- and post-translationally cleaved into 3 structural and 7 nonstructural proteins (Chambers et al., 1990).

To study flavivirus replication, genetic engineering has been used to generate self-replicating flavivirus genomes (replicons) lacking the structural protein-encoding region (Gehrke et al., 2003; Jones et al., 2005; Khromykh and Westaway, 1997; Pang et al., 2001; Shi et al., 2002). In the case of WNV, we have recently shown that cells expressing high levels of the missing WNV structural proteins (C, prM, and E) produced by a genetically engineered Sindbis virus replicon, could be used to package WNV replicons into infectious virus-like particles (VLPs) capable of undergoing a single round of infection in normal cells (Scholle et al., 2004). Other systems have been described for packaging flavivirus replicons, including Kunjin virus replicons (Harvey et al., 2004; Khromykh et al., 1998), yellow fever virus replicons (Jones et al., 2005), tick-borne encephalitis virus (TBEV) replicons (Gehrke et al., 2003), and WNV replicons (Hanna et al., 2005; Puig-Basagoiti et al., 2005). In some cases, these packaging systems have utilized cell lines (Gehrke et al., 2003; Harvey et al., 2004) expressing the flavivirus structural proteins under control of eukaryotic promoters. In both of these reports, spread of infection by replicons on packaging cell lines was noted (Gehrke et al., 2003; Harvey et al., 2004), and one reported the ability to repeatedly repackage replicons (Gehrke et al., 2003). These VLP-generating systems have been useful for packaging VLP genomes encoding various reporter genes (Jones et al., 2005;
Pierson et al., 2006; Puig-Basagoiti et al., 2005) or antigens (Harvey et al., 2003), the study of virus tropism in an arthropod vector (Scholle et al., 2004), and various aspects of viral assembly and entry (Hanna et al., 2005). The use of VLPs for some applications can be limited by modest titers that are produced by various packaging approaches and/or lengthy and inconvenient VLP production protocols.

Here we describe the development of cell lines selected to persistently harbor noncytopathic subgenomic replicons of Venezuelan equine encephalitis virus (VEEV) encoding puromycin N-acetyl transferase (pac) and the WNV C/prM/E cassette. Using these replicons, cell lines that expressed high levels of WNV structural proteins were rapidly generated. These cell lines were shown to be useful for amplifying VLP stocks and comparing the replication capacity of WNV replicons with different genetic structures. In addition, we have used these packaging cell lines to demonstrate that WNV replicons encoding reporter genes are genetically unstable, losing reporter gene activity upon repeated rounds of infection and amplification in packaging cell lines.

**Results**

**Correlation of VLP burst size on packaging cell lines with replicon RNA levels**

Multiple laboratories have shown that flavivirus subgenomic replicons lacking the structural protein genes are unable to spread cell-to-cell. In contrast, when introduced into packaging cells, subgenomic replicons can undergo repeated packaging and infection cycles, utilizing the structural proteins supplied by the cell, producing virus-like particles (VLPs) that result in a spreading infection similar to that observed for live WNV (Gehrke et al., 2003; Jones et al., 2005; Khromykh and Westaway, 1997; Pang et al., 2001; Shi et al., 2002) (see Introduction). To generate cells capable of packaging WNV, we constructed plasmid DNAs encoding noncytopathic VEEV replicons expressing pac and the WNV C/prM/E cassette (see Fig. 1B), and then introduced their in vitro-synthesized VEEV replicon RNAs into BHK cells and selected C/prM/E-expressing cells by treatment with puromycin (Pur). As expected, the selected cells expressed both WNV and VEEV antigens (results not shown). Our initial characterization of Pur-resistant (PurR) VEErep/C-E/Pac-transfected BHK cells (BHK(VEErep/C-E/Pac)) demonstrated that these cells produced VLPs when transfected with either of three different WNV replicon RNAs: (1) a synthetic RNA of a minimal WNV replicon (WNR NS1-5; see Fig. 1A), (2) a synthetic RNA of a WNV replicon containing an antibiotic resistance gene (WNR NS1-5ET2AN; see Fig. 1A), or (3) a replicon RNA (designated WNR cl 1.1) which was harvested from Huh7 cl 1.1 cells harboring a cell-adapted derivative of WNR NS1-5ET2AN which replicates to a very low level (see Fig. 1A).

To characterize the infection of the packaging cells with these three different replicons, monolayers of BHK(VEErep/C-E/Pac) cells were infected at low multiplicity with these three VLPs, overlaid with a semisolid overlay to slow VLP diffusion, and then immunostained with an antibody specific for WNV NS1. Evaluation of the immunostained foci formed by these VLPs on BHK(VEErep/C-E/Pac) cells revealed that focus size increased with incubation time (Fig. 2A), and that the size of the foci correlated with our previously reported levels of WNV antigen expression observed in cells transfected with these replicons (Rossi et al., 2005). Specifically, the WNR NS1-5 foci were much larger than the WNR NS1-5ET2AN foci, which, in turn, were larger than the foci obtained with the WNR cl 1.1 replicon (Fig. 2A). Interestingly, cytopathic effect (CPE) was visible in BHK(VEErep/C-E/Pac) cultures infected with VLPs containing WNR NS1-5 (results not shown) and cytopathic plaques were visible within the immunostained foci formed on these cells with the WNR NS1-5 and WNR NS1-5ET2AN VLPs (Fig. 2A), but not in the foci formed with WNR cl 1.1. To further characterize the phenotype of these three WNV replicons, we calculated the WNV genome copy number in cells infected with these VLPs, by a quantitative dot-blot assay (see Materials and methods). Fig. 2B shows the linear relationship between the genome copy number of these replicons in BHK cells (abscissa) and focus size (ordinate) on monolayers of packaging cells. Growth curves of these VLPs on BHK(VEErep/C-E/Pac) cells (Fig. 2C) confirm the poor replication properties of the cl 1.1 VLPs, consistent with the low level of replication of the Huh7 cl 1.1-adapted replicon, which we hypothesized is the basis for its ability to persist in Huh7 cells (Rossi et al., 2005).

