Increased expression of capsid protein in trans enhances production of single-round infectious particles by West Nile virus DNA vaccine candidate

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West Nile virus (WNV; genus Flavivirus, family Flaviviridae) is an emerging pathogenic arbovirus responsible for outbreaks of encephalitis around the world. Whilst no vaccines are currently available to prevent WNV infection of humans, the use of cDNA copies of flavivirus RNA genomes with large internal deletions within the capsid (C) appears promising. C-deleted vaccines are able to replicate and secrete large amounts of non-infectious immunogenic subviral particles (SVPs) from transfected cells. We have previously generated a WNV DNA vaccine candidate pKUNdC/C where C-deleted WNV cDNA was placed under the control of one copy of the cytomegalovirus (CMV) promoter and the C gene was placed under the control of a second copy of the CMV promoter in the same plasmid DNA. This DNA was shown to generate single-round infectious particles (SRIPs) capable of delivering self-replicating C-deleted RNA producing SVPs to surrounding cells, thus enhancing the vaccine potential. However, the amounts of both SRIPs and SVPs produced from pKUNdC/C DNA were relatively low. In this investigation, we aimed at increasing SRIP production by optimizing trans-C expression via incorporating different forms of C and the use of a more powerful promoter. The construct containing an elongation factor EF1α promoter encoding an extended form of C was demonstrated to produce the highest titres of SRIPs and was immunogenic in mice. Additionally, SRIP and SVP titres were further improved via incorporation of a glycosylation motif in the envelope protein. The optimized DNA yielded ~100-fold greater titres of SRIPs than the original construct, thus providing a promising candidate for further vaccine evaluation.

INTRODUCTION

West Nile virus (WNV; a member of the Japanese encephalitis serogroup within the genus Flavivirus, family Flaviviridae) is naturally transmitted in a sylvatic cycle between genus Culex mosquitoes and avian species (Lindenbach et al., 2007). Occasional non-transmissible infection of mammals (including humans and horses) results in the development of a febrile illness that may progress to include neurological manifestations in ~1% of infections (Gyure, 2009). WNV has been exhibiting a dramatic geographical expansion into the Americas early this century associated with the outbreak of a particularly virulent strain and a resultant epidemic of encephalitis (Lanciotti et al., 1999; Mostashari et al., 2001; Nash et al., 2001). This has driven the need for a greater understanding of the biology of the WNV life cycle and the nature of the host immune response to the virus, and has promoted the generation of effective vaccines. Although a few vaccine candidates are currently in clinical trials (Dayan et al., 2012; Ledgerwood et al., 2011), no vaccine has yet been approved to prevent WNV infections in humans.

WNV has a non-segmented ssRNA genome of positive polarity encoding a single polyprotein, and flanked by 5' and 3' UTRs. The polyprotein is cleaved co- and post-translationally to generate three structural proteins [capsid (C), pre-membrane (prM) and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Roby et al., 2012). The non-structural proteins are involved primarily in genomic RNA (gRNA) replication and in antagonism of the host antiviral response in mouse models (Roby et al., 2012; Suthar et al., 2013). The structural proteins assemble to form infectious virions and non-infectious subviral particles (SVPs). The C protein interacts with the gRNA and lipid membranes to form the nucleocapsid core of WNV virions. The prM and E proteins contribute to the formation of the viral envelope,
with prM acting as a chaperone (Konishi & Mason, 1993), and E constituting the major antigenic glycoprotein and mediator of host cell binding and fusion (Allison et al., 2001; Davis et al., 2006; Dowd & Pierson, 2010; Lee & Lobigs, 2000; Miller et al., 2008). Both the prM and E proteins are able to assemble into immunogenic SVPs independently of nucleocapsid incorporation (Chang et al., 2008; Hanna et al., 2005; Roby et al., 2013).

Replicating pseudo-infectious vaccines with large internal deletions within the C gene offer the combined benefit of the safety of non-infectious vaccines (they cannot package RNA and thus form a spreading infection) as well as the robust immune response generated by the replication of live vaccines (Roby et al., 2011). This approach for vaccine creation has been investigated by a number of groups and has led to the successful generation of protective vaccine candidates against a number of pathogenic flaviviruses (Ishikawa et al., 2008; Kofler et al., 2004; Mason et al., 2006; Pang et al., 2014; Roby et al., 2011; 2013; Rumyantsev et al., 2013; Seregin et al., 2006; Suzuki et al., 2009; Widman et al., 2008). Initial iterations of this strategy consisted solely of C-deleted flavivirus genomes delivered to cells/animals as nucleic acid either directly as cDNA copies (Seregin et al., 2006) or as in vitro transcribed RNA (Kofler et al., 2004). Whilst these investigations were successful at preventing lethal infection with virulent flaviviruses in mice, the effectiveness was limited by the number of cells transfected initially with the vaccines and the amount of SVPs able to be produced from the constructs. The RepliVAX series of vaccines extended the utility of the C-deleted genome approach by utilizing a packaging cell line that provided full-length C in trans, allowing the production of virus-like particles (VLPs) (Ishikawa et al., 2008; Mason et al., 2006; Rumyantsev et al., 2013; Suzuki et al., 2009; Widman et al., 2008). These VLPs were purified directly from culture fluid of transfected cells and utilized as particulate vaccines. The RepliVAX approach was advantageous as the delivery was easier and the VLPs could themselves assist in stimulating a flavivirus-specific immune response as VLPs have an envelope consisting of immunogenic prM and E antigens. However, the particulate vaccines require cold-chain storage, and the culturing conditions and purification methods are difficult and expensive to establish and scale up for commercial production.

