A new Defective Helper RNA to produce Sindbis virus that infects neurons but does not propagate

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Abstract

Recombinant Sindbis viruses are important tools in neuroscience because they combine rapid and high transgene expression with large payloads. Currently, two packaging systems are available for making neurotropic virus, but both produce a fraction of viral particles that can propagate beyond the primary infected neuron. When injected into mouse brains, viruses produced using these systems label neurons at the injection site, but also elsewhere in the brain. Such ectopic labeling caused recombinant Sindbis viruses to be classified as anterograde viruses with limited retrograde spread, and can complicate the interpretation of neuroanatomical and other experiments.

Here we describe a new packaging system, DH-BB(5'SIN;TE12ORF), that produces virus that is both neurotropic (able to infect neurons) and propagation-incompetent. We show in mice that DH-BB(5'SIN;TE12ORF)-packaged virus eliminates infection of cells outside the injection site. We also provide evidence that the ectopically labeled cells observed in previous experiments resulted from secondary infection by propagation-competent virus, rather than from inefficient retrograde spread.

Virus produced with our new packaging system retains all the advantages of previous recombinant Sindbis viruses, but minimizes the risks of confounding results with unwanted ectopic labeling. It should therefore be considered in future studies in which a neurotropic, recombinant Sindbis virus is needed.

Introduction

Sindbis virus is an enveloped, positive strand RNA virus from the family of the togaviridae. Its 11703 base pair genome contains two open reading frames (ORF), one coding for the nonstructural proteins, the other for the structural proteins (Fig 1a)[1].

In recombinant Sindbis vectors, the structural protein ORF is replaced with the gene of interest[2]. Upon infection, the recombinant virus expresses the gene of interest instead of the structural proteins, and thus becomes a vehicle for transgene expression. Because the region encoding the essential structural proteins has been deleted, a cell infected by only recombinant Sindbis does not produce infectious viral particles. Thus to produce virus from a recombinant genome, packaging systems have been developed in which the structural proteins are supplied in *trans* by a second RNA, the Defective Helper (DH) RNA[3].

There are three classes of DH RNAs. In the first class, derived from the wild type Sindbis genome, the nonstructural protein coding region has been deleted using convenient restriction enzymes[3]. The second class is similar to the first, but carries a tRNA sequence at its 5' end rather than the usual 5' sequence of Sindbis virus; this tRNA sequence is also found in certain naturally occurring defective interfering Sindbis particles[3]. In the third class of DH RNAs, derived from naturally occurring defective interfering particles, the structural protein coding region has been reinserted[3,4].

A recombinant Sindbis genome does not encode all the information necessary to produce infectious viral particles. However, propagation-competent viral particles can emerge if the DH RNA is co-packaged with the genome into a single particle. If this occurs, Sindbis functions effectively as a bipartite RNA

virus, where the genome and DH RNA complement each other to expresses the nonstructural and structural proteins[4]. The extent of propagation competence of recombinant Sindbis virus then depends on the rate of co-packaging of the genome with the DH RNA. Packaging of the genomic RNA is always favored over the DH RNA[3,4], but presence of the Sindbis packaging signal[5] or a 5' tRNA sequence in the DH RNA increases the rate of co-packaging, and thus of the propagation competence of the resulting virus[3].

Sindbis virus has a remarkably wide host range, infecting insects and many species of higher vertebrates and many different cell types. Nonetheless, different Sindbis strains have different tropisms, determined by the structural proteins. Notably, the common laboratory strain Toto1101 does not infect neurons, whereas the TE12 strain does[6]. Different DH constructs are derived from different strains, and therefore produce viruses with different tropisms[3,6].

The DH RNA conventionally used in neuroscience for Sindbis virus production is the interfering particle based DH(26S)5'SIN vector[3,4,7,8]. It expresses the TE12 structural proteins, making the resulting virus neurotropic[3,6]. Importantly, it also contains the Sindbis packaging signal, and produces virus that contains a fraction of propagation-competent virions[3]. Previous studies have reported that, when injected into mouse brains, Sindbis virus prepared with DH(26S)5'SIN labels cells far away from the injection site, and have interpreted this as evidence for retrograde labeling[9] (where retrograde labeling is defined as infection of the axon, and labeling of cell bodies, but no trans-synaptic spread). Despite this unintended and potentially confusing ectopic labeling, viruses produced using this DH construct have been extensively used in anterograde tracing experiments[10] (where anterograde labeling is defined as

infection of the somatodendritic compartment and labeling of cell bodies and axons, but no trans-synaptic spread) and for transgene expression[7,11,12].

