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Short Communication

Development and characterization of promoterless helper RNAs for the production of alphavirus replicon particle

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Alphavirus-based replicon systems are frequently used as preclinical vectors and as antigen discovery tools, and they have recently been assessed in clinical vaccine trials. Typically, alphavirus replicon RNAs are delivered within virus-like replicon particles (VRP) that are produced following transfection of replicon RNA and two helper RNAs into permissive cells *in vitro*. The non-structural proteins expressed from the replicon RNA amplify the replicon RNA *in cis* and the helper RNAs *in trans,* the latter providing the viral structural proteins necessary to package the replicon RNA into VRP. Current helper RNA designs incorporate the alphavirus 26S promoter to direct the transcription of high levels of structural gene mRNAs. We demonstrate here that the 26S promoter is not required on helper RNAs to produce VRP and propose that such promoterless helper RNAs, by design, reduce the probability of generating replication-competent virus that may otherwise result from RNA recombination.

Alphavirus (family *Togaviridae*)-based replicon vectors are well-described and widely used in many biological fields of research (Atkins *et al.*, 2008) and have recently been assessed in clinical vaccine trials (Bernstein *et al.*, 2009). Virus-like replicon particles (VRP) are produced by supplying the alphavirus structural proteins *in trans* to cells that also contain a replicon RNA; the replicon RNA is recognized and packaged by the structural proteins, resulting in production of particles. Replicable helper RNA transcripts can be provided by transfection (Berglund *et al.*, 1993; Bredenbeek *et al.*, 1993; Frolov *et al.*, 1997; Geigenmuller-Gnirke *et al.*, 1991; Liljestrom & Garoff, 1991; Pushko *et al.*, 1997; Smerdou & Liljestrom, 1999; Volkova *et al.*, 2006) or as pol II transcripts from stably transfected packaging cell lines (Polo *et al.*, 1999).

Replication-competent alphaviruses (RCV) can be generated by recombination of helper RNAs with replicon RNA (Hill *et al.*, 1997; Raju *et al.*, 1995; Weiss & Schlesinger, 1991). The probability of generating RCV was reduced when the structural protein genes were separated onto two different helper RNAs (Frolov *et al.*, 1997; Pushko *et al.*, 1997; Smerdou & Liljestrom, 1999). These RNA designs contain the 5′ and 3′ sequences required for replication as well as an alphavirus 26S subgenomic promoter that directs the transcription of a structural protein mRNA. The 26S promoter is thought to be critical to these helper RNAs because of the significant mRNA amplification effect it imparts for the production of the subgenomic transcript. This promoter element is present on all current split helper RNA designs (Frolov *et al.*, 1997; Pushko *et al.*, 1997; Smerdou & Liljestrom, 1999; Volkova *et al.*, 2006). Here, we demonstrate that the 26S promoter is not required by split helper RNAs to generate VRP, and we propose that helper RNAs with this design may have advantages in reducing the probability of generating functional recombination events.

Standard 26S promoter-based capsid and glycoprotein (GP) helpers (Fig. 1) used to package Venezuelan equine encephalitis virus (VEEV) replicon RNA were modified to remove the 26S promoter (Δ26S). PCR primers were engineered to amplify the structural genes with unique *Rsr*II (5′) and *Sph*I (3′) restriction sites from helper plasmids that have been described previously (Pushko *et al.*, 1997). A plasmid containing cDNA for a VEEV replicon was digested with *Rsr*II and *Sph*I to remove most of the nsP1 gene and the entire remaining non-structural coding region (nsP2–nsP3–nsP4). The structural gene PCR products were then digested with *Rsr*II and *Sph*I enzymes and ligated individually into a similarly linearized cDNA plasmid containing a replicon vector, generating plasmids corresponding to dHcap(FL) and dHgp(FL) helpers (Fig. 1). Analysis of the replication efficiency of these Δ26S helpers was conducted by Northern blot. RNA was transcribed *in vitro* from plasmids containing cDNA for a replicon vector and each helper construct, and the RNAs were purified and co-electroporated into Vero cells as

A supplementary figure is available with the online version of this paper.

