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Comparison of vRNA and cRNA based reporters for detection of influenza replication

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ABSTRACT

In this study, RNA polymerase I expressed replicons containing EGFP and luciferase reporter genes controlled by influenza vRNA or cRNA promoters were compared side-by-side in the ability to detect influenza RNA-dependent RNA polymerase activity as an indicator of influenza replication. Results showed the vRNA based Luc reporter was more sensitive to early detection of influenza virus at 6 h post infection ($p < 0.05$), and at 10-fold lower titer (MOI = 0.001). Lower sensitivity of cRNA based Luc reporter constructs was due to its background expression, 2-fold lower expression, and around 4 h delay in expression of luciferase. Despite these differences, both cRNA- and vRNA-based reporters demonstrated strong correlation between MOI and luciferase signal, and can be used for effective and early detection of influenza infection *in vitro*. Further, we demonstrated that these reporters can be used successfully to study the kinetics of antiviral drugs including siRNA. Our results also suggest that progeny vRNAs might participate not only in secondary transcription but also in secondary replication. The developed cRNA and vRNA reporters may help with further elucidation of the replication model of influenza A virus.

1. Introduction

Influenza A, a member of the *Orthmyxovirdae* family, is a negative strand RNA (−) virus with an eight segmented single-stranded genome that encodes 11 or 12 viral proteins (Lamb and Krug, 2001; Lamb and Choppin, 1983; McCauley and Mahy, 1983). Most human disease is caused by type A influenza, which circulates naturally in many animal hosts, such as birds, pigs, horses and dogs. The seasonal H1N1 and H3N2 viruses are responsible for the annual influenza A outbreaks which cause 250,000–500,000 deaths around the world (Anonymous, 2003; Thompson et al., 2010). Rarely, due to unpredictable and sudden antigenic shifts influenza also causes dangerous global outbreaks; pandemics. The recently emerged 2005 H5N1 highly pathogenic avian influenza and 2009 H1N1 swine origin influenza viruses raised serious concerns about the potential for a deadly pandemic similar to the 1918 Spanish virus, which worldwide caused 20–40 million deaths (Richman, 2004). Furthermore, recent research on H5N1 has demonstrated the ease with which deadly strains can be produced in the laboratory (Herfst et al., 2012).

During outbreaks a multitude of tools and animal models must to be rapidly deployed to detect influenza infection and characterize its genomic sequence, antigenic characteristics, antiviral susceptibility, and pathogenic potential. Detection of influenza virus commonly requires first amplification of the whole virus by replication in chickens eggs or cell cultures, or amplification of specific genomic regions by nucleic acid amplification technologies. Subsequently, influenza protein (hemagglutination, antibody based detection) levels, viral nucleic acid (RT-PCR, Loop-mediated isothermal amplification (LAMP), next generation sequencing) levels or infectious particles (plaque assay, 50% Embryo Infectious Dose (EID_{50}) or 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay) are detected (Wang and Taubenberger, 2010). Cell culture in canine MDCK cells, incubation in chicken eggs and terminal animal challenges are still the gold standard for diagnosis of influenza infection and pathogenic characterization of novel strains, but results from these tests can take weeks (Allwinn et al., 2002). Thus there is a great need for fast high-throughput real-time methods of diagnosis and characterization of influenza infection and for development of novel antiviral drugs.

Early studies have found that foreign genes flanked by influenza 5′ and 3′ RNA promoters could be expressed under the control of influenza RNA-dependent RNA polymerase (RdRp) (Fig. 1) (Flick

Abbreviations: cRNA, complementary RNA; hpi, hours post infection; Pol, polymerase; RdRp, RNA-dependent RNA polymerase; RNAi, RNA interference; siRNA, short interfering RNA; vRNA, virion genomic RNA.

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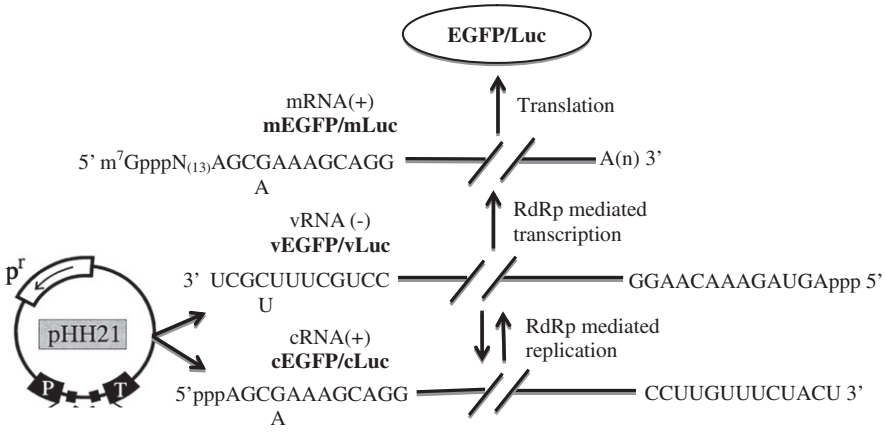

Fig. 1. Schematic expression of foreign proteins by using influenza vRNA or cRNA promoters. Three RNA forms (vRNA, cRNA, mRNA) produced from the reporter gene during influenza infection. EGFP, enhanced green fluorescent protein; Luc, luciferase; RdRp, influenza RNA-dependent RNA polymerase.