Recently, a new approach has been developed to extend the effectiveness of C-deleted vaccines. Incorporation of the full-length C gene under a separate promoter into a plasmid that also encodes a C-deleted Kunjin virus (KUNV; a naturally attenuated WNV sublineage) genome has created a vaccine that is able to generate single-round infectious particles (SRIPs) via C trans-complementation in vivo, thus enhancing the magnitude of the host immune response and reducing the protective dose (Chang et al., 2008). The first-generation SRIP-producing DNA construct pKUNdC/C, henceforth referred to as pKUNdC18-100/CMV-C, was able to secrete some SRIPs and SVPs, and so could elicit protective immunity in vaccinated mice. However, the titres of these particles appeared to be less than expected (Chang et al., 2008). We identified two possible causes of this low secretion: the internal C deletion may have been suboptimal for processing at the C–prM junction and/or the levels of trans-C expression may have been too low for optimal SRIP packaging. Indeed, production of trans-C was notably limited within transfected cell populations – certainly less than C production in cells transfected with the infectious clone pKUN1 (Chang et al., 2008). Recently, we demonstrated that modification of the internal C deletion is able to increase the amount of SVP production from a C-deleted KUNV DNA (Roby et al., 2013). Thus, the nature of the internal C deletion is a critical determinant for the efficient secretion of SVPs. However, this relationship does not address the relatively low efficiency of SRIP packaging exhibited by our original SRIP-producing DNA pKUNdC18-100/CMV-C.

We hypothesized that the level of trans-C expression and the cleavage efficiency at the C–prM signal sequence junction could be the limiting factors in the efficiency of SRIP assembly and/or secretion. Recent evidence with Murray Valley encephalitis virus (MVEV) has demonstrated that trans-complementing C protein containing a cleavable transmembrane anchor at the C terminus, effectively the signal sequence and first few residues of prM, is more efficient at packaging than the mature soluble form of C (Lobigs et al., 2010). Thus, we hypothesized that increasing the degree of trans-C expression in addition to the inclusion of prM sequences to this gene cassette would facilitate a greater degree of gRNA packaging into nucleocapsids, leading to an increased efficiency in assembly and secretion of SRIPs. To investigate this, C-deleted KUNV cDNA trans-complemented by expression cassettes encoding different C-terminally extended forms of C protein under control of either the cytomegalovirus (CMV) or elongation factor EF1α promoter was transfected into human embryonic kidney HEK-293 cells and the efficiency of SRIP production and immunogenicity in mice were assessed.

RESULTS

Human EF1α promoter generates higher levels of C expression in HEK-293 cells than the CMV promoter

In order to facilitate expression of a form of C more closely reminiscent of that produced initially as part of the KUNV polyprotein, C genes with various 3′ extensions were cloned into the CMV promoter-driven pcDNA3.1(+) vector. Extended forms of C were designed to encode the prM signal sequence and downstream residues derived from the N-terminus of prM (Fig. 1a). The mature form of C was also cloned as a control (CMV-C). (See Methods for details of plasmid nomenclature.)

Additionally, we wished to investigate whether changing the promoter in pcDNA3.1(+) to the human EF1α
The promoter would drive higher levels of C expression in cells (Fig. 1b). In conjunction, we also sought to modify the codons of selected forms of the extended C genes to make them as dissimilar as possible to the WT nucleotide sequence without altering amino acid residues (Fig. 1d). This codon modification was performed solely with the purpose of preventing any possibility of RNA recombination between complementary sequences present in trans-C mRNAs and in C-deleted viral gRNAs – a technique used by another group (Widman et al., 2008).

The constructs encoding various forms of C were transfected into HEK-293 and Vero cells to ascertain expression (Fig. 2). Immunofluorescence assays determined that EF1α-promoter-driven expression of C in HEK-293 cells (Fig. 2a) was much stronger than expression from the CMV promoter. Expression of C constructs in Vero cells, however, appeared to be efficient from both promoters, although transfection efficiency appeared lower than in HEK-293 cells.

Western blot analysis of lysates from these two transfected cell lines confirmed that expression of all of the CMV promoter-driven forms of C in HEK-293 cells (Fig. 3a) was less efficient compared with expression from the EF1α promoter, whereas expression in Vero cells (Fig. 3b) was similar from both promoters. C-terminal extension of C led to production of larger forms of this protein than mature C, as was expected. Codon modification did not affect the levels of C expression or the ability of C to be detected by anti-WNV C antibodies.

**Human EF1α promoter also generates higher levels of C expression than the CMV promoter in murine cells**

As the SRIP-producing vaccine plasmids generated in this study will be assessed in mouse immunization models, it was important to ascertain whether the phenotype of enhanced C expression from the human EF1α promoter observed in Figs 2 and 3 would be maintained in murine cells. To achieve this, mouse embryonic fibroblast (MEF) cells were transfected with plasmids expressing mature C under control of either the CMV promoter (CMV-C) or EF1α promoter (EF1α-C), as well as the CMV-driven control plasmid pcDNA3-eGFP (CMV-eGFP) and the empty pcDNA3.1(+) vector. MEFs were fixed for immunofluorescence or lysed for Western blot analyses at 1 day post-transfection (Fig. 4). Immunofluorescence analysis indicated that MEF cells expressed greater levels of C than the CMV-promoter-driven forms of C in HEK-293 cells (Fig. 2a) was much stronger than expression from the CMV promoter. Expression of C constructs in Vero cells, however, appeared to be efficient from both promoters, although transfection efficiency appeared lower than in HEK-293 cells.
Efficient expression of WNV C improves SRIP production

promoter (Fig. 4a). This appears to be a specific property of WNV C as eGFP expression (also driven by the CMV promoter) was not qualitatively different from EF1α-driven C. Analysis of C expression by Western blot confirmed a greater level of C expression from the human EF1α promoter (Fig. 4b).