A second neurotropic DH system was developed more recently by replacing the structural proteins in the DH-BB(tRNA) construct[3], which were derived from the non-neurotropic Toto1101 strain, with the neurotropic TE12 structural proteins from DH(26S)5'SIN[13]. This change resulted in the production of neurotropic virus from the new DH-BB(tRNA;TE12) construct. However, a fraction of virions packaged with the original DH-BB(tRNA) were propagation competent[3], and the addition of TE12 structural proteins to form DH-BB(tRNA;TE12) did not appear to influence the rate of co-packaging of the DH construct. Thus, a fraction of virions produced with DH-BB(tRNA;TE12) are propagation competent.

Here we present a new Defective Helper RNA, DH-BB(5'SIN;TE12ORF), which produces a propagation-incompetent, neurotropic virus. When injected into mouse brains, virus produced with this new DH RNA only very rarely labels neurons outside the injection site, suggesting that the ectopic labels observed for Sindbis viruses derived from other DH RNAs arose from secondary infection by propagation-competent particles. Our new DH RNA therefore provides precise spatial control of Sindbis virus-based expression in the mouse brain, and removes ectopic infection as a confounding factor in neuroanatomical and physiological experiments.

Results and Discussion

Generation of a propagation-incompetent neurotropic Sindbis packaging system

To investigate the replication competence and neurotropism of different Sindbis packaging systems, we generated a recombinant genome that expressed GFP, and packaged it with three different DH RNAs (Fig 1b): (i) the commonly used neurotropic DH(26S)5'SIN[3], (ii) the neurotropic DH-BB(tRNA;TE12)[13] and (iii) the Toto1101 derived DH-BB(5'SIN)[3] (Fig 1 b). As expected[3,13], plaque assays in BHK cells revealed that both DH(26S)5'SIN and DH-BB(tRNA;TE12) produced propagation-competent virus. In contrast, DH-BB(5'SIN)-derived virus showed no evidence of replication competence (Fig 1c). Conversely, the propagation-competent DH(26S)5'SIN and DH-BB(tRNA;TE12)-derived viruses infected primary mouse hippocampal cultures, whereas propagation-incompetent DH-BB(5'SIN)-derived virus failed to infect neurons (Fig1 d)[3,6].

We sought to combine the propagation-incompetence of the DH-BB(5'SIN) system with the neurotropism of the other two packaging systems. Tropism is controlled by the structural proteins[6], replication competence by the probability of co-packaging of the helper RNA with the genomic RNA[3,4]. We therefore replaced only the structural protein ORF of the DH-BB(5'SIN) vector with the structural protein ORF of the DH-BB(tRNA;TE12) construct (Fig 1e). As predicted, virus produced from the resulting DH-BB(5'SIN;TE12ORF) construct does not show evidence of secondary infection in a plaque assay (Fig 1f), but does infect neurons (Fig 1g).

Use of DH-BB(5'SIN;TE12ORF) eliminates labeling of cells outside the injection site

We next characterized the behavior of viruses packaged with DH(26S)5'SIN and DH-BB(5'SIN;TE12ORF) *in vivo*. To do so, we injected equal amounts of virus (as determined by qPCR titering; see Materials and Methods) into the locus coeruleus of 3 mice per virus and examined the pattern of infection.

Virus packaged with DH(26S)5'SIN efficiently infected neurons at the primary injection site. It also labeled numerous cells spread out across the brain far away from the injection site (228 +/- 166 cells per animal). Such spread has previously been reported for virus packaged with DH(26S)5'SIN, and was attributed to some low level of retrograde infection[9]. However, close inspection of those putatively retrogradely labeled cells revealed GFP expression not only in isolated projection neurons with long axons that might have reached the site of injection (Fig 2a), but also in interneurons whose axons remain local and do not extend to the injection site (Fig 2b) and therefore cannot be labeled by retrograde infection. Furthermore, we also observed clusters of 10 to 50 GFP-expressing neurons (Fig 2c). Such dense labeling is again difficult to explain by inefficient retrograde infection, which inherently favors sparse labeling over large areas, but would be expected from occasional propagation of infective particles from the axons of neurons infected at the injection site.

