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An improved *in vitro* and *in vivo* Sindbis virus expression system through host and virus engineering

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ABSTRACT

The Sindbis viral expression system enables the rapid production of high levels of recombinant protein in mammalian cells; however, this expression is typically limited to transient production due to the cytotoxicity of the virus. Limiting the lethality inherent in the Sindbis virus vector in order to enable long term, sustained expression of recombinant proteins may be possible. In this study, modifications to virus and host have been combined in order to reduce the cytopathic effects. Non-cytopathic replication competent viruses of two Sindbis viral strains, TE and 633, were developed using a non-structural protein (nsP) P726S point mutation in order to obtain persistent heterologous gene expression in infected Baby Hamster Kidney (BHK) cells and Chinese Hamster Ovary (CHO) cells. Cells infected with the P726S variant viruses were able to recover after infection, while cells infected with normal virus died within 3 days. The P726S mutation did not reduce the susceptibility of 5- and 14-day-old mice to 633 and TE viruses *in vivo*. In addition, animal survival with the P726S variant viruses was increased and GFP expression was sustained for at least 14 days while the 633 and TE infection resulted in short-term GFP expression or an earlier mortality. Modifications to the host BHK and CHO cells themselves were subsequently undertaken by including the anti-apoptotic gene Bcl-2 and a deletion mutant of Bcl-2 (Bcl-2Δ) as another method for limiting the cytopathic effects of the Sindbis virus. The inclusion of anti-apoptotic genes permitted higher production of heterologous GFP protein following Sindbis virus infection, and the combination of the TE-P726S virus and the CHO-Bcl-2Δ cell line showed the greatest improvement in cell survival. Sindbis virus infection also induced ER stress in mammalian cells as detected by increased PERK phosphorylation and ATF4 translation. Overexpression of Parkin, an E3 ubiquitin ligase that can protect cells against agents that induce ER stress, suppressed Sindbis virus-induced cell death in both BHK cells and *in vivo* studies in mice. Such findings show that viral and host modifications can improve cell survival and production of heterologous proteins, change viral behavior *in vitro* and *in vivo*, and assist in the development of new expression or gene delivery vehicles.

1. Introduction

Alphavirus expression vectors have been employed to produce recombinant proteins in mammalian cells (Boorsma et al., 2003; Bredenbeek et al., 1993; Frolov et al., 1996; Garoff and Li, 1998; Huang et al., 1989; Liljestrom and Garoff, 1991; Lundstrom, 1997; Schlesinger and Dubensky, 1999; Xiong et al., 1989). Sindbis virus is a single-stranded positive sense RNA virus belonging to the alphavirus family that can infect a broad range of cells and replicate to high titers (Strauss and Strauss, 1994). Despite the high-level expression of recombinant proteins, Sindbis virus infected cells rapidly undergo apoptosis limiting protein expression to a transient system (Levine et al., 1993; Mastrangelo et al., 2000).

The Sindbis virus RNA contains two open reading frames. The first is located in the 5′ terminal two-thirds of the genomic RNA translating the nonstructural proteins (nsP 1–4) required for transcription and replication of the viral RNA. The second is located in the 3′ terminal one-third of the genome and codes for structural proteins including capsid, glycoproteins E1 and E2, and a small hydrophobic membrane (6K) protein (Strauss and Strauss, 1994). Several approaches have used the Sindbis virus replication machinery for recombinant protein production. The replicon and defective helper system consists of the replicon RNA encoding the non-structural proteins and the recombinant protein of interest, and the helper RNA encoding the structural proteins (Schlesinger and Dubensky, 1999). The replicons are self-replicating due to the

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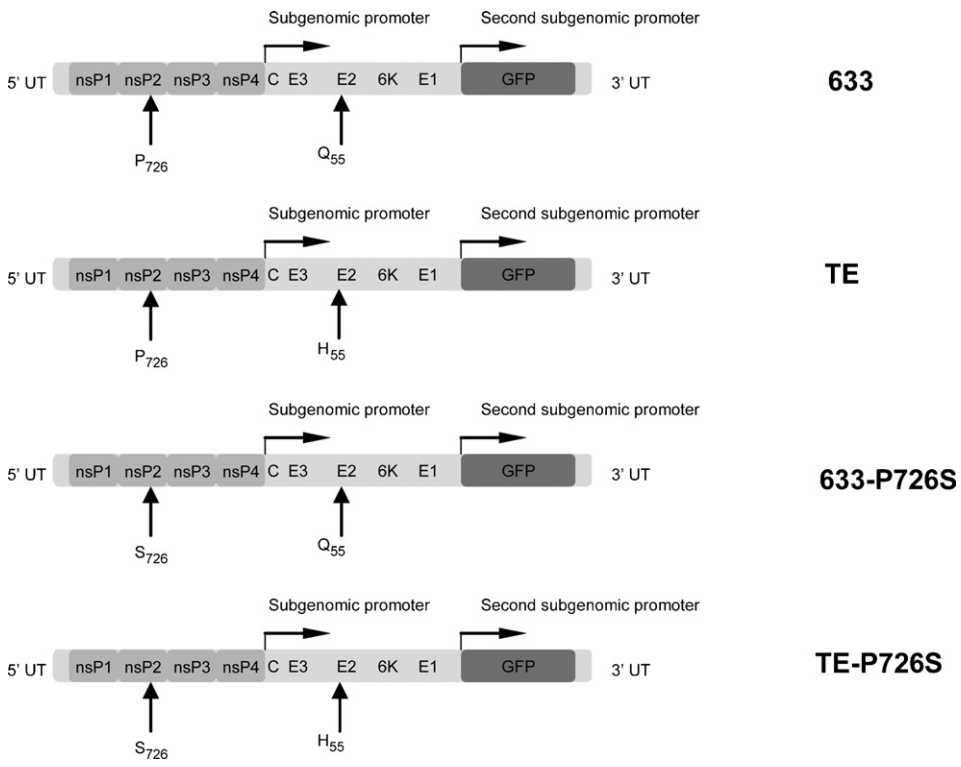