Fig. 2. Immunofluorescence analysis of C expression in (a) HEK-293 and (b) Vero cells transfected with plasmids encoding different versions of C under the control of the CMV and EF1α promoters. Cells transfected with each indicated plasmid were immunolabelled at 1 day post-transfection for WNV C. Nuclei were counterstained using DAPI. Results are representative of at least three independent experiments. Mock, mock transfection; Empty vector, pcDNA3.1(+). Bar, 50 μm.
SRIP-producing plasmid constructs containing the dC33-100 deletion (the additional 15 aa of C, corresponding to α helix 1 in this deletion) was determined previously to be superior to the dC18-100 deletion; see Roby et al., 2013) to incorporate human EF1α promoter-driven expression of the three different forms of codon-modified trans-C (Fig. 1c). The plasmids pKUNdC33-100/CMV-C, pKUNdC33-100/EF1α-cm.C, pKUNdC33-100/EF1α-cm.extC and pKUNdC33-100/EF1α-cm.Cpr were transfected into HEK-293 cells in parallel with the first-generation construct pKUNdC18-100/CMV-C to investigate any positive effect of these trans-C modifications on SRIP production. In addition, an F156S glycosylation mutation, demonstrated previously to lead to increased NS2B/3 protease derived from the KUNdC33-100 replicon encoded on the same plasmid, although the cleavage was not 100% efficient (Fig. 6a). Western blot analysis of the culture fluid demonstrated that pKUNdC18-100 secretes very low amounts of E protein, whilst strong E secretion was detected from all other constructs.

E protein capture ELISA was performed on the culture fluid to quantitatively assess the levels of SVP secretion (Fig. 6b). The trans-C modifications did not appear to lead to a significant change in the levels of total E secretion compared with either pKUNdC33-100/CMV-C or the original construct pKUNdC18-100/CMV-C. Secretion of E protein (SVPs) from cells transfected with the glycosylation mutant pKUNdC33-100/gE/EF1α-cm.extC was significantly higher than from those transfected with pKUNdC18-100/CMV-C (P=0.0009). The construct pKUNdC18-100 that was not trans-complemented by C was shown to lead to poor SVP secretion, as we reported previously (Chang et al., 2008; Roby et al., 2013).

Infected assays at limiting dilution were utilized to determine the titre of SRIPs/virions obtained from each of the transfected plasmids (Fig. 6c). Each of the constructs pKUNdC33-100/EF1α-cm.C, pKUNdC33-100/EF1α-cm.extC and pKUNdC33-100/EF1α-cm.Cpr secreted more SRIPs than pKUNdC18-100/CMV-C, with pKUNdC33-100/EF1α-cm.extC appearing to secrete the highest amount of SRIPs. Statistical analysis of these titres revealed that they were not significantly different from each other despite being up to ~1.5 log different. Transfection of pKUNdC33-100/gE/EF1α-cm.extC, however, led to a statistically significant
88-fold increase in the titre of secreted SRIPs compared with the original pKUNdC18-100/CMV-C construct ($P = 0.0089$).

**Single immunization with the second-generation SRIP-producing DNA vaccine elicits an antibody response in mice**

In order to ascertain the ability of SRIP-producing DNA vaccines to illicit an immune response, female CD1-Swiss outbred mice (4–5 weeks old) were inoculated with 1 $\mu$g plasmid intradermally. Serum was collected from immunized mice at 21 days post-vaccination and analysed for the presence of total anti-KUNV/gE IgG (Fig. 7). Single vaccination with all three plasmids, pKUN1, pKUNdC18-100/CMV-C and pKUNdC33-100/EF1a-cm.extC, induced high levels of anti-KUNV/gE antibodies in most animals. Only two mice failed to engender an immune response – one each from the pKUNdC18-100/CMV-C and pKUNdC33-100/EF1a-cm.extC groups (Fig. 7). Vaccination with pKUNdC33-100/EF1a-cm.extC appeared to induce around threefold higher mean titres of antibodies than vaccination with pKUNdC18-100/CMV-C (Fig. 7); however, statistical analysis showed that the differences between each of the experimental group were not significant, likely due to the low number of animals used.

**DISCUSSION**

C-terminal extension of the trans-C protein was predicted to be beneficial for SRIP production based on several lines...
of evidence. C is normally expressed at the endoplasmic reticulum (ER) membrane as part of the flavivirus polyprotein, covalently linked to the prM signal peptide at its C terminus (Lindenbach et al., 2007). Such a position in close concert with prM and E may assist in directing the nucleocapsid into the budding virion, and indeed extension of C to include pr did improve MVEV genome packaging in a split-genome system (Lobigs et al., 2010). Ectopic production of C protein anchored by a signal sequence was also shown to lead to binding of I2PP2A to a greater extent than mature C, in turn leading to greater activity of the phosphatase PP2A (Hunt et al., 2007). As PP2A negatively regulates activator protein AP-1 activity, this may thus lead to decreased AP-1-mediated transcription of IFN and IFN-stimulated genes (ISGs), and so facilitate more efficient genome replication and (in the case of these constructs) increased SRIP production. In addition, C localization to lipid droplets is important for virus particle formation (Samsa et al., 2009). As lipid droplets are in close association with and are derived from the ER (Braasamle & Wolins, 2012), targeting trans-C expression to this organelle may assist subsequent lipid droplet localization. Mature flavivirus C has previously been demonstrated to reside within ER-associated membrane fractions mediated by the lipophilic nature of the internal hydrophobic domain (Markoff et al., 1997). Indeed, both mature and extended forms of trans-C have been demonstrated in our laboratory to co-localize to the ER membranes (data not shown).