**Expression of WNV structural cassette by a single subgenomic VEEV replicon is stable in BHK cells**

When RNAs encoding the double-subgenomic promoter VEEV replicon (VEErep/C-E/Pac) were introduced into BHK cells using electroporation, a high percentage of the cells was immunopositive for both VEEV and WNV proteins. Selection of these transfected cultures with Pur resulted in the loss of cells that did not express the VEEV replicon, and efficient formation of PurR colonies as expected (Fig. 3A). However, these PurR cultures also accumulated cells that no longer expressed WNV antigens (Fig. 3B), suggesting genetic instability of the double-subgenomic VEEV replicon in these pooled cell cultures, and a selective advantage of WNV antigen-negative cells. In an attempt to overcome this problem, individual cell clones were isolated, and their analysis revealed that clones expressing high levels of WNV antigen grew more poorly than clones lacking WNV antigen expression (results not shown). These findings were consistent with the possibility that WNV structural proteins were toxic, as we previously observed with cells expressing fusion-competent forms of the JEV prM/E (Konishi et al., 2001). However, we predicted that the fusion-competent forms of prM/E would not be efficiently produced in the BHK (VEErep/C-E/Pac) cells, since NS2B/NS3 is required for efficient prM/E particle formation if prM/E are co-expressed with C (Konishi and Mason, 1993; Yamshchikov and Compans, 1994). Thus, the C/prM/E polyprotein or fragments thereof may exhibit some level of toxicity in these cells, or a low level of signal peptidase cleavage at the C-prM junction may produce a
To overcome the genetic instability problem observed in the VEErep/C-E/Pac double-subgenomic promoter VEEV replicon, we created a single-subgenomic promoter VEEV replicon (VEErep/C*-E*-Pac) in which the WNV structural genes were fused to the pac gene (see Materials and methods and Fig. 1B). When introduced into BHK cells, replicon VEErep/C*-E*-Pac conferred Pur resistance (Fig. 3C), and essentially all of the cells in PurR colonies were immunopositive for WNV antigens (Fig. 3D), in contrast to PurR colonies produced by replicon VEErep/C-E/Pac, where E-negative cells were present in every PurR colony (Fig. 3B). Colonies expressing the VEErep/C*-E*-Pac replicon (Fig. 3C) were generally smaller in size than colonies expressing the VEErep/C-E/Pac replicon (Fig. 3A), consistent with the toxic effects of expression of the WNV cassette (see above). When assayed for the amount of WNV antigen expressed at different time-points after electroporation, cultures transfected with VEErep/C*-E*-Pac RNA produced more WNV E antigen than cultures transfected with VEErep/C-E/Pac RNA (Fig. 3E); this finding is explained by increased synthesis from the subgenomic promoter driving E expression due to removal second promoter driving Pac synthesis.

Clonal cell lines expressing single or double-subgenomic promoter replicons produce high titers of VLPs and express high levels of E

To examine the properties of VEEV replicon bearing cells in more detail, we isolated individual cell clones from cultures transfected with VEErep/C-E/Pac and VEErep/C*-E*-Pac replicons. Immunostaining of the cloned cells revealed that over 95% of the cells present in each of the BHK (VEErep/C*-E*-Pac) clones expressed WNV E, whereas, some of the BHK(VEErep/C-E/Pac) clones contained a

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**Fig. 1.** Schematic diagrams showing the replicons used in this study. (A) WNR NS1-5, WNR NS1-5ET2AN, and a cell-adapted form of WNR NS1-5ET2AN (WNR cl 1.1) have been described (see Materials and methods). Replicon WNR C-Luc2A NS1-5 and WNR C-bgal NS1-5 were created from a genome-length infectious cDNA of WNV using standard techniques (see Materials and methods). (B) VEEV replicons were derived from replicon VEErep/Pac (5′VEErep/S/Pac), a noncytopathic replicon that was described in Petrakova et al. (2005). * indicates the position of 36 clustered silent mutations at start of C (found in VEErep/C*-E-Pac and VEErep/C*-E*-Pac) or 32 clustered silent mutations in the signal peptide of NS1 (found in VEErep/C*-E*-Pac).
substantial fraction of cells that did not express E (see above, Fig. 3B). To simplify evaluation of the BHK(VEErep/C-E/Pac) clonal cells, all clones that contained more than 20% WNV E-negative cells were discarded from subsequent analyses. Once these clones were excluded from consideration, all remaining clones obtained from the double-subgenomic construct produced similar amounts of E-antigen as that detected in the clones of cells expressing the single-subgenomic construct (Fig. 4). Furthermore, high titers of VLPs were produced by all of the clones tested (except for clone a of BHK(VEErep/C-E/Pac)—see below; Fig. 4). The small variations in VLP titers observed among all the clones (except BHK(VEErep/C-E/Pac)clone a) indicated that there were no detectable differences in the packaging efficiencies of the proteins produced by either the single- or double-subgenomic VEEV replicons. Furthermore, there was no correlation between the level of WNV E-antigen and the titer of packaged VLPs. Taken together, these results suggest that the levels of E antigen produced in these cells are at levels that are saturating with respect to those needed to package our WNV replicons, and that slight differences in the amount of E detected did not result in detectable differences in the titers of WNV VLPs produced.

