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Short Communication

# Optimized P2A for reporter gene insertion into Nipah virus results in efficient ribosomal skipping and wild-type lethality

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Incorporation of reporter genes within virus genomes is an indispensable tool for interrogation of virus biology and pathogenesis. In previous work, we incorporated a fluorophore into a viral ORF by attaching it to the viral gene via a P2A ribosomal skipping sequence. This recombinant Nipah virus, however, was attenuated *in vitro* relative to WT virus. In this work, we determined that inefficient ribosomal skipping was a major contributing factor to this attenuation. Inserting a GSG linker before the P2A sequence resulted in essentially complete skipping, significantly improved growth *in vitro*, and WT lethality *in vivo*. To the best of our knowledge, this represents the first time a recombinant virus of *Mononegavirales* with integration of a reporter into a viral ORF has been compared with the WT virus *in vivo*. Incorporating the GSG linker for improved skipping efficiency whenever functionally important is a critical consideration for recombinant virus design.

The insertion of reporter genes into recombinant viruses is an important tool not only for the study of virus biology and pathogenesis, but also for the development of faster screening assays for the evaluation of antivirals. For the henipaviruses Nipah virus (NiV) and Hendra virus (HeV) (genus *Henipavirus*), emerging and zoonotic paramyxoviruses (family *Paramyxoviridae*, order *Mononegavirales*) with negative-sense, ssRNA genomes, reporter genes have been inserted either as independent ORFs or as part of existing viral ORFs (Lo *et al.*, 2012; Marsh *et al.*, 2013; Yoneda *et al.*, 2006; Yun *et al.*, 2015). In the former strategy, conserved gene start and stop signals present within virus intergenic sequences are used to drive reporter mRNA expression; however, insertion of additional intergenic sequences can disturb the polar transcriptional gradient of the virus (Conzelmann, 1998; Falzarano *et al.*, 2014). As an alternative, we have used a ribosomal skipping sequence to incorporate the reporter gene into an existing viral ORF, thus avoiding the use of additional intergenic sequences (Yun *et al.*, 2015). While this strategy indeed did not affect the natural transcriptional gradient of NiV, the recombinant reporter virus was significantly attenuated for *in vitro* growth as compared with the parental NiV (Yun *et al.*, 2015).

There are several commonly used '2A' ribosomal skipping sequences, derived from porcine teschovirus-1 (P2A), foot-and-mouth disease virus (F2A), Thosea asigna virus (T2A) and equine rhinitis A virus (E2A). All translate to short 18–22 aa peptides that mediate ribosomal skipping between a C-terminal glycine and proline, thus resulting in separate polypeptides (Donnelly *et al.*, 2001). For our recombinant NiV (based on the Malaysia prototype strain), we previously appended the mCherry fluorophore to the N-terminal end of the NiV matrix gene via a P2A sequence (Fig. 1 and Yun *et al.*, 2015), which appears to be the most efficient of the skipping sequences in widely used human cell lines (Kim *et al.*, 2011). Even so, any inefficiency in skipping would result in a fusion product, which

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might inhibit the function of the fused viral protein. A large N-terminal fusion is known to impede the budding function of NiV matrix (Wang *et al.*, 2010). Therefore, we considered whether any inefficiency of P2A skipping in our recombinant NiV was a factor in its attenuation relative to WT NiV.

We infected human umbilical vein endothelial cells (HUVECs) with our recombinant NiV incorporating mCherry at the N terminus of the matrix (M) gene via a P2A sequence (henceforth labelled rNiV-mCh$^{M}$). Western blotting for NiV-M revealed that P2A ribosomal skipping was indeed inefficient, with about a third of the matrix present as a mCherry fusion protein (Fig. 2a, see lane 2 at various time points). As the efficiency of 2A skipping is known to be dependent on the amino acid context (Minskaia & Ryan, 2013), it appears that the amino acid sequence adjacent to the P2A peptide in our recombinant NiV is not conducive to efficient skipping. It is known that addition of a GSG linker immediately upstream of the skipping sequence improves skipping efficiency (Holst *et al.*, 2006; Szymczak *et al.*, 2004), presumably by allowing the 2A peptide to favour the conformation that induces ribosomal skipping. We therefore added this linker to our reverse genetics construct (Fig. 1a), rescued the modified virus (rNiV-mCh$^{M}$-opt) as previously described (Yun *et al.*, 2015), and compared skipping efficiencies. Addition of the GSG linker resulted in essentially complete ribosomal skipping (Fig. 2a, compare lanes 2 and 3 at various time points).