et al., 1996; Kim et al., 1997; Neumann and Hobom, 1995). Currently, two reporter approaches based on the influenza promoter have been tested; (i) a reporter virus is produced through reverse genetics by replacing non-essential influenza genes, such as fusing or replacing NS1 or neuraminidase proteins with EGFP (Kittel et al., 2004; Manicassamy et al., 2010; Shinya et al., 2004). However, this approach does not allow detection of influenza infection in clinical samples, characterization of novel strains requires construction of new reporter viruses, and selection pressures during passages *in vitro*/*in vivo* of the recombinant viruses can lead to loss of the reporter protein (Manicassamy et al., 2010). Additionally, genes that might be non-essential *in vitro* are likely to be important *in vivo*, and introduction of reporter protein often seriously attenuated the viral pathogenicity *in vivo* (Kittel et al., 2004; Shinya et al., 2004). (ii) Reporter RNA with the conserved influenza vRNA promoter is produced by cellular DNA-dependent RNA polymerase I (Pol I) and is transcribed by viral RdRp and NP proteins provided by super infection with helper influenza virus in suitable cell lines (Lutz et al., 2005; Machado, 2003). Using influenza promoters, which are the highly conserved sequences found in all influenza strains, allows sensitive and broad based detection of even novel influenza strains. Furthermore the virus itself is not modified allowing the replication and pathogenicity of novel strains to be studied under more natural conditions, and real-time detection is also possible. This approach is also suitable for high-throughput screening of multiple clinical samples, and can be applied to the identification of novel anti-influenza drugs.

The objectives of the present study were to compare influenza reporters using vRNA or cRNA promoters. We hypothesized that there might be a higher level of expression of the reporter mRNA using the cRNA promoter. Each cRNA can produce 10–100 molecules of vRNAs which in turn can participate in transcription (vRNA > mRNA) (Kawakami et al., 2011), and it was hypothesized that the higher level of reporter mRNAs might be achieved for cRNA reporter. Two constructs were compared in detection of influenza A virus infection in cell culture by time-course and dose–response study. We also evaluated two reporter proteins, enhanced EGFP (EGFP) and luciferase. Further, we demonstrated that these constructs could be successfully applied to study kinetics of antiviral siRNA.

2. Materials and methods

2.1. Cells and transfection

Human embryonic kidney 293T (HEK293) cells were obtained from the American Type Culture Center (ATCC; Manassas, VA). Cells were grown in Dulbecco's modified Eagles' medium (DMEM; Corning, Tewksbury, MA) supplemented with 10% heat-inactive fetal bovine serum (Thermo Scientific; Logan, UT) and 1% Penicillin (10,000 units/ml)-Streptomycin (10,000 μg/ml)-Fungizone (25 μg) mixture (Walkersville, MD). Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used for reverse transfection. It was diluted and mixed with plasmid for transfection following manufacture's protocol. Lipofectamine™ 2000 (0.25 μl) and 100 ng plasmids were both diluted in serum-free DMEM and after 5 min, mixed and allowed to react for 15 min. 100 μl HEK293 cells with 4×10^5/ml were plated directly on transfection reaction mixture in a 96 well plate by using the reverse transfection method. siRNA kinetics was studied by reverse cotransfection of 100 ng of vLuc reporter plasmid with 5 pmol/10 pmol (final concentration of 33 nM/66 nM) of siRNA in 4×10^5 HEK293 cells in a 96 well plate. Transfection efficiency was evaluated by transfecting pCAG-EGFP plasmid (Addgene), achieving ~80% transfection efficiency.

2.2. Virus and infection

Infections were conducted with influenza A/Puerto Rico/8/1934 (PR8) H1N1 strain. Virus stock was prepared by propagation in the chorioallantoic cavity of 11-day-old embryonated chicken eggs and incubated at 37 °C for 48–72 h. The allantoic fluids were harvested and stored at −80°C. Viruses were titrated by hemagglutination assay and 50% tissue culture infectious dose ($TCID_{50}$) assay. Infection in the 96 well plate/24 well plate was carried out as follows: A/Puerto Rico/8/34 (H1N1) was diluted in the virus growth medium with 2 μg/ml TPCK-trypsin (Sigma–Aldrich, St. Louis, MO) (Szretter et al., 2006). Subsequently, cell growth medium was removed and replaced with 100 μl/200 μl of medium diluted PR8 strain ~18 h after transfection in a 96 well plate/24 well plate.

2.2.1. Hemagglutination (HA) assay and 50% tissue culture infectious doses ($TCID_{50}$)

The chorioallantoic fluids from eggs were harvested and titrated by the hemagglutination (HA) assay using 0.5% chicken red blood cells in PBS (Killian, 2008). Briefly, 50 μl allantoic fluids were serially diluted 2-fold in PBS in a U bottom 96-well plates followed by adding 50 μl of a 0.5% suspension of chicken erythrocytes prepared in PBS on top of each well. The plates were incubated for 30 min at room temperature and plates were observed. Wells were scored for hemagglutination positive/negative visually by whether those erythrocytes were settled and form a solid button.

The infectious titer of virus stock was determined by 50% tissue culture infectious dose assay (TCID50 assay) (Szretter et al., 2006). $TCID_{50}$/ml was calculated by the Reed and Muench method and

was converted into PFU (plaque forming units) (O'Reilly et al., 1992; Reed and Muench, 1938).

2.3. Reporter constructs

The EGFP reporter gene was amplified from pCAG-GFP plasmid (Addgene), while the firefly luciferase (*luc2*) reporter was amplified from the pmirGLO Dual luciferase miRNA target expression vector (Promega). The reporter genes were flanked by portions of the 5′ and 3′ UTR from the A/WSN/33 NP. vRNA or cRNA segment using tagged primers (Suppl. Table 1) to produce vEGFP, vLuc, and cEGFP, cLuc transcript respectively (Lutz et al., 2005). Genes were amplified by PCR using Phusion High-Fidelity PCR Master Mix (Thermo Scientific). The PCR amplicon was purified from agarose gel using QIAquick Gel extraction kit (Qiagen) and cloned into pHH21 plasmid kindly provided by Dr. Yoshihiro Kawaoka (Madison, WI) to produce pHH21-vEGFP, pHH21-cEGFP, pHH21-vLuc and pHH21-cLuc (Lutz et al., 2005). The finished constructs were confirmed by sequencing.