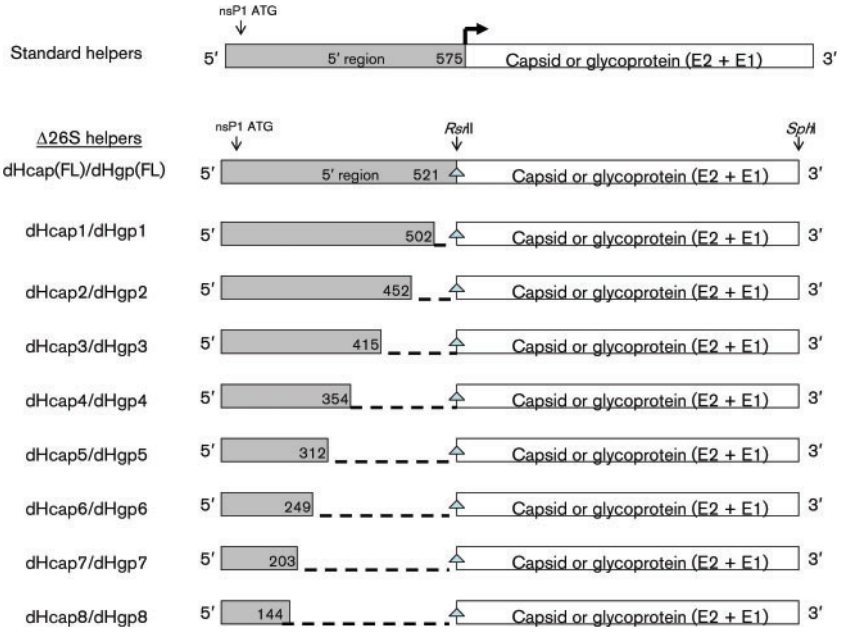

Fig. 1. Design of standard and Δ26S helper RNAs. The number located in each shaded box represents the nt length of the 5′ region upstream of the structural gene for each construct. The △ symbol indicates removal of the 26S promoter sequence. The dark-hyphenated lines indicate the 5′ region deleted from each Δ26S helper. The right-facing arrow represents the VEEV 26S promoter.

described previously (Kamrud *et al.*, 2007). After overnight incubation, total cellular RNA was collected and analysed by Northern blot using probes specific for the genomic and subgenomic transcripts by using methods described previously (Kamrud *et al.*, 2007). The standard helper RNAs demonstrate both a genomic and a subgenomic transcript as expected, whereas the Δ26S helpers each show replication of a single transcript, demonstrating the removal of the subgenomic promoter (Supplementary Fig. S1, available in JGV Online).

To determine which 5′ nt sequences (in addition to the conserved 5′ terminal 44 nt) are required for efficient helper replication, eight consecutive deletions were made in the region 5′ of the structural genes in both the dHcap(FL) and dHgp(FL) helpers (Fig. 1). First, eight different reverse primers were designed complementary to various positions within the region 5′ of the structural genes in the Δ26S(FL) helpers. These primers also contained a unique *Rsr*II restriction site (Fig. 1). A forward primer was designed, which when combined with any of the reverse primers would amplify fragments with unique 5′ *Xba*I and 3′ *Rsr*II restriction sites. Second, the amplified 5′ regions were cloned into plasmids representing the dHcap(FL) and dHgp(FL) helpers linearized with *Xba*I (located in the plasmid backbone sequence) and *Rsr*II. This generated eight sets of 5′ region truncated helper plasmids, designated dHcap1–8 and dHgp1–8 (Fig. 1).