We previously determined that rNiV-mCh$^{M}$ had slower replication kinetics relative to WT NiV in permissive cell lines such as Vero (Yun *et al.*, 2015) and HeLa (unpublished data) as well as primary human cells such as human neural stem cell derived neurons/astrocytes and HUVECs (Yun *et al.*, 2015). The defect appeared most pronounced in HUVECs. We therefore infected HUVECs with WT NiV, rNiV-mCh$^{M}$ and rNiV-mCh$^{M}$-opt and found that rNiV-mCh$^{M}$-opt infection resulted in significantly higher released virus titres relative to rNiV-mCh$^{M}$ at 12 h post-infection (p.i.) (Fig. 2b). rNiV-mCh$^{M}$-opt titres remained similar to those of WT NiV until 24 h p.i., peaking at about 0.5 log lower titres (Fig. 2b).

We then determined whether the improved growth kinetics of rNiV-mCh$^{M}$-opt relative to rNiV-mCh$^{M}$ would translate to *in vivo* lethality more similar to WT NiV. Using a widely used small animal model for NiV pathogenesis, we inoculated Syrian golden hamsters (10 3- to 8-week-old females

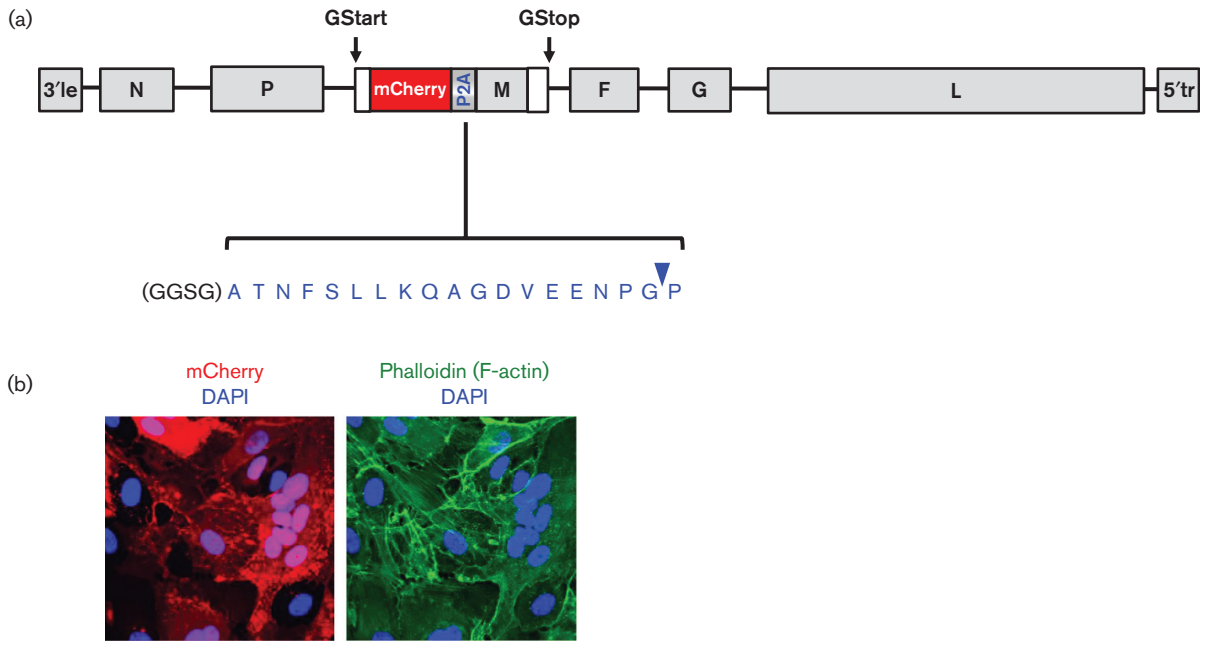


**Fig. 1.** Schematic of recombinant NiV genome with the mCherry reporter expressed in the same transcriptional unit as the matrix (M) gene (rNiV-mCh$^{M}$). (a) mCherry expression is driven by the gene start (GStart) and gene stop (GStop) signals that flank the M ORF. mCherry and M are expressed as individual proteins via the P2A ribosomal skipping sequence (blue letters). Arrowhead (blue) indicates the 'cleavage' site between the glycine (G) and proline (P) residues. The M protein thus retains an additional N-terminal proline residue. For rNiV-mCh$^{M}$-opt, a GSG linker was inserted preceding the P2A sequence, with an additional glycine to preserve the rule of six for paramyxovirus genomes. (b) Human umbilical vein endothelial cells (HUVECs) were infected with rNiV-mCh$^{M}$ and imaged by confocal microscopy. Left panel, mCherry can be seen in multinucleated syncytia caused by NiV-induced cell–cell fusion. Right panel, same field stained with phalloidin to detect F-actin for cell demarcation.