Interestingly, the single clonal packaging cell line which showed a normal level of E production and a low level of VLP production {BHK(VEErep/C-E/Pac)clone a; Fig. 4} was found...
to have a nonsense mutation in the initiation site of the WNV C protein. This AUG was mutated to AUA in BHK(VEErep/C-E/Pac)clone a, requiring the WNV polyprotein to initiate at an in-frame AUG 16 codons downstream, producing a truncated C protein. Although this finding was not pursued, it suggests that a truncated C can function in flavivirus assembly, albeit less efficiently.

Clonal cell lines expressing a single-subgenomic VEEV replicon can be passaged without a loss in packaging ability

To investigate differences in genetic stability of the WNV insertions in the mono- and double subgenomic VEEV replicons in their corresponding packaging cell lines in more detail, we compared the ability of different passage levels of
clonally derived cell lines to replicate VLPs containing WNR NS1-5. For these studies, we selected two clones: BHK (VEErep/C*-E*-Pac)cl.2.9 (mono-subgenomic replicon) and BHK(VEErep/C*-E/Pac)cl.8.2 (harboring double-subgenomic replicon VEErep/C*-E/Pac), which has a genetic structure that differed from our original replicon VEErep/C-E/Pac by clustered silent mutations at the start of C that were introduced to reduce the chance of recombination; indicated by the “*”, see Materials and methods and Fig. 1B). These two cell lines were passed side-by-side for 3 weeks, and cells were cryopreserved at the starting passage level (passage 2, p2) and at the end of each subsequent week (p5, p9, p13). Following thawing, the cells were compared side-by-side for percentage of WNV E-negative cells (Fig. 5A), production of VLPs (Fig. 5B), and focus size formed by WNR NS1-5 VLPs (Fig. 5C).

During passaging, BHK(VEErep/C*-E/Pac)cl.8.2 became enriched with E-negative cells, whereas BHK(VEErep/C*-E*-Pac)cl.2.9 maintained the same high proportion of E-positive cells (Fig. 5A). Fig. 5B shows that both cell lines produced high levels of VLPs at lower passage levels (where the percentage of E-positive cells was similar), but at higher passage levels, the BHK(VEErep/C*-E/Pac)cl.8.2 cultures produced lower titers of VLPs (Fig. 5B). These data are consistent with the prediction that E-negative cells present in the high-passage BHK(VEErep/C*-E/Pac)cl.8.2 cultures were unable to participate in VLP production, and that these E-negative cells could serve to limit VLP production, by acting as substrates for VLP infection that were unable to produce progeny VLPs. The high-passage BHK(VEErep/C*-E/Pac)cl.8.2 cultures also produced smaller numbers of infectious foci than lower-passage cultures (Fig. 5C), and the foci sizes were smaller (Fig. 5C), consistent with the idea that the nonpackaging cells in these cultures that were infected during the initial adsorption period were unable to produce progeny VLPs (resulting in a lower number of foci formed) and that nonpackaging cells surrounding packaging cells infected during the initial adsorption infection soaked up progeny VLPs hindering spread (and thus reducing focus size).

![Graph A](image1.png) ![Graph B](image2.png) ![Graph C](image3.png)

**Fig. 5.** Passaging of selected BHK clones harboring VEErep/C*-E/Pac or VEErep/C*-E*-Pac replicons revealed the instability of the double-subgenomic (VEErep/C*-E/Pac) construct. BHK(VEErep/C*-E/Pac)cl.8.2 and BHK(VEErep/C*-E*-Pac)cl.2.9 clones were passaged for 13 passages (with splits of 1:10 at each passage level) with weekly cryopreservation. The cultures were then thawed and assayed side-by-side by IHC to determine the number of E antigen-expressing cells, and used as substrates for VLP production and in focus forming assays. (A) Percent of cells expressing E antigen. (B) VLP production at 72 h postinfection with WNR NS1-5 VLPs at MOI = 0.1. (C) Photograph of foci formed by WNR NS1-5 VLPs on cells [(1) BHK(VEErep/C*-E/Pac)cl.8.2, (2) BHK(VEErep/C*-E*-Pac)cl.2.9] at different passage levels.
Replication of WNV genome is not affected in cells expressing VEEV replicons

To investigate whether the presence of VEEV replicons (and/or the replicon-encoded WNV proteins) interfered with WNV replication, we assessed WNV focus morphology on BHK, BHK(VEErep/C*-E/Pac) cl.8.2, and BHK(VEErep/C*-E*-Pac) cl.2.9 cells. The side-by-side comparison shown in Fig. 6A failed to reveal large differences in focus size and morphology on monolayers of BHK and BHK replicating VEEV replicons VEErep/C*-E/Pac or VEErep/C*-E*-Pac. Fig. 6A also shows that cells harboring VEErep/Pac replicon (which does not express any WNV proteins) produce WNV foci similar to those seen on BHK.