Investigations into the coordinated cleavages at the C–prM junction have revealed that the presence of a cleavable transmembrane anchor at the C terminus of C is important for efficient gRNA packaging when expressed in trans (Lobigs et al., 2010). HEK-293-based packaging cell lines inductively expressing mature MVEV C, anchored C or Cpr upstream of an IRES (internal ribosome entry site)-driven prM–E gene cassette were compared with cells expressing an uninterrupted C–prM–E gene cassette for their ability to package MVEV replicon RNA. Without the presence of any prM sequence downstream, anchored C appeared unable to package replicon RNA, presumably due to a severe deficiency in NS2B/3-mediated maturation, as demonstrated previously (Lee et al., 2000; Lobigs & Lee, 2004). However, when the C terminus was extended to include the pr sequence, this anchored form of C was able to package RNA with greater efficiency than the construct expressing mature C, at levels indistinguishable from uninterrupted C–prM–E by 2 days post-infection. A recent report demonstrating trans-encoded Cpr allows efficient packaging of C-deleted Dengue virus genomes further corroborates the utility of C-terminal extension in the construction of these vaccines (Pang et al., 2014). These results suggest that whilst NS2B/3-mediated cleavage maturation of C is important for nucleocapsid formation, initial localization of C to the ER membrane in an anchored form also contributes to efficient packaging. Extension of the trans-encoded C protein to include the prM signal sequence and 10 ER luminal residues in our experiments led to maturation of ~50% of the expressed protein (presumably by NS2B/3) and a small enhancement of SRIP secretion, although this increased SRIP production was not statistically significant (Fig. 6). Extension of the trans-encoded C to include the entire pr sequence did not enhance SRIP production compared with mature C alone.

Extended forms of C ectopically expressed from the CMV promoter could be produced in Vero and HEK-293 cells (Figs 2, 3, 5 and 6), although production in HEK-293 cells was less efficient. The CMV immediate-early promoter/enhancer, in situ, drives expression of the major immediate-early proteins (IE1 and IE2), which are essential for early gene expression and virus replication (Boshart et al., 1985; Dorsch-Hasler et al., 1985; Isomura & Stinski, 2003). The CMV promoter is used routinely to drive the expression of various recombinant genes in vitro and in vivo, and is characterized as displaying high levels of expression in cells of many different origins (Fabre et al., 2006; González-González et al., 2010; Londrigan et al., 2007; Qin et al., 2010; Zheng & Baum, 2005). However, some reports have indicated that the CMV promoter may undergo silencing via CpG hypermethylation in mammalian cells when constitutively overexpressing a foreign gene (Brooks et al., 2004; Mehta et al., 2009; Prösch et al., 1996; Teschendorf et al., 2002; Vanniasinkam et al., 2006) or display expression patterns that are heavily restricted by cell type (Baskar et al., 1996). This poor expression of C from the CMV promoter may be due to the stressful nature of the C protein in mammalian cells (Hunt et al., 2007; Limjindaporn et al., 2007; Yang et al., 2002, 2008), which may in turn lead to suppressed transcription. Indeed, the core protein from the related hepatitis C virus (HCV) – a known modulator of the cellular stress response and protein transcription (Kim et al., 1994; Otsuka et al., 2000) – has been demonstrated to express poorly from the CMV promoter during construction of a stable cell line. The use of an EF1α promoter was able to overcome this silencing effect, allowing durable expression of the HCV core (Tokushige et al., 1997). Additionally, the CMV promoter was able to drive efficient expression of eGFP in MEF (Fig.

Fig. 5. Immunofluorescence analysis of cells transfected with different constructs and/or infected with culture fluids from transfected cells. HEK-293 cells transfected with each plasmid were immunolabelled for WNV C (red) and WNV E (green). Culture fluid derived from this transfection was diluted 1:10 and inoculated onto Vero cells for analysis of infectious particle content via WNV E immunolabelling. Secondary passage was performed by diluting culture fluid from the first passage 1:10 and using this to infect fresh Vero cells. Nuclei were counterstained using DAPI. Results are representative of at least three independent experiments. Mock, mock transfection. Bar, 50 μm.
Fig. 6. Analyses of protein expression, and SRIP and SVP secretion following transfection of HEK-293 cells with second-generation plasmid constructs. (a) Western blot analysis of E and C expression in transfected HEK-293 cell lysates and E secretion into culture fluid at 2 days post-transfection. Blots were immunolabelled using antibodies specific for WNV C and
4) and HEK-293 cells (data not shown) in experiments in our laboratory. Thus, the observed lack of KUNV C expression from CMV constructs is likely a consequence of the nature of the C protein rather than a fault in the CMV promoter per se. Why the nature of the promoter should affect C expression in HEK-293 and MEF cells (Figs 2, 3, 4, 5 and 6) and not in Vero cells is at this point unknown, although Vero cells are recognized as harbouring malfunctioning signalling pathways such as a deficiency in IFN secretion (Desmyter et al., 1968; Emeny & Morgan, 1979). Thus, it is possible that the Vero cells are less equipped to recognize and respond to the foreign DNA that may in turn lead to lesser ability to suppress CMV promoter-driven transcription. Future experiments comparing KUNV C expression from the CMV and EF1α promoters in transfected WT and type 1 IFN receptor (IFNAR)−/− or IFN regulatory transcription factor (IRF) 3/7−/− MEF cells should resolve whether the IFN response is involved in CMV promoter suppression.