2.4. siRNA

Synthetic anti-influenza siRNA NP-1496 targeting NP mRNA of influenza A virus was designed as previously reported (Ge et al., 2003) and anti-GFP siRNA was used as a negative control. Both were ordered as annealed siRNA from Integrated DNA Technologies (Coralville, IA).

2.5. Fluorescence detection and luciferase assay

A Nikon Eclipse TS100 fluorescence microscopy was utilized for observation of EGFP expression, and a SPOT Insight 2Mpixel Monochrome FireWire Digital Camera (SPOT Image, Sterling Heights, MI) was used for fluorescence imaging. EGFP fluorescence density was obtained using a Synergy TM 2 multi-mode microplate reader (Biotek, Winooski, VT). For luciferase assay, cells were grown on a white 96 well plate. Before reading, supernatant was removed and cells were lysed using the Glolysis buffer (Promega, Madison, WI) following manufacture's instruction. Cell lysates were analyzed using the Steady-Glo® Luciferase Assay System (Promega, Madison, WI) and signals were read in a Synergy TM 2 multi-mode microplate reader (Biotek, Winooski, VT) following manufacturer's instruction.

2.6. Strand-specific real time PCR quantification of influenza A virus RNAs

HEK293 cells were reverse transfected with vLuc/cLuc constructs and seeded in a 24 well plate. 20hr post transfection, HEK293 cells were infected with MOI = 0.1 A/Puerto Rico/8/1934 influenza. Cells were collected for RNA extraction at 4, 12, 24, and 48 hpi post infection using RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer protocol. The total RNA concentration was quantified by Nanodrop 1000 spectrophotometer (Thermo Scientific, Barrington, IL). Equal amounts of RNA were used for cDNA synthesis with High Capacity cDNA Reverse Transcription Kit (Applied Bioscience, Carlsbad, CA). Strand-specific primer were modified from Ge et.al. (Ge et al., 2003) to produce vLuc/cLuc primers, and oligo-dT primers were used for mRNA reverse transcription (Suppl. Table 1). After three strands were reverse transcribed, 1 μl of each cDNA product were mixed with luciferase real time PCR detection primers (Suppl. Table 1) and iQ™ SYBR® Green Supermix (Biorad, Hercules, CA). Human GAPDH gene was selected as the reference gene and Real Time PCR detection was conducted in the MyiQ2 two-color Real-time detection system (Biorad, Philadelphia, PA). The 2-step PCR thermo cycling conditions were as follows: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 55 °C for 30 s. The specificity of amplification during real-time PCR was confirmed by checking the melting curve. All PCR reactions were run in duplicates. The Ct values that varied by >0.5 unit (cycle) between duplicates were discarded and resultant Ct values were averaged. The amount of each strand (vRNA/cRNA/mRNA) in each sample was normalized to the GAPDH gene using the delta Ct method (Schmittgen and Livak, 2008).

2.7. Statistical analysis

Data was analyzed as a completely randomized block design using JMP statistical software (SAS Institute). The Student *t*-test was used to compare means of two groups. Tukey's test was used to perform multiple comparisons among several group means. Pearson's correlation analysis was used to estimate correlation between luciferase expression/RNAs/cRNA/mRNA. All experiments were repeated in triplicate or quadruplicates. The level of significance chosen to determine whether the data points obtained from several different samples were significantly different was $p < 0.05$.

3. Results

3.1. Detection of influenza A virus replication in HEK293 cells by using influenza-induced EGFP expression

HEK293 cells were transfected with the reporter constructs pHH21-vEGFP orpHH21-cEGFP and later infected with A/PR/8/34 (H1N1) strain. Infection with influenza resulted in significant accumulation of EGFP protein inside of cells, reflecting the influenza RdRp mediated primary and secondary transcription (vEGFP > mEGFP) for vEGFP reporter and replication/transcription (cEGFP > vEGFP > mEGFP) for cEGFP reporter (Fig. 2). Influenza induced EGFP expression became detectable at around 8 hpi. At later time points more cells exhibited the EGFP signals as influenza replicated in the cells throughout the plate. Unexpectedly, the negative control transfected with pHH21-cEGFP but not infected with influenza showed sporadic EGFP expression indicating that low level translation of Pol I expressed cRNA have occurred. In contrast, the vEGFP reporter did not produce any visible EGFP expression in the absence of influenza. No significant differences were noticed between vEGFP and cEGFP based constructs at later time points.

3.2. Time course study of influenza A virus replication in HEK293 cells using influenza-induced luciferase expression

The EGFP reporter was replaced with firefly luciferase gene (*luc2*) codon optimized for more efficient expression in mammalian cells. New vLuc and cLuc reporters were tested using the same transfection/infection protocol in the time course study (Fig. 3). The vLuc uninfected control remained at the same level as background signal from non-transfected cells. The cLuc uninfected control produced significantly higher signal ($p < 0.05$) than the background, indicating low level translation of Pol I expressed cLuc cRNAs. This data corresponds to the previous observations with cEGFP uninfected control.