To verify that packaging cells replicated WNV genomes similarly, we infected BHK as well as BHK(VEErep/Pac), BHK (VEErep/C*-E/Pac), and BHK(VEErep/C*-E*-Pac) with VLPs containing WNV replicon expressing firefly luciferase (Luc) (WNR C-Luc2A NS1-5, see Fig. 1A). As we and others have previously published (Rossi et al., 2005; Shi et al., 2002), expression of Luc produced from replicons is directly proportional to the level of genome replication and offers a simple method to measure genome replication. As shown in Fig. 6B, the WNR C-Luc2A NS1-5 replicon replicated equally well on all cell lines. Taken together, these data indicate that neither the presence of replicating VEEV RNA nor VEEV proteins reduced WNV replication, in agreement with previously published results (Petrakova et al., 2005), and further demonstrated that the expression of high levels of WNV structural proteins in these cells had no deleterious effect on WNV genome replication.

Comparisons of VLP and virus yield revealed that VLPs were produced more poorly than virus on the packaging cell lines, suggesting that cis expression of the structural genes and/or inhibition of assembly by the truncated C protein produced by WNR NS1-5 could result in lower packaging. Fig. 7A shows that in side-by-side electroporations of packaging cells with WNV RNA and WNR NS1-5 RNA, virus grew to higher titers than VLPs. Since there is a lag in the production of infectious virus from electroporated cells (see Fig. 7A, and results not shown), we compared production of virus and VLPs in this packaging cell line following infection with VLPs and WNV. At both multiplicities of infection (MOI) used in this experiment, VLPs harboring WNR NS1-5 were produced at lower titers than live virus (Figs. 7B, C). The growth advantage of virus was also observed in focus-formation assays. Specifically, WNR NS1-5 foci formed on monolayers of low-passage BHK(VEErep/C*-E/Pac) cl.8.2 cells were smaller than those formed by WNV (Figs. 7D, E).

Reporter genes are rapidly lost from WNV replicons during serial passage

We have previously shown that once introduced into cells, WNV replicons can be stably maintained for up to 40 passages (Rossi et al., 2005). However, our previous efforts to make recombinant WNV with reporter genes in the 3′ UTR met with rapid loss of reporter gene activity during passage and deletion of portions of the reporter gene cassette (Zhao and Mason, unpublished), consistent with a recent report by Doms and co-workers (Pierson et al., 2005). To examine the stability of reporter-expressing replicons in our packaging cells, we examined the effects of serial passage of VLPs on titer and reporter gene activity. VLPs could be passaged in packaging cells for multiple passages, achieving titers similar to those obtained in original infections (results not shown). However, when VLPs were assayed for reporter activity, a rapid loss of activity was observed with each passage (Figs. 8A, B). This loss
of reporter activity was so rapid that we were only able to obtain VLPs with a high level of reporter gene activity from direct electroporations (p0 in Figs. 8A, B). Despite this limitation, high titers of reporter gene-encoding VLPs could be produced from the samples collected from WNV replicon-electroporated cultures of every packaging cell line we tested.

At the same time that the reporter gene activity was lost from serial passages of VLPs containing replicons WNR C-Luc2A NS1-5 and WNR C-bgal2A NS1-5 (see Fig. 1A), a change in their focus-forming phenotype was observed. Examination of focus sizes for our minimal replicon (WNR NS1-5) demonstrated no change between passages p0 and p6 (labeled [1] in Fig. 8C). However, large foci were present in p0 of WNR C-Luc2A NS1-5 and WNR C-bgal2A NS1-5, and became the only detectable population in passage 6 (compare [2] and [3] p0 and p6 in Fig. 8C). Using an in situ β-galactosidase (βgal) assay, we were able to clearly demonstrate that the VLPs producing the large foci in the p0 population of WNR C-bgal2A NS1-5 were missing the βgal activity, since staining of the monolayer with X-Gal ([3] in Fig. 8D) revealed that only small foci produced functional βgal. When the same monolayer was immunostained, large foci that were βgal-negative ([3] in Fig. 8D) were also detected ([3] in Fig. 8E). These data are consistent with reports by Mandl and co-authors that TBEV replicons lose reporter activity (Gehrke et al., 2005), although other labs have not reported loss of replicon reporters during transient (Harvey et al., 2004; Jones et al., 2005; Khromykh et al., 1998; Varnavski and Khromykh, 1999) or continuous packaging of flavivirus replicons (Harvey et al., 2004).