The eukaryote EF1α promoter is an endogenous transcriptional regulator which drives high levels of expression of the EF1α protein (Kim et al., 1990; Wakabayashi-Ito & Nagata, 1994). EF1α protein itself is involved in tRNA recruitment during mRNA translation, regulation of the cytoskeleton and inhibition of p53-mediated apoptosis (Blanch et al., 2013; Condeelis, 1995). The EF1α promoter has been demonstrated to be effective for the construction of stable cell lines and transgenic animals constitutively expressing foreign genes as this promoter is endogenous to the host cell (Chung et al., 2002; Gill et al., 2001; Gopalkrishnan et al., 1999; Hong et al., 2007; Sinici et al., 2006; Teschendorf et al., 2002; Tokushige et al., 1997). The EF1α promoter has been shown to be more efficient than the CMV promoter in driving transient expression of IFNAR1 in COS cells (Goldman et al., 1996), and GFP in HEK-293T cells (Chung et al., 2002) and MEFs, among other cell lines (Qin et al., 2010). The versatility of this promoter was confirmed in the present study as exchange of the CMV promoter for the human EF1α promoter was able to facilitate much higher levels of expression of all investigated forms of KUNV C when transfected into HEK-293 and MEF cells, whilst not improving C expression in Vero cells (Figs 2–6). This was an important discovery as the achievable transfection efficiency of HEK-293 cells is far superior to that of Vero cells, as observed in our laboratory, and therefore HEK-293 cells are ideal for the relatively difficult and inefficient transfection required to characterize the large SRIP-producing plasmids.

The benefit of using the human EF1α promoter to drive trans-C expression was demonstrated by transfection of pKUNdC33-100/EF1α-cm,extC into HEK-293 cells. This led to secretion of more SRIPs (~1.5 log10 greater) than did the equivalent plasmid with CMV promoter-driven trans-C expression, pKUNdC33-100/CMV-C (Fig. 6c). A similar pattern was observed upon pKUNdC33-100/gE/EF1α-cm,extC transfection, which led to secretion of significantly more SRIPs (~2 log10 greater) than the first-generation construct pKUNdC18-100/CMV-C (Fig. 6c). Thus, enhancing the amount of trans-C expressed per cell appears to be a critical determinant of trans-packaging vaccine design as it leads to a greater titre of secreted SRIPs. The level of C production may thus have been a limiting factor in packaging efficiency of the first-generation SRIP-producing DNA.

Improvements to the C-deleted genome which increase SVP secretion have been identified previously (Roby et al., 2013). Modifying the internal C deletion from dC18-100 to dC33-100 significantly improved E secretion from non-glycosylated constructs and addition of an F156S glycosylation mutation to E further significantly improved secretion of this protein into the culture fluid of transfected cells (Roby et al., 2013). Combining each of these modifications in the second-generation plasmid construct pKUNdC33-100/gE/EF1α-cm,extC led to significant improvements in E secretion and SRIP production (~2 log10 greater titres) compared with the first-generation construct pKUNdC18-100/CMV-C (Fig. 6). Changing the C deletion from dC18-100 to dC33-100 slightly reduced the production of SRIPs in CMV-C trans-complemented plasmids (Fig. 6). Interestingly, the production of SVPs was similar for pKUNdC18-100/CMV-C and pKUNdC33-100/CMV-C plasmids (Fig. 6). It would seem that the SVP secretion deficiency in the dC18-100 construct is entirely corrected by the addition of functional C in trans, suggesting a complex interplay between capsid expression and SVP secretion from the C-deleted genome.

Preliminary assessment of the non-glycosylated second-generation SRIP-producing DNA pKUNdC33-100/EF1α-cm,extC in the mouse model demonstrated that vaccination appeared to generate a humoral immune response in vivo comparable with the first-generation vaccine plasmid pKUNdC18-100/CMV-C at a single 1 μg dose (Fig. 7). This experiment was designed to assess the capacity of the second-generation vaccine to induce an immune response rather than to compare the magnitude of this response between different constructs. Thus, the observation of a slight albeit not statistically significant increase in KUNV-specific antibody titres derived from vaccination with pKUNdC33-100/EF1α-cm,extC compared with pKUNdC18-100/CMV-C remains to be confirmed and appropriately characterized.
in future experiments with larger numbers of animals. An antibody response comparable with that derived from pKUNdC18-100/CMV-C immunization has also been observed in initial experiments utilizing mice vaccinated with the glycosylated variant pKUNdC33-100/gE/EF1a-cm.extC (data not shown).

The lack of any dramatic difference in performance in vivo between pKUNdC18-100/CMV-C and pKUNdC33-100/EF1a-cm.extC is unlikely to be due to a difference in the ability of CMV and EF1x promoters to drive trans-C expression between murine cells and HEK-293. Experiments with transfected MEFs demonstrated that the human EF1x promoter is indeed more efficient at expressing C in murine cells than the CMV promoter (Fig. 4). Future experiments with larger numbers of animals will aim to reduce the amount of DNA vaccine administered per dose in order to determine the minimum threshold for efficient immunization and protection from lethal WNV challenge, and will further characterize the nature of the immune response generated for these promising vaccines.

