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Genome rearrangement of influenza virus for anti-viral drug screening

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ABSTRACT

Rearrangement of the influenza A genome such that NS2 is expressed downstream of PB1 permits the insertion of a foreign gene in the NS gene segment. In this report, the genome rearranged strategy was extended to A/California/04/2009 (pH1N1), and *Gaussia* luciferase (GLuc) or GFP was expressed downstream of the full-length NS1 gene (designated GLucCa04 and GFPCa04, respectively). In growth kinetics studies, culture of amantadine sensitive GLucCa04 (Sens/GlucCa04) in the presence of amantadine significantly decreased GLuc expression and viral titers for 48 h post-infection (hpi). When Sens/GlucCa04 was subsequently used in an *in vitro* anti-viral screening assay, amantadine treatment significantly decreased GLuc expression from amantadine sensitive compared to amantadine resistant GLucCa04 (Res/GlucCa04) as early as 16 hpi. In *in vivo* screening studies, DBA mice were treated daily with amantadine from 1 day prior to infection and inoculated with either Sens/GlucCa04 or Res/GlucCa04 alone or as a co-infection with the parental strain. On days 3 and 5 post-infection, lung samples were collected and amantadine treatment was shown to decrease GLuc expression by two orders of magnitude ($p < 0.05$) in Sens/GlucCa04 infected mice. Furthermore, while both Sens and Res/GlucCa04 were highly attenuated, addition of the parental strain to the inoculum yielded clinical disease indicative of GLuc expression and pulmonary viral titers. These findings indicate that the use of GLucCa04 can potentially accelerate *in vitro* and *in vivo* anti-viral screening by shortening the time required for virus detection.

1. Introduction

Influenza A viruses cause annual epidemics and occasional pandemics (Jhung et al., 2011; Molinari et al., 2007; Morens et al., 2010). To control influenza, vaccines are administered prior to seasonal outbreaks, and anti-viral drugs are administered after the presentation of clinical signs. Influenza A belongs to the family *Orthomyxoviridae* and, as such, has an 8-segmented negative polarity single stranded RNA genome (Palese and Shaw, 2007). Using a genome rearrangement strategy, we have previously shown that an H9N2 vaccine strain could be modified to express a second HA protein (H5 HA) from Segment 8 (NS gene segment). When this virus was administered as a vaccine, both mice and ferrets were protected against lethal highly pathogenic H5N1 challenge (Pena et al., 2013). According to this genome rearrangement strategy, NS1 was truncated to NS1(1–99) (Talon et al., 2000) and NS2 was deleted from Segment 8. The foot-and-mouth-disease virus (FMDV) 2A protease was cloned downstream of NS1(1–99) and followed by the transgene of interest (*i.e.* H5 HA, GFP, or luciferase). To re-introduce NS2, Segment 2 was modified such that FMDV 2A protease was cloned downstream of PB1 followed by the NS2 open-reading frame (ORF). In both Segment 2 and 8, the segment specific packaging signals were reconstituted at the end of the inserted gene sequence (Fujii et al., 2005; Liang et al., 2005). Herein, the genome rearrangement was applied to an additional virus strain, A/California/04/2009 (pandemic H1N1), and *Gaussia* luciferase (GLuc) expressing variants maintaining the full-length NS1 gene were evaluated for anti-viral screening.

To date, only two groups of compounds have been licensed for treatment of influenza: the adamantanes (amantadine and rimantidine) and the neuraminidase (NA) inhibitors (oseltamivir and zanamivir). Unfortunately, most circulating strains of influenza are resistant to adamantanes, and oseltamivir-resistant virus strains continue to be isolated (Hurt et al., 2011; Lackenby et al., 2011; Ujike et al., 2011). Consequently, there is a need for rapid development of novel anti-viral compounds. For antiviral screening, there are two conventional assay systems: (1) cytopathic effect (CPE) assay or plaque reduction assay, and (2) NA inhibitor assays (Buxton

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et al., 2000; Hayden et al., 1980; Kao et al., 2010; Potier et al., 1979; Severson et al., 2008; Su et al., 2010). Each assay has specific drawbacks. For example, the CPE assay requires culturing the virus in the presence of a compound for 3–5 days, and NA assays are specific only to compounds that target the viral NA.

More recently, several high throughput-screening (HTS) assays have been developed. These assays are cell-based and include the generation of stable cell lines (MDCK, Hela, or 293T) expressing influenza driven *Renilla* luciferase (RLuc) or *Firefly* luciferase (FLuc) reporter constructs (Hossain et al., 2010; Martinez-Gil et al., 2012; Zhang et al., 2011), and a 293T cell line expressing the viral ribonucleoprotein genes (Ozawa et al., 2013). Importantly, these assays take advantage of luciferase expression that can be quickly assayed from cell lysates. With the introduction of secreted *Gaussia* luciferase (GLuc), luciferase activity can be assayed directly from cell culture supernatant (Tannous et al., 2005), and with a 1000 fold increase in sensitivity of GLuc compared to RLuc or FLuc (Zhu et al., 2011), there is the potential to improve upon existing assays. Furthermore, as a result of the cell-based nature of existing assays, expression of the reporter gene is an indirect measure. In contrast, the use of a virus carrying the reporter gene has the potential to give a direct representation of viral replication and anti-viral efficacy (Heaton et al., 2013; Manicassamy et al., 2010; Pena et al., 2013).