Analyses of the sequence of the region from the 5′UTR through the middle of NS1 of passage 6 of our minimal replicon (WNR NS1-5) showed that no changes from the original cDNA sequence were detected during 6 passages, consistent with our inability to detect a change in focus phenotype between p0 and p6 ([1] in Fig. 8C). However, analyses of the packaged reporter gene-encoding replicons indicated that the reporter genes were so rapidly truncated that deletions were identified in cells infected with VLPs harvested from electroporated cultures. These analyses included the demonstration that PCR reactions obtained from the reporter-encoding fragments of cDNAs derived from cells infected with the p0 VLPs containing either WNR C-bgal2A NS1-5 or WNR C-Luc2A NS1-5 contained a ladder of amplification products smaller than the size obtained from the parental in vitro transcribed RNA (see Supplemental
Furthermore, sequencing of several of these fragments revealed that all contained in-frame deletions eliminating most of the reporter gene. Interestingly, the entire C protein-coding region was maintained in the last passage examined (p6; see Supplemental data). These sequence data and the data showing that these p6 replicons produced infectious foci larger than WNR NS1-5 (Fig. 8C) suggest that a full-length C gene rather than the truncated C in WNR NS1-5 could have a positive effect on replicon replication or packaging. This finding was further supported by results from a side-by-side VLP yield experiment showing that VLPs from p6 reporter gene replicons were released from packaging cells at titers approximately 10-fold higher than those produced by p0 or p6 WNR NS1-5 VLPs (results not shown).

The loss of genetic material from the replicon genomes is consistent with internal recombination to eliminate the reporter gene construct, indicating both a strong selective pressure for reporter-deleted replicon genomes and recombination potential of the WNV replicon genome. Although we cannot be 100% certain that the deletions were produced in vivo (rather than in the in vitro T7 reactions), we were unable to detect any evidence of truncated genomes in our in vitro transcripts (see Supplemental data). Furthermore, attempts to “clone” packaged replicons that maintained reporter activity failed, sequential staining of foci of infection of WNR C-bgal2A NS1-5 for βgal...
and antigen revealed that X-Gal-negative cells that reacted strongly with our antibody to NS1 often appeared within these foci, suggesting a high frequency of recombination events.

Despite the generation of intra-molecular recombinants in our WNV replicons, we were unable to detect recombination between any of the WNV replicons and the VEEV replicons that produced live virus. Specifically, two blind-passages on Vero cells of undiluted passage-6 harvests from all three VLPs shown in Fig. 8C failed to produce any self-propagating WNV detectable with antibodies to the WNV E protein using a highly sensitive immunohistochemical (IHC) staining method.

Discussion

In this study, we describe the construction and application of novel VEEV replicon-based cell lines that can be used to package WNV replicons. We have previously described a highly efficient alphavirus replicon-derived packaging system for WNV subgenomic replicons (Scholle et al., 2004) that utilizes sequential electroporations of WNV replicon RNA, followed by a Sindbis replicon RNA encoding the WNV structural proteins. The VEEV replicons used to drive the WNV structural protein expression used in the current study have several advantages over our previously described Sindbis replicon system. Specifically, VEEV replicons are less cytoxic, and PurR cell lines expressing high levels of C-prM-E can be rapidly selected from cultures electroporated with these replicons. Furthermore, the resulting cell lines can be used for side-by-side analyses of WNV replicons with altered genetic structure, allowing more precision in evaluation of WNV genome function than available by using our previously described dual-electroporation system. Stable packaging cell lines have been described previously in CHO cells where expression of TBEV prM/E proteins was under control of a CMV promoter (Gehrke et al., 2003) and in another study using an inducible promoter system to package Kunjin virus replicons (Harvey et al., 2004). Our system circumvents the lengthy identification of inducible clones with appropriate tightness of promoter regulation. Our packaging cell lines produce high levels of packaged replicons (VLPs) and have proven useful for characterizing levels of replicon expression, producing reporter protein-encoding VLPs, and characterizing WNV genome instability.

The VEEV replicons described in our study are able to establish persistent replication in BHK cells and express the WNV E protein, at levels comparable to those expressed in WNV-infected cells. Although these packaging cell lines produce slightly lower levels of WNV VLPs than those obtained by us in sequential electroporation using Sindbis replicons (Scholle et al., 2004), the VEEV replicon cell lines are easier to work with, and by using them, we have been able to readily characterize the genetic stability of a variety of WNV replicon constructs. The most significant advantage of packaging cell lines compared to the use of sequential electroporations is the ability to amplify existing VLPs stocks (see below), reducing the need for template preparation and in vitro transcriptions.

WNV replicon RNAs introduced into our VEEV-replicon-based packaging cells produced infectious foci and were able to undergo multiple rounds of replication and packaging, displaying properties similar to the system reported by Mandell and coworkers (Gehrke et al., 2003). As expected, the packaging cell line produced by this group encoded the prM and E proteins in the absence of C, so it could only package TBEV replicons that contained a complete C gene (Gehrke et al., 2003). Our packaging cell lines contain the C-coding region and efficiently packaged replicons with either partial or complete C-coding region. Interestingly, replicons with a full-length C-coding region yielded higher titers of infectious VLPs than partial-C genomes. These results demonstrate that C expressed in trans is able to function in genome packaging in the absence of cis-expressed C, but that C expressed in cis increases packaging efficiency, due to either enhanced genome replication and/or more efficient genome encapsidation (currently under investigation).