The luciferase signal from vLuc or cLuc reporter transfected cells showed a similar overall trend during time course, with both constructs demonstrating significant increase in luciferase signal compared to the uninfected control. For vLuc, a significant difference with uninfected control was detected as early as 6 hpi ($p < 0.05$), while for cLuc only after 17 hpi the luciferase signal became significantly different from the uninfected control ($p < 0.05$). Signal for both constructs increased exponentially, peaked at 25 hpi and decreased gradually afterwards. At the peak, the signal was 6767 fold higher than uninfected control for vLuc ($p < 0.05$) and 736 fold higher for cLuc ($p < 0.05$). The vRNA promoter-driven luciferase

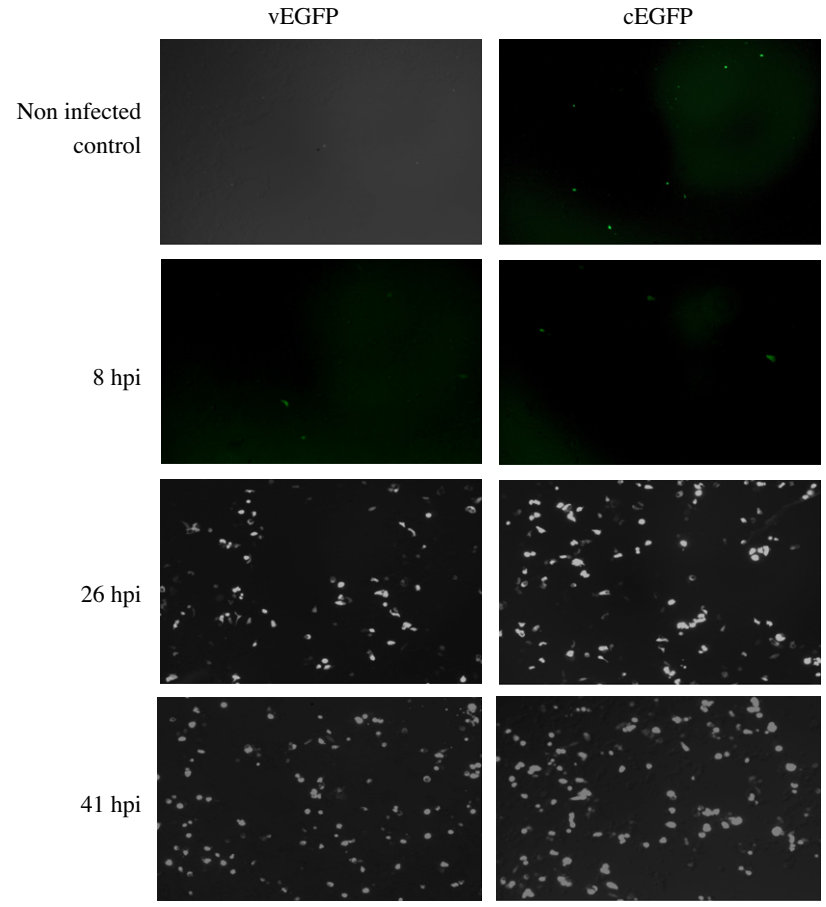

Fig. 2. Time course study of influenza induced EGFP expression in HEK293 cells. HEK293 cells were reverse transfected with vEGFP or cEGFP constructs in 96 well plate and infected with A/Puerto Rico/8/1934 (MOI = 1) 18 h post transfection. Experiment was repeated twice. Non-infected control, negative control transfected with construct but not infected with influenza.

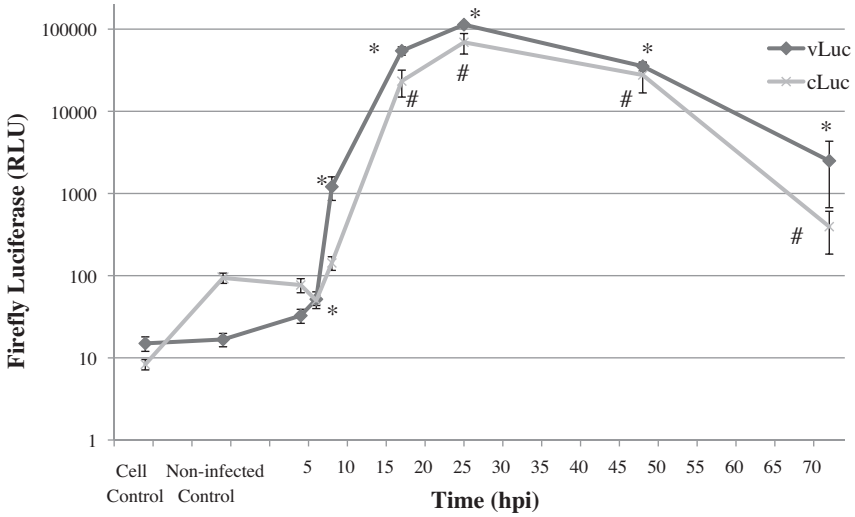

Fig. 3. Time course study of influenza induced luciferase expression in HEK293 cells. HEK293 cells were reverse transfected with vLuc or cLuc constructs in 96 well plate and at 18 h post transfection, infected with A/Puerto Rico/8/1934 at MOI = 1. Cell control, no treatment negative cell control; Uninfected control, negative cell control transfected with construct but not infected. The error bars represent standard error ($N = 3$ biological replicates). Student t test was used for statistical analysis. *Indicates significant differences between vLuc reporter and uninfected control ($p < 0.05$); #indicates significant differences between cLuc and uninfected control ($p < 0.05$).

expression started to increase at least 4hr earlier resulting in 8-fold higher vLuc signal at 8 hpi ($p < 0.05$). However, cLuc signal increased faster and the differences between two was only 1.6-fold at 25 hpi ($p > 0.05$).

3.3. Time course study of stability of reporter proteins

The luciferase signal was measured only in lysed cells in the time course study. To determine if luciferase proteins are lost in the cell culture supernatant during influenza induced cell apoptosis, the luciferase coming from both lysed cells and supernatant has been separately quantified (Suppl. Fig. 1A). Data indicated that only around 1% of luciferase was present in the supernatant even at later time points. Luciferase signal dropped gradually after 24 hpi around 4-fold at 48 hpi and 7-fold at 72 hpi for both lysed cells and the supernatant. It is suggested that the decrease in luciferase signal was likely due to the decreased expression and the increased degradation of luciferase at the later stages of influenza infection.