Virus packaged with our newly engineered DH-BB(5'SIN;TE12ORF) packaging system labeled neurons at the injection site with an efficiency similar to that of DH(26S)5'SIN. However, in contrast to DH(26S)5'SIN-packaged virus, virus packaged with DH-BB(5'SIN;TE12ORF) showed almost no ectopic infection. In a total of 3 mice, we detected only a single labeled cell outside the injection site,

compared with hundreds of cells per animal using the previous system. Thus use of DH-BB(5'SIN;TE12ORF) eliminates the co-packaging of helper RNA, and thereby eliminates the secondary infection responsible for ectopic spread beyond the injection site. Accordingly, we classify recombinant Sindbis virus as purely anterograde viruses in the mouse brain.

Conclusion

We here describe a new Sindbis virus packaging system that produces a neurotropic virus that does not propagate beyond *in vitro* or *in vivo*. Secondary infection is hard to control and can have unintended effects in neuroanatomical and physiological studies. Our new packaging system should therefore be used in all future studies in which secondary infection can confound the interpretation of results.

Materials and Methods

Defective Helper constructs

The three DH constructs used in this study were generous gifts from the following laboratories: Robert Malinow (UCSD; DH(26S)5'SIN), Jinny Kim (KIST; DH-BB(tRNA;TE12)) and Charles Rice (Rockefeller University; DH-BB(5'SIN)). To produce DH-BB(5'SIN;TE12ORF) we performed PCR for the TE12 ORF on DH-BB(tRNA;TE12) using forward primer 5'-TAA AGC ATC TCT ACG GTG GTC C-3' and reverse primer 5'-CAT CGA GTT TTG CTG GTC GGA-3'. We amplified the DH-BB(5'SIN) backbone excluding the structural ORF by PCR using forward primer 5'-CCG CTA CGC CCC AAT GAT CC-3' and reverse primer 5'-TAT TAG TCA GAT GAA ATG TAC TAT GCT GAC-3'. We then

assembled the two PCR fragments using Gibson assembly master mix (New England Biolabs) according to the manufacturer's protocol.

Sindbis virus production

To produce high-titer Sindbis virus, we first linearized both the genomic construct and the DH construct with PacI or XhoI (New England Biolabs), respectively. We then *in vitro* transcribed genomic and helper RNAs using the mMessage mMachine SP6 *in vitro* transcription kit (Life Technologies). We transfected BHK-21 cells with a 1.7:1 weight ratio of genomic to helper RNA using Lipofectamine 2000 (Life Technologies) in complete media (alpha-MEM, 5% heat inactivated FBS, 1% PennStrep, 1% MEM vitamins, 1% 200mM L-Glutamine (all Life Technologies)) according to the manufacturer's instructions. Forty hours after transfection, we removed the supernatant and concentrated the virus by ultracentrifugation, as previously described[7].

Sindbis virus titering

To obtain a measure of Sindbis titer independent of the virus's tropism, we quantified the number of RNaseI-protected genomic RNAs per ml of virus (GC/ml). Briefly, we digested 1μl of virus with 1U of RNaseI (Epicentre) in 50μl total volume at 37C for 30 minutes and then extracted protected RNA using Trizol reagent (Life Technologies) according to the manufacturer's protocol. We reverse transcribed the genomic RNA using the gene specific primer 5'-GGG TCG CCT TGC TTG AAG TG-3' that falls into the nsp1-4 region of the genomic RNA using SuperScriptIII reverse transcriptase (Life Technologies) according to the manufacturer's protocol. We then measured the number of cDNA molecules relative to a plasmid standard by qPCR using SYBR Green power Mastermix (Life Technologies) and primers 5'-TAT CCG CAG TGC GGT TCC AT-3' and 5'-TGT CGC TGA GTC CAG TGT TGG-3'.

Primary hippocampal neuron culture

We prepared dissociated hippocampal neurons from E18 CD1 mouse pups as previously described[14] and plated them in XONA Microfluidic chambers (SND450, Xona Microfluidics) according to the manufacturer's instructions. At 14-21 DIV we infected cultures with 1μl of 10^{10} GC/ml virus as previously described[15]. We fixed the cells in 4% PFA (Electron Microscopy Sciences) in 0.1M PB 24 hours after infection and stained them using Neurotrace 640/660 Deep Red Fluorescent Nissl Stain (Life Technologies) according to the manufacturer's protocol. We then imaged the cells on a Zeiss LSM780 confocal microscope using a 40x objective.