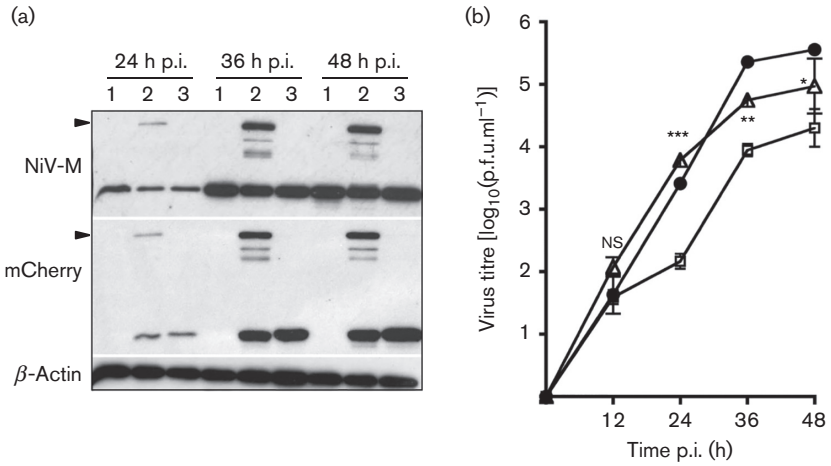


**Fig. 2.** rNiV-mCh$^M$ with an optimized P2A sequence has improved growth kinetics *in vitro*. (a) HUVECs were infected with WT NiV (1), rNiV-mCh$^M$ (2) or rNiV-mCh$^M$-opt (3) at m.o.i. 0.1, and cell lysates were collected at the time points indicated post-infection (p.i.). Western blot analysis with anti-NiV-M (Wang *et al.*, 2010) and anti-mCherry (Abcam, ab125096) shows that P2A ribosomal skipping in the mCherry-P2A-M cassette was inefficient for the original rNiV-mCh$^M$ virus, and that insertion of the GSG linker in rNiV-mCh$^M$-opt results in essentially complete ribosomal skipping. The arrowheads indicate the uncleaved fusion product. The approximate apparent molecular masses are 42 kDa for NiV-M, 33 kDa for mCherry and 75 kDa for the fusion product. (b) HUVECs were infected with WT NiV (●), rNiV-mCh$^M$ (□) or rNiV-mCh$^M$-opt (△) at m.o.i. 0.1, and supernatants were collected and replenished every 12 h, with subsequent titrating on Vero cells by plaque assay. Error bars represent SD of triplicate samples. rNiV-mCh$^M$-opt (P2A with the GSG linker) grew to significantly higher titres than the original rNiV-mCh$^M$ (P2A without the GSG linker). Two-way ANOVA followed by Bonferroni post-tests: NS, not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

per condition, intraperitoneal route) with $10^3$ or $10^4$ p.f.u. of WT NiV, rNiV-mCh$^M$ or rNiV-mCh$^M$-opt. As suggested by its attenuated growth *in vitro*, rNiV-mCh$^M$ was significantly attenuated *in vivo* relative to WT NiV at the higher dose ($P=0.037$, log-rank Mantel–Cox test) (Fig. 3a, right panel). At the lower dose, there was a clear difference in survival (although just beyond statistical significance, $P=0.067$ for rNiV-mCh$^M$ vs WT NiV), with only 4/10 animals infected with rNiV-mCh$^M$ succumbing by 25 days p.i. vs 7/10 for rNiV-mCh$^M$-opt and 8/10 for WT NiV (Fig. 3a, left panel). In contrast, rNiV-mCh$^M$-opt had no significant difference from WT NiV at either dose ($P>0.05$). *Ex vivo* mCherry imaging of tissues from hamsters inoculated with $10^4$ p.f.u. rNiV-mCh$^M$-opt confirmed virus replication and spread, with predominant replication in the lungs but also in the kidneys, spleen and liver, and the beginnings of detectable brain infection at the olfactory bulb by day 7 p.i. (Fig. 3b). Immunohistochemistry with anti-NiV-M on lung and brain tissues from WT NiV- vs rNiV-mCh$^M$-opt-inoculated hamsters showed similar detection of viral antigen in blood vessel endothelial cells, a hallmark of henipavirus pathogenesis (Fig. 3c).