The phenotypic mechanisms driving the effectiveness of SRIP-producing DNA and other C-deleted vaccines are the expression and secretion of high amounts of SVPs and the replication of gRNA in a manner imitating virus infection (Mandl, 2004; Roby et al., 2011). The use of self-replicating C-deleted RNA genomes allows engagement of host cell pattern recognition receptors such as TLR3, RIG-I and MDA5 (Chang et al., 2006; da Concejao et al., 2013; Daffis et al., 2008; Frederiksen et al., 2008; Nasirudeen et al., 2011; Tsai et al., 2009), and thus the induction of ISGs, cytokines/chemokines and IFN, which serves to attract antigen-presenting cells and so stimulate adaptive immunity (Bourne et al., 2007; Diamond et al., 2003b; Lim & Murphy, 2011; Ma et al., 2009; Ramos et al., 2012; Suthar et al., 2013). The viral polyprotein provides a source of peptides to stimulate T-cell proliferation (Brien et al., 2007; Shrestha & Diamond, 2004; Sitati & Diamond, 2006), and the secreted SRIPs and SVPs drive the formation of a protective neutralizing antibody response against E (Diamond et al., 2003a; Larena et al., 2011). Accordingly, as the second-generation SRIP-producing DNA (pKUNdC33-100/gE/EF1a-cm.extC) created in this study secretes significantly more SRIPs and SVPs than the first-generation construct (pKUNdC18-100/CMV-C) (Fig. 6b, c), it represents a promising candidate for future evaluation.

METHODOLOGY

**Construction of plasmid DNA.** The C protein expression plasmids pcDNA3.1-C, pcDNA3.1-extC and pcDNA3.1-Cpr (referred to as CMV-C, CMV-extC and CMV-Cpr, respectively) were generated via insertion of KUNV cDNA fragments generated using the primers listed in Table 1 into the vector pcDNA3.1 (+) (Life Technologies) utilizing the KpnI and XhoI or XbaI restriction sites. All constructs expressing mature C terminate translation at a point corresponding to the first NS2B/NS3 (NS2B/3) cleavage site of the KUNV polyprotein. The C terminus of extC constructs extends beyond the NS2B/3 cleavage site to include the prM signal sequence and the first 10 ER luminal residues. Cpr constructs were extended even further to include 92 ER luminal residues, terminating at the furin cleavage site in prM (Fig. 1a).

The pEF1a-C plasmid (referred to as EF1a-C) was generated via replacement of the CMV promoter in pcDNA3.1-C with the human EF1x promoter utilizing the BanHI and HindIII restriction sites. Quick-change PCR was performed on this plasmid to introduce unique Asel and Sfhi restriction sites flanking the C gene (Fig. 1b).

Custom gene synthesis and codon modification (indicated by ‘cm’) were performed on the KUNV Cpr sequence by GenScript, obtaining the sequence outlined in Fig. 1(d). The synthetic gene was incorporated into the pUC57 vector plasmid prior to shipping. The plasmids pEF1a-cm.C, pEF1a-cm.extC and pEF1a-cm.Cpr (referred to as EF1a-cm.C, EF1a-cm.extC and EF1a-cm.Cpr, respectively) were generated via PCR amplification of codon-modified C genes from the pUC57 vector (see Table 1 for primers), and cloning these products into pEF1a-C using the AseI and Sfhi restriction sites (Fig. 1b).

Construction of the KUNV cDNA-based plasmids pKUN1, pKUN1/gE, pKUNdC18-100, pKUNdC33-100 and pKUNdC18-100/CMV-C has been described previously (Chang et al., 2008; Hall et al., 2003; Roby et al., 2013). The trans-complemented plasmid pKUNdC33-100/CMV-C was generated in an analogous fashion to pKUNdC18-100/CMV-C via the addition of the CMV-C expression cassette to the pKUNdC33-100 cloning process. A unique Sfhi restriction site was introduced downstream of the trans-C gene in pKUNdC33-100/CMV-C via PCR and cloning back into the template (Table 1). The

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**Fig. 7.** Humoral immune responses in immunized mice. Fixed-plate ELISA-determined reciprocal anti-KUNV/gE IgG titres at 21 days after intradermal immunization of mice with 1 μg of each of the indicated DNA plasmids. Dotted lines represent the cut-off point of detection in this assay. Non-responding samples were assigned a value of 1.0 to facilitate visualization on the graph.
second-generation SRIP-producing plasmid constructs pKUNdC33-100/EF1a-cm.C, pKUNdC33-100/EF1a-cm.extC and pKUNdC33-100/EF1a-cm.Cpr were generated via the PCR amplification of EF1a-cm.C, EF1a-cm.extC and EF1a-cm.Cpr expression cassettes (Table 1) to replace CMV-C in pKUNdC33-100/CMV-C using the MluI and SbfI restriction sites. The glycosylation mutant pKUNdC33-100/gE/EF1a-cm.C was generated by the process described above; however, the initial template for pKUNdC33-100/gE/CMV-C construction already incorporated the F156S in E, as in pKUN1/gE (Fig. 1c).