Plaque assays

We infected 90% confluent BHK-21 cells with Sindbis virus in 200μl volume for 1 hour in 24-well plates. We then overlaid the cells with 0.4% SeaPlaque Agarose (Lonza) in complete culture media. 24 hours post infection we fixed the cells in 4% PFA and imaged them on a Zeiss Observer microscope.

Virus injections

Animal procedures were approved by the Cold Spring Harbor Laboratory Animal Care and Use Committee and carried out in accordance with National Institutes of Health standards.

We pressure injected 180nl of 2×10^{10} GC/ml virus into the right locus coeruleus (5.4mm posterior, 0.8mm lateral, 2.9mm and 3.1mm deep from the surface of the brain; 90nl per depth) of 10-14 week old C57/BL6 males as described[16]. 48 hours post infection we transcardially perfused animals with ice cold saline (9g/l) followed by ice cold 4% PFA (Electron Microscopy Sciences) in 0.1M PB. We post fixed

brains in 4% PFA in 0.1M PB overnight at 4C and then sectioned them on a Vibratome and mounted the sections in Vectashield with DAPI (Vector Labs).

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Author contributions

J.M.K. and A.M.Z conceived the study. J.M.K. and P.G.S. performed the experiments. J.M.K. and A.M.Z. wrote the paper.

Conflict of interests

The authors declare no conflict of interests.