In contrast, for the EGFP reporter there seems to be a significant release of EGFP into the supernatant at all time points (Suppl. Fig. 1B). About 24% of total EGFP signal was found in the supernatant at 24 and 48 hpi, while 47% of the total EGFP signal was located in the supernatant at 72 hpi; indicating significant release of EGFP from cells into supernatant. Furthermore EGFP also accumulated slower than luciferase and achieved maximum signal in cells at 48 hpi compared to 25 hpi for luciferase. It is also more stable as no changes in total fluorescence intensity (cells plus supernatant) have occurred from 48 to 72 hpi.

3.4. Dose response study for vLuc and cLuc constructs

To compare the sensitivity of vRNA and cRNA constructs in detecting influenza A virus replication, HEK293 cells were transfected with vLuc or cLuc constructs and infected with A/PR/8/34 strain at different MOIs (MOI = 0.1, 0.01, 0.001). Luciferase signal was evaluated at different time points (12, 24, 48 and 72 hpi) and correlation between influenza MOI and signal was determined. Both constructs demonstrated strong correlation between MOI and the logarithm of luciferase signal with R^2 for vLuc at 0.91 ($p < 0.05$) and 0.88 for cLuc at 24 h ($p < 0.05$) (Fig. 4) and 48 hpi (not shown). The detection limit at 24 h for vLuc was ~MOI = 0.001 ($p < 0.05$) and ~MOI = 0.01 for cLuc reporter ($p < 0.05$). It appears that the upper detection limit was not reached in this experiment as no saturation plateau was detected even at high MOIs. Due to the lower sensitivity at early phase of infection and the degradation of luciferase at late phase of infection, luciferase signal and MOIs did not correlate well at 12 and 72 hpi (not shown).

3.5. Time course analysis of level of vRNA, cRNA and mRNA for vLuc and cLuc reporters using real time RT-PCR

To study the level of three RNA forms(vRNA, cRNA, mRNA) produced by two luciferase reporters real time RT-PCR using strand specific RT primers was used to quantify different RNA species. For vLuc reporter the level of vRNA is determined by both the Pol I expression and by vRNA replication (Pol I expressed vRNA > cRNA > progeny vRNA). In contrast for cLuc reporter only progeny vRNA produced by replication of Pol I expressed cRNA should be present (Pol I expressed cRNA > progeny vRNA). For both the vLuc and cLuc reporter, high level of vRNA was detected (Fig. 5A). The vLuc reporter had a 2.3-fold higher level of vRNA at 4 hpi and there was significant 6-fold increase in the level of vRNA at 24 hpi for both reporters ($p < 0.05$). Overall distribution of vRNA level across time points was similar for both constructs.

Overall distribution was very different for cRNA produced by vLuc and cLuc reporters. For vLuc reporter the level of cRNA is determined by vRNA replication (Pol I expressed vRNA > cRNA). For cLuc reporter only Pol I expressed cRNA should be present as progeny vRNA cannot participate in replication (Pol I expressed cRNA > progeny vRNA). The cRNA level at 4 hpi was 115-fold higher with the cLuc reporter than vLuc reporter ($p < 0.05$) (Fig. 5B). At 24 hpi cLuc reporter showed only 1.7-fold increase in the level of cRNA compare to 4 hpi, while for vLuc reporter there was 16-fold increase indicating vRNA replication (vRNA > cRNA). There was overall 12-fold higher level of luciferase cRNA for cLuc reporter. At 48 hpi, the cRNA level dropped 5-fold ($p < 0.05$) for cLuc reporter while no significant changes were found for vLuc reporter.

For vLuc reporter the level of mRNA is determined by both the primary transcription of Pol I expressed vRNA and by secondary transcription of replicated progeny vRNA (Pol I expressed vRNA > - primary mRNA; progeny vRNA > secondary mRNA). For cLuc reporter only progeny vRNA can participate in secondary transcription (Pol I expressed cRNA > progeny vRNA > secondary mRNA).There was 4-fold increase in the level of mRNA at 24 hpi for cLuc reporter and 100 fold for vLuc reporter ($p < 0.05$) (Fig. 5C). Overall 2-fold

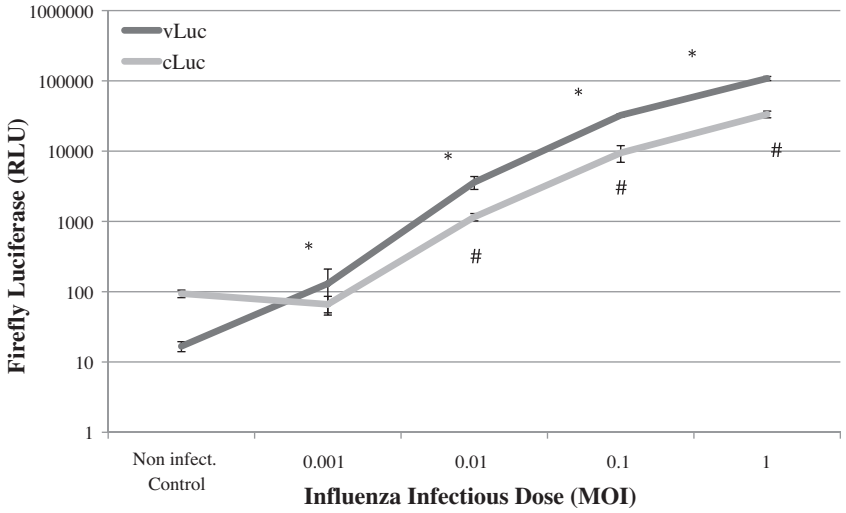

Fig. 4. Dose response study of influenza induced lucferase expression in HEK293 cells. HEK293 cells were reverse transfected with vLuc or cLuc constructs in 96 well plate and infected 18 h postransfection with A/Puerto Rico/8/1934 at MOI = 0.001, 0.01, 0.1, 1. Cell control, no treatment negative cell control; Non-infected control, negative cell control transfected with construct but not infected with influenza. The error bars represent standard error ($N = 3$ biological replicates). R-square of linear fit for vLuc is 0.91 and 0.88 for cLuc ($p < 0.05$). *Indicates significant differences between vLuc reporter and uninfected control ($p < 0.05$); #indicates significant differences between cLuc and uninfected control ($p < 0.05$).