Using our cells, we were able to demonstrate differences in VLP focus size of packaged replicons containing no reporter (WNR NS1-5), a 3' UTR reporter (WNR NS1-5ET2AN), and a derivative of this latter replicon that had been adapted to replicate persistently in Huh7 cells, by reducing its replicative capacity (Rossi et al., 2005). Thus, the size of these foci correlated with the RNA replication ability of the respective WNV replicon introduced. Since the packaging cells provide constant amounts of viral structural proteins with similar supply, assembly, and cell binding properties, the WNV replicon RNA becomes the rate-limiting component that determines efficiency of VLP synthesis and spread of infection. Thus, our packaging cells should be useful for rapidly evaluating mutations in the noncoding and nonstructural coding regions of the WNV genome that alter replication, by merely comparing the size of foci formed on packaging cells. Interestingly, the presence of high levels of trans-expressed WNV C/prM/E protein in these cells did not appear to have any effect on the growth of WNV or the replication of WNV replicons.

Our analyses also showed that these packaging cells produced higher yields of virus than VLPs following either synthetic RNA transfection or infection with virus or a minimal VLP (WNR NS1-5) generated from the same reverse genetics system. There are several possible reasons for this difference. One is that the trans-expressed forms of C, prM, or E (even though they are expressed at levels higher than that found in WNV infection) were not as efficiently incorporated into VLPs as cis-expressed virally encoded structural proteins. Alternatively, the fragments of C and/or signal sequence to NS1 encoded in our “minimal” replicon interfere with genome encapsidation, or there are some specific packaging signals in the region deleted from our replicon genome that contribute to packaging efficiency.

Our prototype packaging cell lines used noncytopathic, double-subgenomic VEEV replicons to express antibiotic resistance needed to maintain the VEEV replicon and to produce the WNV structural proteins. Although cells with these replicons were very useful for packaging, sequential passaging of the cells resulted in the loss of the WNV structural protein...
from some cells. In one case, we noted the introduction of a stop codon in the WNV coding region (results not shown) of the replicons present in one WNV antigen-negative cell clone, but it is also possible that nonsense mutations in the WNV coding region and/or deletion of the WNV coding region could all produce cell lines that would be WNV antigen-negative and thus unable to package WNV replicons. To overcome this problem, we fused the WNV structural proteins with an antibiotic resistance gene to create a noncytopathic VEEV replicon expressing a monocistronic message that was capable of maintaining the WNV proteins for long-term passaging. The cell line containing this construct showed a slight growth defect relative to parental BHK cells, but readily packaged replicons for multiple passages. Nevertheless, the growth retardation of this cell line and the loss of WNV antigen from cells expressing the double-subgenomic VEEV replicon suggest that the WNV C/prM/E proteins have some toxic effects on cells. Other investigators have circumvented toxicity problems with flavivirus structural proteins through the use of inducible promoter systems (Harvey et al., 2004) or the identification of cell lines that are less sensitive to these toxic effects, such as CHO cells (Gehrke et al., 2003).

Flavivirus replicons expressing heterologous genes have a wide range of potential applications from monitoring changes in RNA replication via reporter readout to their use as effective genomes. VLPs can serve as a convenient delivery vehicle for those replicons, displaying infectious properties very similar to those of the virus they are derived from (Scholle et al., 2004). Our data and those of other investigators demonstrate that encapsidation of reporter-containing replicons into VLPs is possible at titers high enough to be useful for a variety of applications. However, in this manuscript, we identified a significant selective pressure against encapsidation of reporter gene expressing replicons when they are passaged on a packaging cell line. Even after just one passage, approximately 90% of reporter activity was lost, despite continued efficient VLP production for multiple passages. These results can be explained by a strong selection for reporter-deficient replicons that replicate to higher levels than their reporter-containing counterparts that are, therefore, preferentially packaged and transduced to naive cells in each subsequent passage. After a few rounds of passaging, all reporter-expressing replicons were eliminated from our preparations. Loss of the reporter gene activity correlated with a deletion of the genes encoding the reporter genes. This loss is easily explained by the enhanced fitness and spreading of the reporter-deleted replicons; these replicons produced larger foci on cells than their parental reporter gene-encoding replicons, consistent with their apparent superior fitness.

Despite the fact that WNV replicons lose their reporter genes, this problem can be readily overcome by using a highly efficient transfection or high MOI infection to introduce flavivirus replicons into the packaging cells and produce VLPs in a single reaction without the need for multiple passages. Using this method, along with polyethylene glycol precipitation, we have readily obtained preparations of reporter gene-bearing replicons with titers of over 10^8 infectious units (iu) per ml (data not shown).

The results of these studies clearly demonstrate the significant selective pressures to eliminate unneeded genetic material within WNV genomes that are repeatedly repackaged. This loss of information is not strictly related to genome packaging constraints since some of our reporter-encoding WNV replicon genomes are similar in size to the WNV genome. The loss of reporter genes in these VLP passaging experiments stands in stark contrast to the maintenance of reporter genes in cell lines bearing cell-adapted WNV replicons (Rossi et al., 2005). In the case of these cell-adapted replicons, the replicon-encoded reporter genes have been maintained for dozens of passages in the presence of the selective antibiotic (Rossi et al., 2005), and the resistance marker genes are also maintained for weeks in the absence of antibiotic application (results not shown). Taken together, these findings demonstrate that the selective pressures placed on WNV replicon genomes are significantly different in cell-restricted (persistent infection) and spreading (VLP transmission) environments. These studies therefore confirm the elegant genetic simplicity of these agents, and the continuous genetic pressure that maintains compact and effective genomes.