Studies were then conducted to determine whether matched combinations of deletion-mutant Δ26S helper RNAs could replicate, express structural proteins and package VRP. RNA was transcribed *in vitro* from plasmids containing the cDNA for a replicon expressing an influenza HA gene (Hubby *et al.*, 2007) and each helper construct. The RNAs were purified and co-electroporated into Vero cells as described above. After overnight incubation, VRP were harvested for titration, and from the electroporated cells total cellular RNA and cytoplasmic lysates were collected for capsid- and GP-specific Northern and Western blot analyses (Kamrud *et al.*, 2007). For Northern blots, positive-sense transcripts were detected with an RNA probe specific for nsP1 sequences present on both helper and the replicon RNAs. Northern blot analysis demonstrated efficient helper replication with the detection of progressively shorter transcripts corresponding to the respective truncation of the 5′ nt sequence (Fig. 2a). The dHcap8 and dHgp8 Δ26S helpers demonstrated markedly reduced replication. This truncation removes the 51 nt conserved sequence element found in all alphaviruses (Ou *et al.*, 1983), and others have also shown that this element is important for replication (Frolov *et al.*, 2001; Monroe & Schlesinger, 1984; Niesters & Strauss, 1990; Tsiang *et al.*, 1988). VRP production with each of the Δ26S helpers was reduced compared with the VRP yields produced with standard helper RNAs (Fig. 2a). Western blot analysis indicated that Δ26S capsid helpers expressed either very little protein or larger proteins relative to the expected molecular mass for capsid, which presumably constitute fusion proteins. Fusion protein expression was not noted with Δ26S GP helpers by using a goat-anti-VEE E2 polyclonal antibody. The low VRP yields observed may be due to reduced expression of structural proteins or production of fusion proteins that do not function efficiently to package VRP. Analysis of the nt sequence upstream of the structural gene open reading frames (ORFs) revealed the presence of in-frame initiation codons that could produce the capsid fusion proteins observed. Of these, only one was in a favourable context for initiation of translation (Kozak, 1984), and it is in all of the helper RNAs. Prominent capsid-reactive proteins were detected,

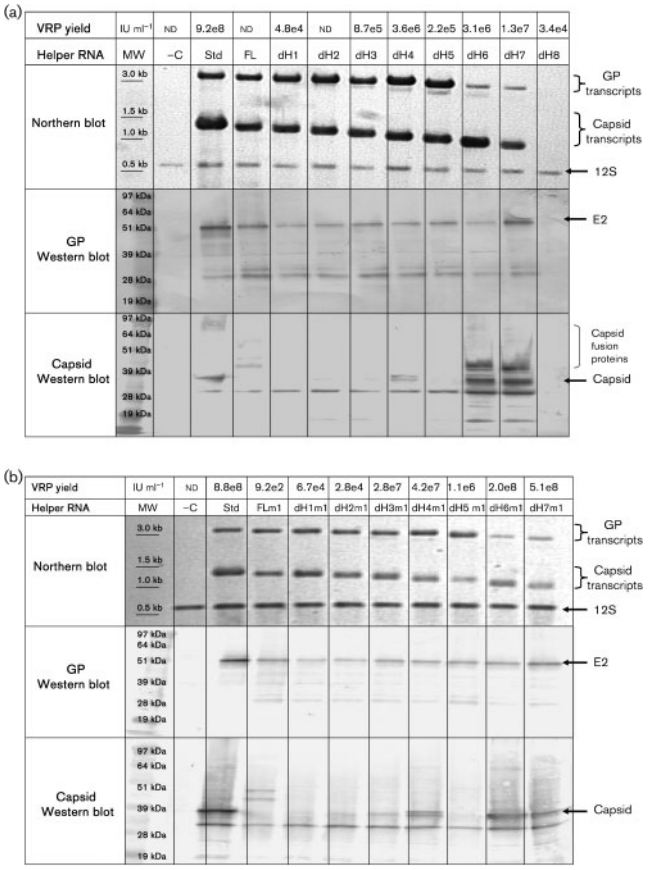

Fig. 2. Summary of VRP yield, Northern and Western blot analysis using matched combinations of deletion mutant Δ26S helper RNAs. (a) Analysis of deletion-mutant Δ26S helper RNAs. (b) Analysis of deletion mutant Δ26S helper RNAs with the nsP1 start codon modified to a stop codon (m1 helpers). VRP yields represented as infectious units (IU) ml^{-1} determined on Vero cells. The average titres determined from three experiments are represented. Total RNA was extracted from electroporated cells and 0.5 μg of each sample was analysed. A 12S rRNA-specific probe was used to demonstrate equivalent amounts of total cellular RNA were analysed by Northern blot. Protein lysates were produced from electroporated Vero cells and 10 μg of each protein sample was separated by SDS-PAGE, transferred to PVDF membranes and analysed with goat anti-VEE capsid or E2-specific polyclonal antibodies. −C, Negative control sample; Std, standard helpers with functional 26S promoters; ND, none detected.