Thus, in this study, the genome rearrangement strategy was applied to A/California/04/2009 while maintaining full-length NS1. In initial studies, we characterized viral growth and luciferase expression from the rearranged viruses, and evaluated growth kinetics in the presence of amantadine and oseltamivir. As a means to develop an alternative anti-viral screening assay, amantadine sensitive and resistant GLuc expressing variants were further evaluated in the presence of amantadine. After pre-incubation and culture of virus with amantadine in MDCK cells, GLuc expression was indicative of drug efficacy from as early as 16 hpi. Subsequently, the stability of GLuc expression was evaluated over serial passage, and the use of GLucCa04 in MN assays was also explored. Lastly, *in vivo*, we demonstrate that when GLucCa04 is given alone or as a co-infection with its parental strain, GLuc expression can be used as an indicator of amantadine sensitivity and anti-viral efficacy.

2. Materials and methods

2.1. Cells and viruses

MDCK cells were kindly provided by Dr. R. Webster (St. Jude Children's Research Hospital) and maintained in 10% fetal bovine serum (Cellgro, Corning) supplemented DMEM (Sigma). The parental virus for these studies was recombinant mouse-adapted A/California/04/2009 (H1N1) for which the 8-plasmid reverse genetics (RG) system has been described (Ye et al., 2010). Mouse-adapted A/California/04/2009 (H1N1), like the original human isolate, is amantadine resistant and for clarity is referred to as Res/Ca04.

To generate an amantadine sensitive strain, site-direct mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) and primers to change M2 N31S in Segment 7. Successful mutagenesis was confirmed by sequencing and the virus was rescued in a co-culture of 293T:MDCK (Hoffmann et al., 2000). All viruses were stored at −80 °C until use. Amantadine sensitive reverse genetics Ca04 was designated Sens/Ca04.

2.2. Cloning and construction of rearranged viruses

To generate GLuc or GFP expressing Ca04 strains, both Segments 2 and 8 were modified as previously described (Pena et al., 2013),

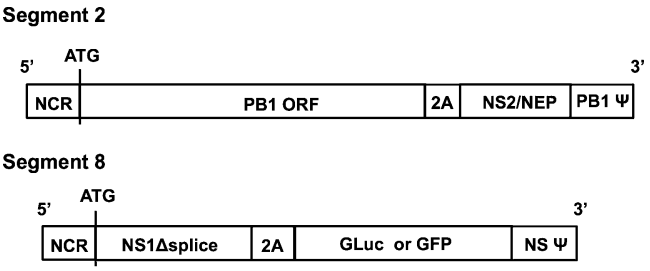

Fig. 1. *Schematic representation of Segment 2 and 8 constructs for the rearranged virus strategy.* NCR denotes non-coding region, ORF denotes open-reading frame, and ψ denotes packaging signal (consisting of both the UTR and a region of the ORF). GLuc and GFP denote *Gaussia* luciferase and green-fluorescent protein, respectively. NS1Δsplice constitutes NS1 with the donor and acceptor splicing sites knocked-out, a stop-codon inserted early in the NS2 ORF, and a truncation to encompass only NS1. Segments are drawn in the positive sense orientation and are not drawn to scale.

with the exception that full-length NS1 was maintained (Fig. 1). To prevent aberrant splicing and prevent NS2 expression, the acceptor and donor splicing sites in NS1 were mutated by site-direct mutagenesis and a stop-codon was inserted out-of frame with NS1 but early in the NS2. Subsequently, NS2 was deleted using inverse PCR, and the resulting NS1 gene was designated NS1Δsplice. Using overlapping PCR, a fragment was generated containing BsmBI sites flanking NS1Δsplice followed by the FMDV 2A protease, the *Gaussia* luciferase (New England Biolabs) or GFP ORF, and the NS gene packaging signal (Fujii et al., 2005). This PCR fragment was subsequently digested with BsmBI and cloned into pDP-2002. To modify Segment 2, a construct containing a C-terminal portion of the PB1 gene followed by the FMDV 2A protease, the NS2 ORF and the PB1 packaging signal (Liang et al., 2005) was produced. This fragment was subsequently sub-cloned into the PB1 gene segment using suitable restriction sites. Upon completion of cloning, both plasmids were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500XL Sequencer (Applied Biosystems).

Amantadine resistant (*i.e.* wild-type) GFP variant (GFPCa04), and both amantadine sensitive (Sens/GlucCa04) and resistant (Res/GlucCa04) strains of the GLuc expressing viruses were rescued by incorporating the appropriate M gene plasmid.

2.3. Viral growth kinetics

Growth kinetics experiments were performed in MDCK cells in 6-well plates using an MOI of 0.01 as described previously (Pena et al., 2013). At 0, 6, 12, 18, 24, 48, and 72 h, 250 μL of supernatant was collected from each well and replenished with OPTI-MEM (Invitrogen) containing 1 μg/mL of TPCK-trypsin (Worthington). From the supernatant, 70 μL was frozen at −20 °C for later analysis of luciferase expression and 180 μL was frozen at −80 °C for viral titration by $TCID_{50}$. GLuc expression was quantified using a BioLux® *Gaussia* Luciferase Assay Kit (New England Biolabs). Briefly, the luciferase substrate was prepared immediately prior to use by performing a 1/1000 dilution of the substrate into the sample buffer. Next, 50 μL of diluted luciferase substrate in sample buffer was added to 20 μL of cell culture supernatant in a white flat bottom 96 well plate (Greiner). Immediately after addition of the substrate, the luminescence was measured over a 1 s integration time on a Victor X4 Luminescence Plate Reader (Perkin Elmer).

Virus growth kinetics/luciferase expression experiments were performed in the presence of amantadine hydrochloride (Sigma) or oseltamivir carboxylate (Toronto Research Chemicals). The Sens/GLucCa04 virus (MOI 0.01) was pre-incubated for 30 min with amantadine hydrochloride or oseltamivir carboxylate at concentrations of 0, 0.1, 1, or 10 μg/mL. After incubation, the virus-drug mixture was overlaid on MDCK cells in 6-well plates and incubated

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