Fig. 1. Replication and packaging competent recombinant Sindbis viruses 633, TE, 633-P726S, and TE-P726S. The 633 and TE viruses differ by an amino acid at the position 55 of the E2 protein that is Glutamine (Q) or Histidine (H). The Proline (P) to Serine (S) mutation is at the amino acid position 726 in the nsP2 domain of the nonstructural proteins. The green fluorescence protein (GFP) is expressed from the second subgenomic promoter.

replicase encoded by the non-structural proteins. The helpers are required to package the replicons to create new virus particles. On the other hand, the replication and packaging-competent system contains a duplicated subgenomic promoter to express heterologous proteins. The RNA vector genome encodes the non-structural proteins, the structural proteins, and the recombinant proteins creating infectious particles (Frolov et al., 1996).

The recombinant Sindbis virus 633 and TE were constructed to study the effects of amino acid changes on neurovirulence (Dropulic et al., 1997). We examined the replication and packaging-competent Sindbis virus vectors 633 and TE engineered to produce green fluorescent protein (GFP) as the heterologous model protein from the additional subgenomic promoter (Fig. 1). *In vivo*, the neurovirulence of Sindbis virus was influenced by the amino acid change from histidine to glutamine at position 55 of the E2 structural protein. The TE virus (His-55) was fully virulent for 14-day-old mice, but the 633 virus (Gln-55) was not (Tucker et al., 1993). The TE virus displayed more efficient infection than the 633 virus in neuronal N18 cells, which was likely due to more efficient virus binding of the TE virus than the 633 virus (Lee et al., 2002). However, in non-neuronal BHK cells, the 633 and TE viruses showed a similar level of virus binding and replication (Dropulic et al., 1997; Lee et al., 2002).

The cytopathic effects of the Sindbis replicons could be reduced by a single amino acid change in the nsP2 non-structural protein domain (Dryga et al., 1997). A small number of BHK cells survived Sindbis virus infection in the presence of defective interfering particles (Weiss et al., 1980). The SIN-1 virus was isolated from the culture and shown to develop persistent infection without the defective interfering particles. The SIN-1 virus was found to contain the P726S mutation (Dryga et al., 1997). Another experiment using Sindbis virus expressing puromycin acetyltransferase as a selectable marker identified an adaptive mutation for noncytopathic infection at the same location in nsP2 protein, P726L (Frolov et al., 1999). This P726L mutation was also used in a Sindbis virus replicon engineered to express heterologous genes (Agapov et al., 1998).

We incorporated the P726S mutation in the 633 and TE viruses and examined cytotoxicity and GFP expression in Baby Hamster Kidney (BHK) and Chinese Hamster Ovary (CHO) cell lines commonly used for recombinant protein expression. Sindbis virus vectors have the potential for investigation of recombinant protein expression in neurobiology due to the ability to express recombinant proteins in neurons both in vitro and *in vivo*. However, cell death induced by Sindbis virus vectors has limited their application. We also tested the 633-P726S and the TE-P726S variant viruses in a mouse model (5- and 14-day-old) to assess toxicity and duration of expression.

Sindbis virus infection is known to be lethal due to activation of the programmed cell death cascade or apoptosis (Levine et al., 1993; Hardwick, 1997; Mastrangelo et al., 1999). Expression of anti-apoptotic genes has been shown to inhibit cell death at least to a limited extent against Sindbis virus infection. AT-3 cells expressing Bcl-2 were protected against apoptosis when infected with the 633 virus. However, the AT-3 Bcl-2 cells were killed following infection with the TE virus (Ubol et al., 1994). Cells over-expressing Bcl-2 or Bcl-x_L were protected against cell death following Sindbis virus or Semliki Forest virus infection (Kiiver et al., 2008; Lundstrom et al., 1997; Mastrangelo et al., 2000). Bcl-2 is a widely studied anti-apoptotic gene that includes four homology domains (BH1–4) and an unstructured loop region susceptible to post-translational modifications and degradation during apoptosis. The Bcl-2 gene has been modified to remove this loop domain to create a variant called Bcl-2Δ (Chang et al., 1997). The Bcl-2Δ protein was more protective than the wild-type Bcl-2 protein against various apoptosis insults in mammalian cell culture including Sindbis virus infection, IL-3 withdrawal, serum withdrawal, and glucose deprivation (Chang et al., 1997; Figueroa et al., 2001). The Bcl-2Δ variant appeared to be more stable and less susceptible to proteolytic cleavage than the