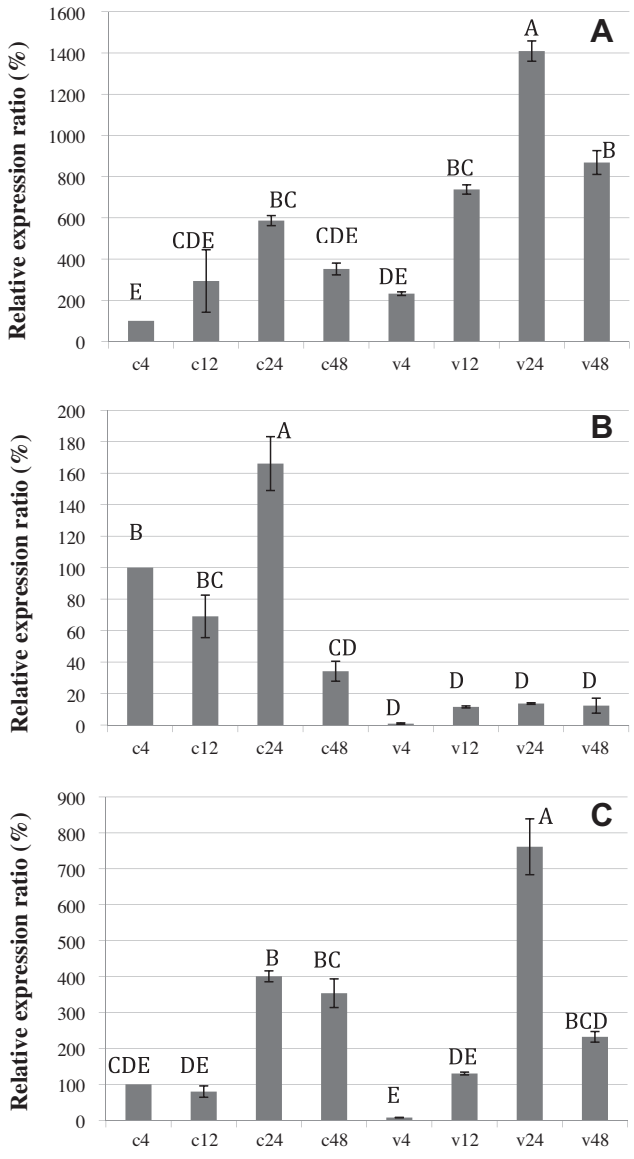

Fig. 5. Time course analysis of level of vRNA (A), cRNA (B) and mRNA (C) for vLuc and cLuc constructs using real time RT-PCR. HEK293 cells were reverse transfected with constructs in 24 well plates and infected 18 h postransfection with A/Puerto Rico/8/1934 (MOI = 0.1).At selected time points (4, 12, 24, 48 hpi) the cells were collected, the total RNA was extracted and real time RT-PCR was used to quantify luciferase RNAs (vRNA, cRNA, mRNA) using strand specific RT primers. The RNA signal for cLuc cRNA 4 hpi was selected as 100%. The error bars represent standard error ($N = 4$ replicates). c4–c48, cLuc reporter at 4–48 hpi; v4–v48, vLuc reporter at 4–48 hpi. Tukey's test was used to compare level of RNA between different time points for both constructs. Samples with different letters (A, B, C, D, and E) showed significant difference between corresponding RNA levels ($p < 0.05$).

higher level of luciferase mRNA was found for vLuc reporter, which correlates well with detected differences in luciferase signal at 24 hpi. The vLuc reporter showed strong significant correlation between levels of vRNA, mRNA and luciferase (Suppl. Table 2). The cLuc reporter showed lower but still significant correlation between levels of cRNA, mRNA and luciferase.

3.6. Kinetic study of anti-influenza NP-1496 siRNA using vLuc reporter construct

The vLuc reporter was tested as a tool to evaluate *in vitro* silencing kinetics of the previously developed anti-influenza NP-1496 siRNA targeting the NP segment (Ge et al., 2003). The vLuc reporter demonstrated that NP-1496 siRNA significantly decreased the influenza RdRp activity by around 335 fold ($p < 0.05$) for 5 pmoles and 862 fold ($p < 0.05$) for 10 pmoles of siRNA by 24 hpi compared to anti-GFP negative control (Fig. 6). There were no significant differences between 5 pmoles and 10 pmoles siRNA treatments ($p > 0.05$).

4. Discussion

Previous studies have demonstrated sensitive detection of different influenza strains using vRNA based reporter (Li et al., 2009; Lutz et al., 2005). In this study, for the first time we compared the detection of influenza replication using constructs with influenza vRNA or cRNA promoters controlling two popular reporter genes (EGFP and luciferase).

4.1. Comparison of vRNA and cRNA based reporters

The vRNA promoter is responsible for both transcription of genomic vRNAs into mRNAs (parental vRNA > mRNA) and for the first step of replication (parental vRNA > cRNA) via mechanism of terminal *de novo* initiation. The cRNA promoter is only responsible for the second step of replication (cRNA > progeny vRNAs) via the internal initiation and realignment synthesis model (Deng et al., 2006). Biochemical and genetic evidence suggests that the vRNA and cRNA replication complexes are structurally and functionally distinct (Gonzalez and Ortin, 1999), and that cRNA synthesis occurs in *cis* using the vRNA-associated RdRp, while progeny vRNA synthesis occur in *trans* by distinct RdRp associated with cRNA (Jorba et al., 2009; Moeller et al., 2012).