from the sixth and seventh helper truncations that correspond to the predicted molecular mass of proteins that would be produced if this start codon was used for translation (Fig. 2a).

To generate Δ26S helpers that have this start codon ablated (mutated to a stop codon), site-directed mutagenesis was carried out (QuikChange kit; Stratagene) on all but the eighth truncation variant, which demonstrated reduced replication. These helpers are identified by an 'm1' designator. Ablation of all 5′ region start codons was conducted but these Δ26S helpers replicated poorly, presumably due to disruption of 5′ RNA structure(s) important for replication (data not shown). Vero cells were electroporated with replicon and m1 Δ26S helper RNAs as described above and the results of VRP production, Northern and Western blot analyses are shown in Fig. 2b. Northern blot analysis demonstrated efficient helper replication. Western blot analysis demonstrated that the majority of capsid proteins expressed from the m1 helpers had the correct molecular mass, suggesting that the m1-modification suppressed the translation of the capsid fusion proteins noted previously. VRP yields with the m1 Δ26S helpers were uniformly higher than those generated with the original Δ26S helper set. VRP yields generated with the sixth and seventh truncation m1-modified Δ26S helpers were similar to the yields produced with standard helper RNAs (Fig. 2b). There was no clear correlation between GP helper replication and VRP production especially for the H6m1 and H7m1 helpers (Fig. 2). Based on the Northern analysis, these GP helpers did not replicate well yet expressed similar amounts of GP to the other constructs analysed. However, the VRP yields with these GP helpers were only slightly lower than those measured with standard helper RNAs. An explanation for the lack of correlation between GP helper replication and GP expression remains unclear. The replication of the H6m1 and H7m1 capsid helpers were less affected than their GP helper counterparts. This may be due to the capsid helpers overall smaller length relative to the GP helpers, thus imparting a replication advantage to the shorter RNAs.

As with standard split helper RNAs, generation of RCV using Δ26S helper RNAs would require a minimum of two independent recombination events. In the absence of the 26S promoter, most recombination events would not result in the generation of a functional transcriptional unit that could express an intact structural protein. Regeneration of a complete structural region with Δ26S helpers requires that recombination events occur in a specific order and in specific nucleotide locations. The initial recombination event must involve the capsid helper coding sequence, since it must be located in a 5′ position relative to the glycoproteins for its autoprotease activity to cleave itself and to generate a functional capsid protein (Strauss & Strauss, 1990). The standard split helpers do not have this constraint, as the presence of the 26S promoter on each helper makes them independent transcriptional cassettes. Furthermore, the Δ26S capsid helper must be recombined with the replicon vector via a near-nt perfect recombination event to achieve a recombinant in which there would be efficient expression of the capsid protein. Only recombination events that are downstream of the replicon 26S promoter and do not result in production of capsid fusion proteins may be viable. The second recombination event, involving the Δ26S GP helper, must occur downstream from the capsid gene to avoid insertional mutagenesis and maintain the structural protein ORF.

Because the capsid protein provided by a split helper RNA system does not need to maintain its cleavage activity, introduction of a stop codon at the 3′ end of the capsid gene, in place of the chymotrypsin-like cleavage site, increases the difficulty of producing functional recombinants with a glycoprotein helper even further. That is, the recombination event would have to be nt perfect to replace the engineered stop codon in the capsid gene, while reconstituting an active capsid cleavage site and maintaining the glycoprotein ORF. Capsid helpers with a stop codon engineered in place of the cleavage site have been generated and they function as well as the cleavage-competent capsid helpers to package VRP (data not shown). Thus, producing VRP using Δ26S helper RNAs would theoretically generate RCV at a lower frequency than with standard split RNA helpers as most double recombination events would yield non-functional recombinants. The order and location of recombination restrictions described above are limited to double recombination events. Recombinants arising due to three or more individual events would not be restricted in the same manner.