In conclusion, we found that incorporation of a GSG linker preceding the P2A sequence resulted in essentially complete ribosomal skipping (Fig. 2a), which in turn significantly improved the replication kinetics as compared with the original rNiV-mCh$^M$ (Fig. 2b). As might be expected from this improvement, the optimized virus also had *in vivo* lethality more similar to WT, with no statistical difference between the two viruses at two different doses of infection (Fig. 3). Although the use of a GSG linker immediately preceding the 2A sequence to promote efficient skipping has been known for some time (Holst *et al.*, 2006; Szymczak *et al.*, 2004), many research groups in virology are not yet aware of this simple improvement. We hope this work draws further attention to the GSG linker and the importance of verifying efficient skipping whenever 2A sequences are used.

Consistent with our findings, another group has shown that a recombinant NiV with similar insertion of GFP upstream of the matrix gene via a P2A sequence had similar replication kinetics to WT NiV in Vero cells (Lo *et al.*, 2014). To maintain the rule of six (by which paramyxovirus genome lengths are exact multiples of six), Lo *et al.* (2014) had inserted a single glycine residue between GFP and the P2A, which may have had the advantage of serving as a linker to provide more efficient ribosomal skipping.

In previous work, we showed that insertion of a reporter gene in between the nucleoprotein (N) and phosphoprotein (P) genes as a separate ORF did not affect either the polar transcriptional gradient or the *in vitro* growth of the virus (Yun *et al.*, 2015). It appears that for NiV, the first two intergenic regions (N to P, and P to M) do not result in attenuation of transcript levels, while the intergenic regions following the matrix gene result in progressive