Each cRNA is used for production of 10–100 molecules of vRNAs which in turn can be used for production of mRNA (Kawakami et al., 2011), and it was originally hypothesized that higher reporter signal might be achieved with cRNA reporter. Dose response and time course studies showed that vLuc reporter lead to an early detection of influenza replication and at low titer. The lower sensitivity of cLuc was due to its background expression, 4 h delay and overall lower level of expression of luciferase. The background signal detected with both cLuc and cEGFP reporters may be due to the presence of cryptic Pol II promoter (Gagnon-Kugler et al., 2009). It has also been reported that a small percentage of Pol I ribosomal transcripts can be polyadenylated and translated at a low level (Grummt and Skinner, 1985; Lo et al., 1998; Surmacz et al., 1986). The background translation of cLuc can be decreased by the eliminating cryptic Pol II promoter or using ribozyme to cleave poly A tail (Feng et al., 2009). Similar background transcription likely also happens in vLuc/vEGFP, but as the gene is in antisense orientation, the functional protein cannot be produced in the absence of influenza RdRp. The delayed production of luciferase from the cLuc promoter could be explained by the requirement for an additional replication step. The vRNA from vLuc reporter can directly participate in transcription (vRNA > mRNA) while cLuc reporter require an additional replication step (cRNA > vRNA > mRNA) before transcription. The progeny vRNA synthesis requires cRNA, newly synthesized NP/RdRp proteins for encapasidation and another RdRp in trans for replication. Thus vRNA replication occurs late in the infection process leading to the delay in luciferase expression for cLuc reporter. Our results seem to indicate that a lower level of expression by cLuc reporter is due to bottleneck in the second step of replication (cRNA > progeny vRNA). Despite high levels (3–115 folds) of cRNAs produced by cLuc reporters, the level of vRNA was 2.4-fold lower for cLuc reporter. This indicates that either not all expressed cRNAs were used for replication, or fewer vRNA molecules were produced from each cRNA. This might be caused by the deficiency in viral or host factors required for second

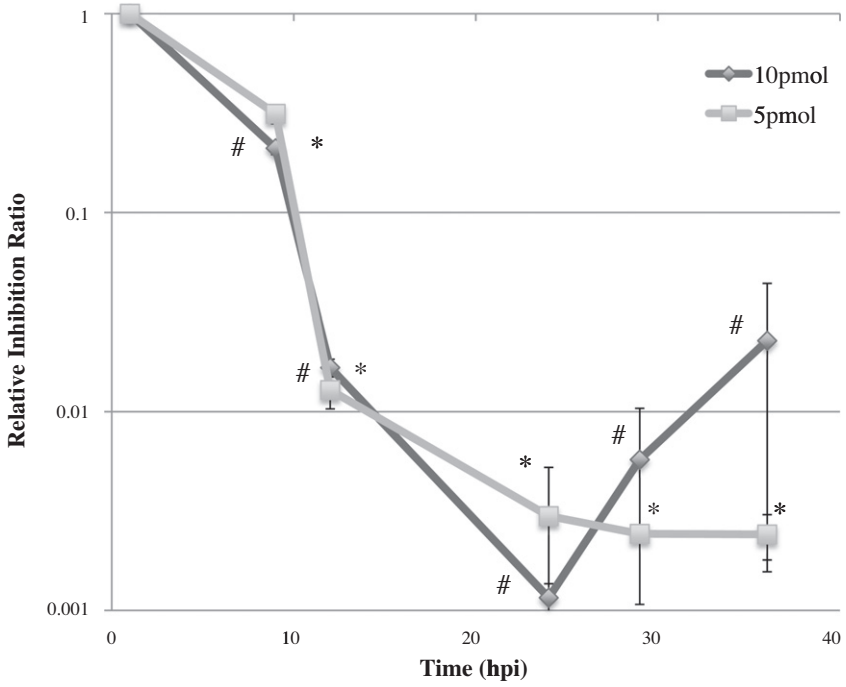

Fig. 6. Kinetic study of anti-influenza NP-1496 siRNA using vLuc reporter construct.HEK293 cells were reverse co-transfected with vLuc constructs and NP-1496 or anti-GFP siRNA (5 or 10pmoles per well) in 96 well plate and infected 20 h postransfection with A/Puerto Rico/8/1934 at MOI = 0.1. Cells were lysed at different time post infection and luciferase activity was determined. Relative inhibition of influenza RNA polymerase was determined by dividing activity of NP-1496 by activity of anti-GFP siRNA treated cells. Each treatment was done in triplicates and statistical analysis was done by Student *t*-test. *5 pmoles; #10 pmoles indicates significant differences ($p < 0.05$) between NP-1496 and anti-GFP treated cells.

step of replication (Karlas et al., 2010). Also high levels of Pol I expressed cRNAs might compete with newly replicated progeny vRNAs for access to RdRp and NP proteins, decreasing stability of progeny vRNAs. Our observation might also explain the lower yield of infectious virus obtained using reverse genetics 'unidirectional' strategy producing positive sense cRNA (Hoffmann and Webster, 2000).

Two types of reporter proteins (EGFP, luciferase) were compared in the current study. Firefly luciferase (*luc2*) is easy to quantify and is more sensitive at earlier time points and at low titer, but it has a short half-life (~3 h), requires expensive reagents, dedicated equipment (luminometer) and does not allow real time monitoring of infection. Application of other luciferases, such as secreted *Gaussia* luciferase, might be beneficial for real time detection (Zhu et al., 2011). EGFP reporter can be easily detected under the microscope and allows real time detection, but at the same time extensive release of EGFP into cell culture was observed which might indicate EGFP induced cell lysis. It has been demonstrated previously that high level of EGFP expression is cytotoxic to mammalian cells leading to cell apoptosis (Liu et al., 1999). As a result, EGFP might be unsuitable for some drug screenings that use cell death as a biomarker.