The current split helper systems reduce the probability of generating RCV (Frolov *et al.*, 1997; Pushko *et al.*, 1997; Smerdou & Liljestrom, 1999; Volkova *et al.*, 2006). Because generation of RCV in these systems is a rare event, it is difficult to demonstrate experimentally a further reduction in the frequency of its occurrence. To demonstrate the enhanced barrier to functional recombination when employing the Δ26S helper design, we constructed a replicon vector in which the capsid gene (with a stop codon engineered in place of the cleavage site) was inserted directly downstream of the 26S promoter. As discussed above, this construct represents an RNA molecule that would be generated after a functional first recombination occurred between a replicon RNA and a capsid helper RNA. By providing the capsid replicon RNA as the requisite first recombinant template, the two helper RNA systems can be directly compared for the ability to generate RCV via a second recombination between capsid replicon RNA and GP helper RNA. For this experiment, capsid VRP were harvested following co-electroporation of Vero cells with capsid replicon RNA and either 26S promoter GP helper or Δ26S GP helper RNA. Following infectivity titration, Vero cell culture flasks were infected with the two capsid VRP preparations at two multiplicities of infection. The two preparations were carried through two blind Vero cell passages to allow for the amplification of functional recombinants, if present, as described previously (Kamrud *et al.*, 2007). Media samples were collected from pass two flasks and analysed by plaque assay. The pass two samples from cultures infected with capsid VRP generated with a 26S promoter GP helper each contained p.f.u. (m.o.i. $0.1=9.8\times10^7$ p.f.u. ml^{-1}, m.o.i. $0.05=7.4\times10^7$ p.f.u. ml^{-1}). However, the pass two samples from cultures infected with capsid VRP generated with the Δ26S GP helper had no detectable p.f.u. Sequence analysis of

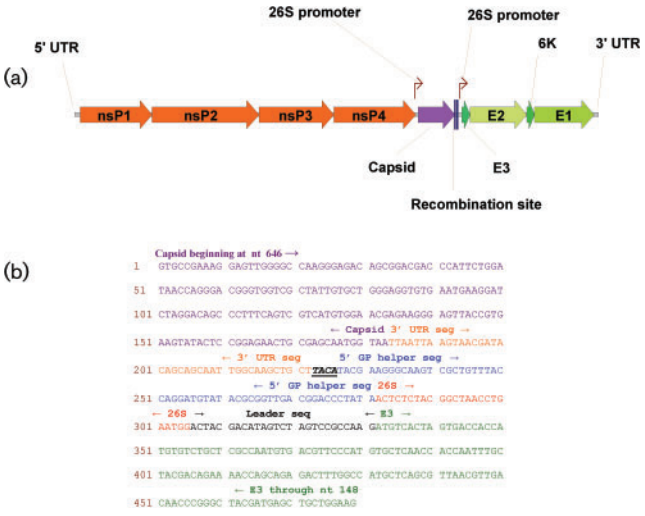

Fig. 3. (a) Schematic representation of a clone identified in RNA extracted directly from particles in p.f.u. material. (b) Nucleotide sequence of the capsid-replicon/GP helper recombination junction from the clone identified in RNA extracted directly from particles in p.f.u. material. The recombination site is identified as the bold italicized and underlined sequence.

RT-PCR amplified cDNAs of RNA extracted directly from particles in the p.f.u.-positive culture fluids revealed that the 26S promoter GP helper had recombined into the 3′ non-coding region of the capsid-replicon RNA, resulting in a recombinant that expressed capsid and GP under separate 26S promoters (Fig. 3). These data support the theoretical predictions outlined above describing the increased constraints on functional recombination when employing the Δ26S helper design. Based on these data, we believe that the Δ26S helper design offers an efficient system for the production of VRP, while providing additional constraints on the generation of functional RNA recombination events.

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