Materials and methods

Cell lines

BHK, Vero, Huh7, and a Huh7-derived cell line (Huh7 cl 1.1) harboring WNR NS1-5ET2AN have been described previously (Rossi et al., 2005).

WNV replicons

A prototype WNV replicon (WNR NS1-5) and a WNV replicon harboring the HIV tat gene fused to the gene for neomycin phosphotransferase II (neo) via the FMDV autocatalytic proteinase 2A (WNR NS1-5ET2AN) have been described (Rossi et al., 2005) (see Fig. 1A). Two similar WNR NS1-5 replicons were used in our studies, both had the same overall genetic structure, but one contained a sense mutation at position 2242 A–G, codons K72E in NS1; in all assays, we detected no difference in replication or packaging of these two replicons. To help in the characterization of WNV replicon packaging and assembly, two additional replicons were constructed from the same cDNAs (Rossi et al., 2005) used to make the other replicons (Fig. 1A). One of these (designated WNR C-Luc2A NS1-5) was created by insertion of the Luc gene (lacZ) between the C and NS1 protein-encoding regions of the WNV genome. This construct contained the sequences encoding all of C and the first 8 codons of the anchor of C (which also serves as the signal sequence for prM), fused in-frame to the initiation codon of Luc. The lacZ gene was then followed by FMDV 2A and the NS1 signal peptide (see Fig. 1A). A βgal construct with a similar structure was generated by insertion of the lacZ gene followed by the FMDV 2A coding region in the same site used to engineer WNR C-Luc2A NS1-5, generating a construct
Development of VEEV replicons expressing WNV structural proteins

Fig. 1B shows the schematic diagrams of the VEEV replicons used in this study; replicon VEErep/Pac is the same as 5′VEErep/S/Pac (Petrakova et al., 2005). Replicon VEErep/C*-E/Pac was created by inserting the WNV C/prM/E cassette from a plasmid encoding SR WN C/prM/E (described in Scholle et al., 2004) downstream of a second subgenomic promoter added to the cDNA encoding VEErep/Pac (see Fig. 1B). The sequence encoding the first 29 amino acids of C of VEErep/C-prM/E cDNA was changed by introduction of 36 clustered silent mutations to yield replicon VEErep/C*-E/Pac. These mutations ablated the cyclization signal needed for flavivirus replication (Corver et al., 2003; Khromykh et al., 2001; Lo et al., 2003), providing a safeguard against production of viable live virus by recombination with WNV replicon genomes that were subsequently expressed in these cells (see below). A single-subgenomic VEEV replicon was created by fusing the WNV C/prM/E cassette of VEErep/C*-E/Pac to the pac gene through a linker consisting of the first 9 codons of NS1 fused to the last 25 codons of NS2B, followed by 2 codons of NS3 fused directly to FMDV 2A (see Fig. 1B). To reduce the chance of recombination between this VEEV replicon and the WNV replicon genomes, 32 clustered silent mutations were introduced into the coding sequence of the signal peptide of NS1 in replicon VEErep/C*-E-Pac. VEEV replicon RNAs were produced by linearizing plasmid DNAs with MluI and generating transcripts by using a MegaScript SP6 kit (Ambion). BHK cells persistently expressing VEEV replicons were selected by culturing cells, electroporated with these synthetic RNAs, in the presence of 10 μg/ml of Pur.

IHC staining and quantitative ELISA

IHC staining and ELISA were performed as previously described using acetone/methanol-fixed cell monolayers or Triton X-100-solubilized cell lysates (Rossi et al., 2005). Immune reagents included mouse hyperimmune ascites fluids specific for WNV or VEEV (supplied by R.B. Tesh, UTMB), or monoclonal antibodies (MAb) to WNV E (7H2; Bioreliance), flavivirus E (D1-4G2; Gentry et al., 1982), or WNV NS1 (6H4; generously supplied by E. Konishi, Kobe University, Kobe, Japan). Affinity purified goat anti-mouse HRP-conjugated IgG (KPL) was used as a secondary antibody for both types of assays. E protein expression levels were determined using ELISA data by comparing values from VEEV replicon-expressing cells to the values obtained with dilutions of a lysate of WNV-infected BHK cells harvested at 30 h after infection (dilutions were prepared in a lysate obtained from uninfected BHK cells, to keep the protein concentration constant; standards were coated on the plates at the same protein concentration as test samples, and run in each assay). These assays produced a linear standard curve that revealed that WNV E antigen levels were nearly constant between 24 and 36 h postinfection with WNV, and increased slightly at later time points. Using the standard curve obtained from the WNV-infected sample, the amounts of E present in the VEEV replicon cell lines were determined; these values are displayed relative to the value of “100” which was assigned to the amount of antigen present in BHK cells 30 h after infection with WNV.