Cells and culture conditions. HEK-293 and immortalized MEF cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Gibco) supplemented with 2 or 10 % (v/v) FBS (Morganate), 10 mM HEPES, 100 µg streptomycin ml⁻¹, 100 U penicillin ml⁻¹, 1 mM sodium pyruvate and 2 mM L-glutamine. Vero (African green monkey kidney) cells were cultured in RPMI 1640 (Gibco) supplemented with 2 or 6 % (v/v) FBS, 10 mM HEPES, 100 µg streptomycin ml⁻¹, 2 or 10 % (v/v) FBS, 10 mM HEPES, 100 µg streptomycin ml⁻¹, 100 U penicillin ml⁻¹ and 2 mM l-glutamine. Vero monkey kidney) cells were cultured in RPMI 1640 (Gibco) supplemented with 2 or 6 % (v/v) FBS, 10 mM HEPES, 100 µg streptomycin ml⁻¹, 2 or 10 % (v/v) FBS, 10 mM HEPES, 100 µg streptomycin ml⁻¹, 100 U penicillin ml⁻¹ and 2 mM l-glutamine. Cells in antibiotic-free media were transfected, medium was replaced and cells were cultured under standard conditions.

Transfection of cells. Cells in antibiotic-free media were transfected with 0.5 µg plasmid DNA per 24-well plate well and 1 µg DNA per 12-well plate well using Lipofectamine LTX transfections, DNA (HEK-293) or DNA and Plus Reagent [1 µl (µg DNA)⁻¹; Vero] were incubated with OptiMEM [Gibco; 200 µl (µg DNA)]⁻¹ for 5 min at room temperature. Lipofectamine LTX was subsequently added to the transfection solution [HEK-293, 3 µl (µg DNA)⁻¹; Vero, 4 µl (µg DNA)⁻¹], and the solution was mixed via inversion and incubated for 30 min at room temperature. The transfection solution was then applied to the cells at 50 µl per 24-well plate well and 100 µl per 12-well plate well, respectively. After 6 h of transfection, medium was replaced and cells were cultured under standard conditions.

Infectious assays. Culture fluid derived from plasmid transfection was treated with RNase A (20 µg ml⁻¹) and RQ1 DNase (4 U ml⁻¹; Promega). Each culture fluid sample was clarified via centrifugation at 100 g for 5 min at 4 °C. Supernatant was transferred to a fresh tube in 250 µl aliquots and stored at −80 °C.

Vero cells were infected with 10-fold serial dilutions of transfected HEK-293 cell culture fluid to determine titres of infectious particles. Infected cells were fixed 2 days post-infection and immunolabelled for the KUNV E antigen for immunofluorescence microscopy. For the appropriate dilutions of each sample, individual E-positive cells (SRIP-producing plasmids) or foci (infectious clones pKUN1 and pKUN1/gE) were counted and multiplied by the dilution factor. These raw counts were then scaled taking into account the proportion of the well surface area covered by the coverslip (59.5 %) and the volume of culture fluid used for infection (200 µl ml⁻¹, effectively 119 µl per coverslip). Thus, the value was multiplied by 8.40 to give the titre as SRIPs/virions (ml culture fluid)⁻¹.

Immunofluorescence assays. Transfected or infected HEK-293, Vero or MEF cells were fixed at 1 day post-transfection/2 days post-infection with 4 % (v/v) paraformaldehyde, 0.1 % (v/v) Triton X-100 in PBS at room temperature for 30 min. Fixed transfected and infected cells were washed once with PBS and incubated with the monoclonal 3.67G antibody (recognizing KUNV E) (Adams et al., 1995) and/or a pre-absorbed cocktail of rabbit polyclonal anti-WNV C antibodies 3E6 and 3G3 (kindly provided by Tom Hobman, University of Arizona, Tucson, Arizona, USA).

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Used to clone</th>
<th>Name</th>
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<tr>
<td>CMV-C, CMV-extC</td>
<td>C-KpnI-F</td>
<td>GGGGTACCGGCCGACCAATGCTAAGAACCAGG</td>
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<tr>
<td>EF1a-cm.extC, dC/C plasmids</td>
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</tr>
</tbody>
</table>

*Underlined regions indicate introduced restriction sites.
University of Alberta, Canada) in 0.3 % (w/v) BSA, 0.1 % (v/v) Triton X-100 in PBS for 1 h at 37 °C. Cells were then washed twice with PBS before incubation with secondary antibodies goat anti-mouse IgG–Alexa Fluor 488 conjugate (Invitrogen) and donkey anti-rabbit IgG–Alexa Fluor 555 conjugate (Invitrogen) in 0.3 % (w/v) BSA, 0.1 % (v/v) Triton X-100 in PBS for 1 h at 37 °C. Following this, nuclei were counterstained with DAPI in PBS for 5 min at room temperature. The coverslips were finally washed three times with PBS before mounting onto slides with Mowiol-4-88. Immunofluorescence images of slides were captured using a Zeiss Axioshot2/Axiocam MRm system and analysed using Axiovision AC software (Carl Zeiss). Minimal enhancement of image intensity and contrast was applied equally to all images per experiment using Photoshop software (Adobe).

**Western blotting.** Proteins in mammalian cell lysates and culture fluid were separated by SDS-PAGE using a Mini-PROTEAN Tetra Cell system (Bio-Rad: HEK-293 and Vero) or Bolt Mini Gel system (Life Technologies; MEFs). Samples of 15 μl per well cell lysate and 20 μl per well treated culture fluid were loaded into the wells of 12 % SDS-PAGE gels (HEK-293 and Vero) or 50 μl per well cell lysate were loaded into the wells of 4–12 % pre-cast gradient Bolt gels (Life Technologies; MEFs). Gels were electrophoresed at a constant voltage of 130 V for ~1.25 h. Electrophoresed proteins were transferred to a nitrocellulose membrane at a constant voltage of 100 V for 1.5 h at room temperature. Post-transfer, membranes were blocked in a solution of 2.5 % (w/v) skim milk powder in PBS overnight at 4 °C with gentle rocking on an orbital shaker.