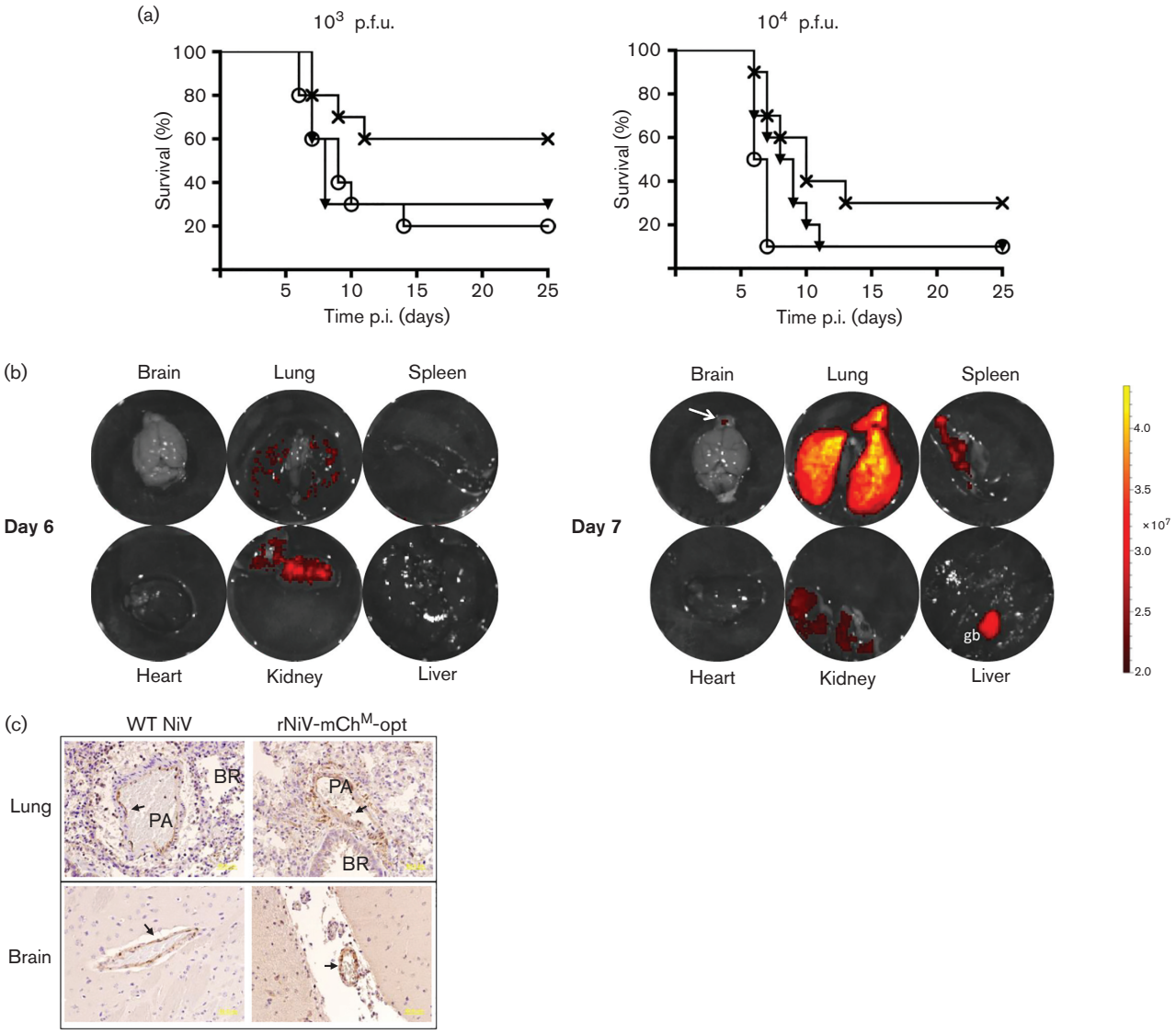


**Fig. 3.** Improved ribosomal skipping results in lethality more similar to WT NiV *in vivo*. (a) WT NiV and recombinant mCherry reporter viruses without (rNiV-mCh$^M$) or with (rNiV-mCh$^M$-opt) the GSG linker for optimal P2A skipping were used to inoculate groups of Syrian hamsters ($n$=10 per group for each virus and dose combination, intraperitoneal route) at $10^3$ p.f.u. or $10^4$ p.f.u. per animal. Kaplan–Meier survival curves show no significant differences between WT NiV (○) and rNiV-mCh$^M$-opt (▼), whereas rNiV-mCh$^M$ (×) exhibited significant attenuation relative to WT NiV that was most apparent at the higher inoculum used ($P$=0.037, log-rank Mantel–Cox test). (b) Two hamsters were inoculated with rNiV-mCh$^M$-opt ($10^4$ p.f.u., intraperitoneal route), and tissues were imaged *ex vivo* for mCherry fluorescence at 6 and 7 days p.i. using an IVIS Spectrum imaging platform. Data are displayed as radiant efficiency on a colour scale from $2\times10^7$ (dark red) to $4.35\times10^7$ (yellow). Gb, Gallbladder. White arrow indicates fluorescence in olfactory bulb. (c) Immunohistochemistry with anti-NiV-M was performed on lung and brain tissues from similarly infected hamsters. Arrows indicate examples of positive antigen staining. PA, Pulmonary artery; BR, bronchiole.

several-fold reductions in relative mRNA levels (Yun *et al.*, 2015). Insertion of a foreign ORF between two paramyxovirus genes is typically accomplished by duplication of the respective intergenic region. Therefore, one might expect that for NiV, insertion of a foreign ORF between N and P, or between P and M, would not result in disturbance of the transcriptional gradient due to the non-attenuating nature of these intergenic regions, consistent with our previous results (Yun *et al.*, 2015). However, intergenic regions may have regulatory functions beyond mRNA transcriptional regulation, and it may thus be preferable to simply insert the foreign ORF within an existing viral ORF. Further, as the complexity of our modifications to paramyxovirus genomes increases, even encompassing combinations of

reporters and exogenous factors within a single genome, making use of both insertion strategies may become increasingly valuable.

As an alternative to using a 2A ribosomal skipping sequence, an internal ribosome entry site (IRES)-like sequence that allows mRNA cap-independent initiation of translation and thus bicistronic expression has been used for Sendai virus (genus *Respirovirus* within the *Paramyxoviridae*) to incorporate a luciferase reporter within the N gene ORF (Touzelet *et al.*, 2009). This recombinant virus was not attenuated *in vitro*, although how it compares with the WT virus *in vivo* has not been evaluated. Further, the strategies differ in that expression of the IRES-dependent gene relative to the cap-dependent gene is highly variable, usually significantly lower than the cap-dependent gene (Mizuguchi *et al.*, 2000), whereas 2A skipping is co-translational and therefore results in similar expression of linked genes. Thus, for high reporter expression, or reporter expression more indicative of virus protein expression, it may be more advantageous to use a 2A-based strategy. As a final consideration, if expression of the reporter is in any way disadvantageous for virus fitness, mutation and loss of reporter expression would be more likely with an IRES-based strategy or insertion of the reporter as an independent ORF, whereas in the P2A insertion strategy, the expression of the downstream virus protein is dependent on continued translation of the upstream reporter. In our case, optimizing the efficient ribosomal skipping of our P2A-based reporter insertion strategy resulted in a recombinant Nipah virus that reflected WT lethality in the currently most widely used small animal model.

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