VLP production

VLPs were produced in packaging cells by infection with previously derived VLPs, electroporation with 5 to 10 μg of WNV replicon RNAs synthesized in vitro (see above), or electroporation with replicon RNAs present in total RNA isolated from cells (HuH7 cl 1.1) harboring a persistently replicating WNV replicon. After infection or transfection, the cells were maintained on media with 1% fetal bovine serum (FBS; low serum concentration was used to prevent cell overgrowth) and VLP-containing supernatants were collected at indicated times and frozen for subsequent analysis.

VLP titrations

VLPs titers were determined by infecting monolayers of HuH7 cells with small volumes of serial dilutions of VLP preparations, and refeeding with media containing 1% FBS after a 60 min adsorption period. Cell layers were fixed 24–30 h later and subjected to IHC staining as described above. The number of immunopositive cells was determined in wells and used to calculate VLP titers which were expressed as iu/ml.

VLP plaque assay

VLP foci (or plaques) were visualized on monolayers of packaging cells BHK(VEErep/C-E/Pac), BHK(VEErep/C*-E/Pac), or BHK(VEErep/C*-E*-Pac) by infection as described above and then refeeding with a semisolid overlay containing 0.6% tragacanth (MP Biomedicals) to prevent VLP diffusion. Cell layers were fixed 2 to 4 days later and immunostained as described above.

Serial passaging of VLPs using packaging cells

Low passage BHK(VEErep/C*-E/Pac)cl.8.2 cells were aliquoted and stored in liquid nitrogen. After thawing, cells were plated in 12-well plates for 16 h before infecting. The first VLP passage was performed with MOI = 0.5 using samples harvested at 60 h postelectroporation. All other passages were performed by adding 200 μl of a 1:10 dilution of VLPs from previous passage to cell layers and rocking plates for 1 h. After inoculums were removed, 1.0 ml of 1% FSB media was added to each well. VLP-containing media were harvested at 3 days after the beginning of infection. Side-by-side titrations completed at the end of the passaging experiment revealed that the
MOI used in these infections was between 0.1 and 0.5 iu/cell at each passage level.

**Determination of WNV genome copies**

Duplicate BHK cell monolayers were infected with VLPs, and 48 h later RNA samples were harvested from one set of wells and the remaining wells were immunostained (see above) to determine the number of VLP-infected cells. Levels of WNV RNA were determined using a dot-blot hybridization assay with a slight modification of previously described methods (O’Donnell et al., 2001), using dilutions of in vitro transcribed WNV replicon RNA as a standard. The number of genome copies detected was then normalized to the number of VLP-infected cells (from the IHC-stained wells) to determine the genome copy number per VLP-infected cell.

**Luc assays**

BHK or Huh7 monolayers prepared in 96-well black-wall plates were infected with dilutions of WNR C-Luc2A NS1-5 VLPs prepared in media with 1% FBS. Plates were rocked for an hour to facilitate binding and then incubated at 37 °C for up to 30 h. Media was removed at the indicated times, and Luc assay reagent containing 40 mM Tricine, 8 mM magnesium acetate, 33 mM of DTT, 0.13 mM EDTA, 0.1% Triton X-100, 0.53 mM ATP, 0.47 mM α-luciferin (Molecular Imaging Biosystems), and KOH to pH 7.8 was added to the cell monolayers. Plates were agitated for 30 s and light output was measured in a TR717 microplate luminometer (Applied Biosystems). To control for differences in individual experiments, assays included side-by-side determinations of light output from a dilution series prepared from a Luc standard (Promega), and the standard curve generated from these samples was used to determine the relative light units (RLU) of Luc present in test samples.

**βgal staining**

Cells infected with WNR C-bgal2A NS1-5 VLPs were fixed using cold acetone/methanol, rehydrated with PBS, and then incubated in X-Gal solution (0.5 mg/ml X-Gal, 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide, 1 mM MgCl₂ in PBS) for 30–60 min. In some cases, the X-Gal-stained monolayers were rinsed with PBS and immunostained as described above.

**Replicon RNA isolation and sequencing**

Sequence analysis of packaged replicons were determined by infecting Vero cells with VLPs and then harvesting total cellular RNA at 24 h postinfection and subjecting it to reverse transcription (RT) and PCR using a high-fidelity polymerase (Accu-Taq: Sigma) by a method similar to one we have previously described (Rossi et al., 2005). PCR amplification was accompanied using sense primer 5’-AGTAGTCCGCT-GTGTGAGC and antisense primer 5’-CCGAGTGCTGTT-GAGACC producing a cDNA fragment that included the 5’ UTR, C, reporter insert, and a part of NS1. The PCR reaction products were resolved by agarose gel electrophoresis, fragments were excised, purified, and then subjected to automated DNA sequencing at the UTMB Protein Chemistry Core Facility.

**Acknowledgments**

We thank Dr. R.B. Tesh, UTMB for providing anti-WNV and anti-VEEV antibodies, and Dr. E. Konishi, Kobe University, Kobe, Japan for providing the anti-NS1 MAb. This work was supported by a grant from NIAID to PWM through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (NIH grant number U54 AI057156).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2006.02.036.

**References**