After overnight blocking, the membranes were probed with antibodies specific for the proteins of interest. Excess blocking solution was removed via washing twice with PBS containing 0.05 % (v/v) Tween 20 (PBS-T) and the membrane was incubated with primary antibody for 1 h at room temperature [3.67 μg anti-KUNV E, rabbit polyclonal 3E6 anti-WNV C and monoclonal anti-GAPDH (glycer-aldehyde 3-phosphate dehydrogenase) (Sigma) antibodies] diluted in 0.25 % (w/v) skim milk powder in PBS. Following three washes with PBS-T and a further 30 min of blocking in 2.5 % (w/v) skim milk in PBS, the membrane was washed twice with PBS-T and incubated for 1 h with secondary antibody (goat anti-mouse IgG–Alexa Fluor 680 conjugate and goat anti-rabbit IgG–Alexa Fluor 680 conjugate; Invitrogen). Excess secondary antibody was removed by three washes with PBS-T and three washes with PBS. Blots were then scanned using an Odyssey infrared imaging scanner (LI-COR Biosciences). Western blots presented in the figures are composites of different membranes treated under identical conditions, with demarcations between membranes indicated by vertical lines.

**E protein capture ELISA.** The relative amount of secreted E protein (i.e., SVPs) in the culture fluid of transfected HEK-293 cells was analysed quantitatively using capture ELISA. Round-bottomed 96-well plates were coated overnight at 4 °C with monoclonal 3.91D ascites fluid (anti-KUNV E) (Adams et al., 1995) in carbonate coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃; pH 9.6) at 50 μl per well. Plates were subsequently washed with PBS-T and blocked for 1 h at room temperature with TNETC buffer [10 mM Tris-base, 0.2 M NaCl, 1 mM EDTA, 2 % (v/v) casein, 0.05 % (v/v) Tween 20] at 100 μl per well. Plates were then washed with PBS-T and incubated with twofold serial dilutions of culture fluid in TNETC buffer at 50 μl per well for 1 h at room temperature. Following another wash with PBS-T, plates were incubated with biotinylated monoclonal 4G2 antibody (cross-reactive anti-flavivirus E) (Gentry et al., 1982) in TNETC buffer at 50 μl per well for 1 h at room temperature. Plates were subsequently washed with PBS-T and incubated for 30 min at room temperature with streptavidin–HRP (Life Technologies) in PBS-T at 50 μl per well. After a final wash with PBS-T, plates were developed for 1 h at room temperature with ABTS substrate buffer [40 mM citric acid, 110 mM Na₂HPO₄, 10 M ABTS, 0.1 % (v/v) H₂O₂] at 100 μl per well and absorbance recorded at 405 nm (A₄₅₀) using a Multiskan EX plate reader (Labsystems).

**Immunogenicity in mice.** Female outbred CD1 mice (4–5 weeks old) obtained from the Animal Resources Centre (Murdoch, WA, Australia) were utilized for all animal experiments. Mice were housed in Physical Containment Level 2 conditions, in groups of three to five in HEPA-filtered isolation cages, and given ad libitum access to clean food and water. All experimental procedures were approved by the University of Queensland Animal Ethics Committee (SCMB/226/12/ NHMRC ‘Evaluation of improved non-infectious DNA-based vaccines against West Nile virus’) in accordance with the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia).

In preparation for intradermal delivery, mice were anaesthetized using an intraperitoneal injection of ketamine/xylozal/PBS (1:1:8, ~100 mg ketamine kg⁻¹ and 10 mg xylozal kg⁻¹) mixture. For each individual vaccine, groups of six mice were given a single dose of 1 μg DNA in PBS per mouse. Once mice were completely unconscious, the intradermal vaccine dose was administered by insertion of a 29-gauge needle intradermally in either ear and gentle depression of the syringe to create a liquid bleb as the epidermis was lifted from the underlying stratum. Mice were then observed until they recovered from the anaesthetic and were housed under standard conditions for the length of the experiment.

For mouse anti-KUNV/gE antiserum titres, direct ELISAs were carried out on fixed infected and uninfected C6/36 (Aedes aegypti mosquito) cell plates in parallel, and the absorbance results compared. Plates were blocked for 1 h at room temperature with TNETC buffer at 100 μl per well. After blocking, contents of the wells were flicked out and duplicate serial twofold dilutions (starting at 1: 20) of mouse antiserum were prepared down the plates at 50 μl per well volume in TNETC buffer. Serum was incubated in the plates for 1 h at room temperature followed by washing with PBS-T. Secondary antibody (goat anti-mouse IgG–HRP conjugate; Promega) was applied in TNETC buffer at 50 μl per well and was incubated for 1 h at room temperature followed by washing with PBS-T. Plates were developed for 1 h at room temperature with ABTS substrate buffer at 100 μl per well and A₄₅₀ recorded using a Multiskan EX plate reader. An infected plate reading of double the corresponding result on the uninfected plate and at least 0.3 was regarded as positive for anti-KUNV/gE antibodies; thus the most dilute reading that fitted these criteria was considered the end point and the titre was represented as the reciprocal of the dilution factor.

**Statistical analyses.** Statistical analysis of data was performed using Prism 6 software (GraphPad). One-way ANOVA was performed on the dataset, with Fisher’s least significant difference test applied to compare the means of second-generation SRIP-producing constructs against pKUNdC18-100-CMV-C with a confidence interval of 95 %. As these were planned comparisons, the P values and confidence intervals were not corrected for multiple comparisons. All column graphs represent the mean ± SEM of the dataset. Bars in scatter plots demonstrate the mean of the datasets.

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