Figure Legends

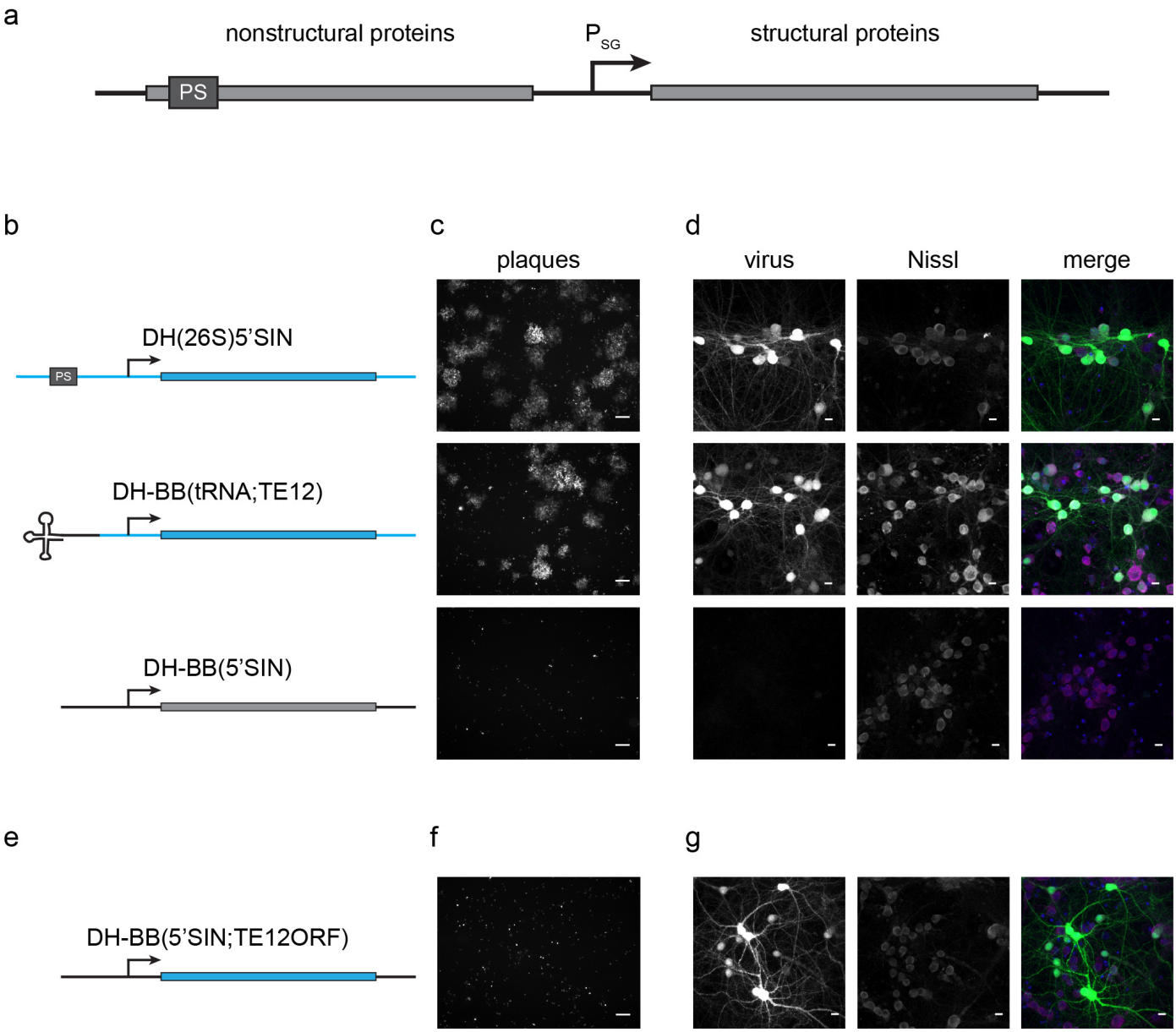

Figure 1: Different DH RNAs and their respective propagation competencies and neurotropism. (a) Overview of the Sindbis virus genome. (b) Different Defective Helper RNAs differ in their 5' end and in the origin of their structural protein-coding region. (c) Whereas both DH(26S)5'SIN and DH-BB(tRNA;TE12) packaged virus produces plaques in BHK cells, DH-BB(5'SIN) packaged

virus does not produce plaques. Only individually infected cells are discernible. Scale bar = 100μm. (d) DH(26S)5'SIN and DH-BB(tRNA;TE12) packaged virus efficiently infect cultured hippocampal mouse neurons, while DH-BB(5'SIN) does not. Scale bar = 10μm. (e) DH-BB(5'SIN;TE12ORF) has the same structure as DH-BB(5'SIN) but carries the same structural protein open reading frame as DH(26S)5'SIN. Virus produced using DH-BB(5'SIN;TE12ORF) does not produce plaques (f) but does infect neurons (g).

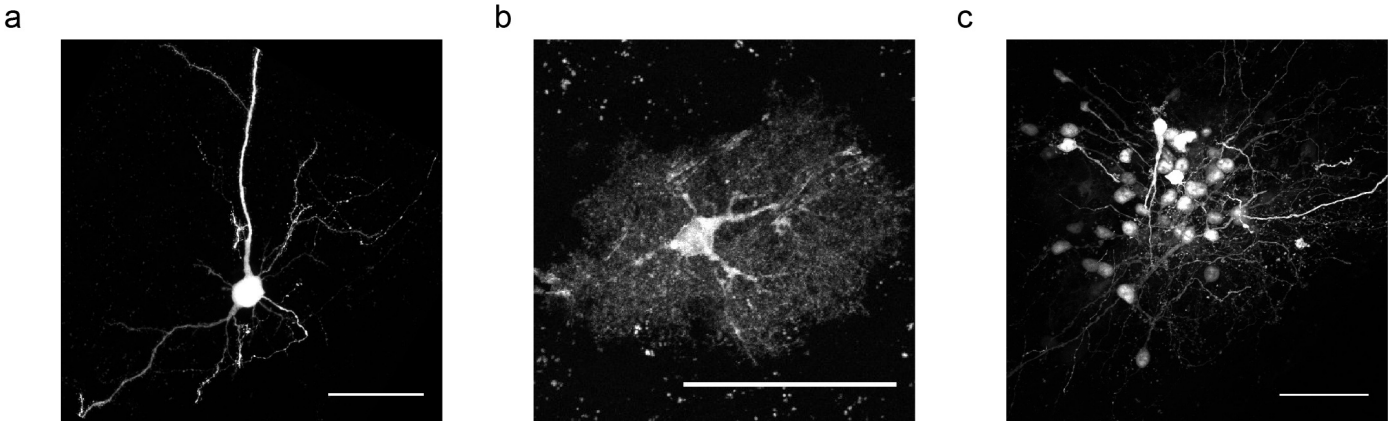

Figure 2: DH(26S)5'SIN packaged virus infects many neurons outside the primary injection site in locus coeruleus, such as pyramidal neurons in cortex (a), but also interneurons in cortex (b) and clusters of local granule cells in the olfactory bulb (c). Scale bar = 50μm.