The anti-influenza drugs have become more important in preparation for an influenza pandemic, yet several recent influenza isolates have developed resistance to existing anti-influenza drugs (Moss et al., 2010). Recently nucleic acid–based approaches, especially RNAi-based drugs which could be easily developed has become a popular approach in targeting influenza replication (Abrahamyan et al., 2009; Ge et al., 2004, 2003; Sui et al., 2009). Despite the importance of the subject few tools are available to conduct high throughput screening for anti-influenza RNAi drugs. We have demonstrated the application of developing a vLuc reporter for the evaluation of kinetics of siRNA-based influenza drug. Eventually high throughput systems with cell-based reporters can be developed for rapid screening and evaluation of novel RNAi based and traditional drugs (Zhang et al., 2012). Developed vRNA and cRNA reporters may be especially useful in screening the activity of novel drugs against specific stages of the viral cycle (vRNA > cRNA, cRNA > vRNA, vRNA > mRNA).

4.2. Study of replication using vRNA/cRNA reporters

The cRNA and vRNA reporters may also shed new light on the replication model of the influenza A virus. Both Pol I expressed vRNA by vLuc reporter and vRNA produced by replication of cRNAs (cRNA > vRNA) expressed by cLuc reporter depend on newly synthesized NP and RdRp for encapsidation and stabilization and in this regard resemble progeny vRNA. In traditional replication model the incoming parental vRNA is responsible for both primary transcription and replication, while progeny vRNA can only participate in secondary transcription and packaging but not in additional replication. Yet our results did detect the presence of luciferase cRNA produced with vLuc reporter that can only arise through replication of progeny vRNA. The detected cRNA is not caused by non-strand-specific amplification as we found no correlation between the level of cRNA and vRNA/mRNA (Suppl. Table 2) and such cRNA was detected previously by others (Regan et al., 2006). This suggests that progeny vRNA encapsidated by newly synthesized NP and RdRp might be able to participate in additional rounds of replication leading to production of secondary cRNA (vRNA > cRNA > progeny vRNA > secondary cRNA) (Fig. 7). It might also lead to production of secondary progeny vRNA as we detect significant 6-fold increase in progeny vRNA with time, although it might be also caused by stabilization of Pol I expressed vRNA by newly synthetized NP/RdRp proteins. We also detected a significant increase in cRNA at 24 h as compare to 4 h for cLuc reporter, but in this case we also cannot determine if increase is caused by secondary replication of progeny vRNA or by increased stabilization of Pol I expressed cRNA.

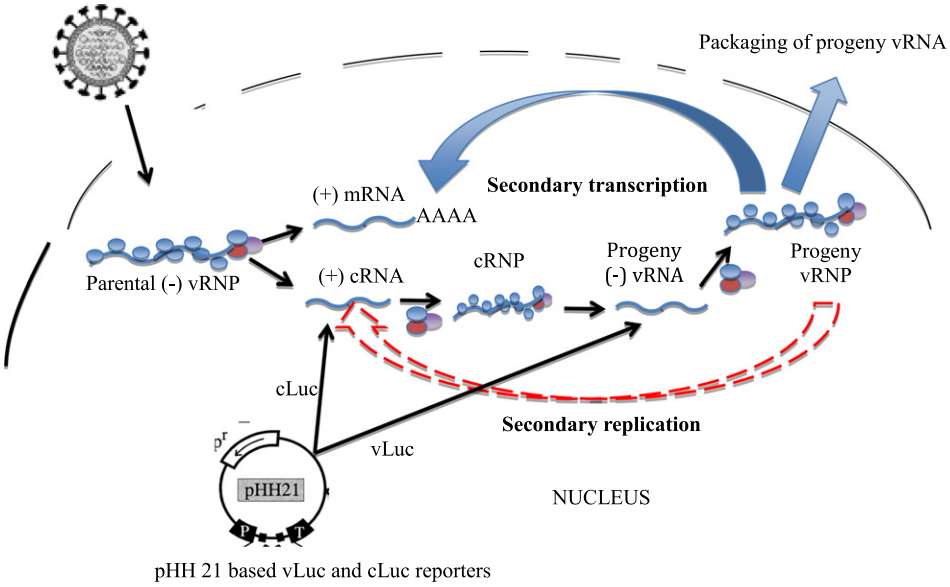

Fig. 7. Hypothesized secondary replication of progeny vRNA of influenza virus.

The inability of progeny vRNA to participate in replication as suggested by traditional model is puzzling. Progeny vRNA interacts with the same NP/RdRp proteins as parental vRNA. Progeny RdRp complex seems functional and is able to participate in secondary transcription (Neumann et al., 2004). Recent studies indicate that same *cis* RdRp is responsible for both transcription and for the first step of replication (vRNA > cRNA), and that both mRNA and cRNA can be produced at the same time from the same vRNA molecule (Vreede and Brownlee, 2007). Also progeny vRNA is able to replicate after it is packaged into virion and transferred to a new cell. For traditional model to function the progeny vRNA must be tagged to prevent its replication but not transcription, and the tag must be removed during packaging. We are not aware of any structural or biochemical differences between parental and progeny vRNAs that would preclude progeny vRNA from participating in replication. Of course *in vivo* probably only small percentage of progeny vRNA could participate in secondary transcription and replication, as majority of progeny vRNA is transported out of the nucleus for packaging into virions.

The idea that most of the cRNA is produced from the parental vRNA comes from experiments with temperature-sensitive NS1 mutants that at restrictive temperature show more than 90% decrease in vRNA synthesis but normal accumulation of cRNA (Falcon et al., 2004; Wolstenholme et al., 1980). Closer look at the detailed results by Wolstenholme et al. (1980) actually indicates that majority of decrease in vRNA is due to cytoplasmic vRNA destined for packaging and not involved in cRNA replication. Decrease in the level of nuclear vRNA involved in cRNA replication is much smaller with close correlation between decrease in vRNA (1.85-fold at 2.5 h) and cRNA (1.54-fold at 2.5 h) at restrictive temperature. As parental vRNA level should not be affected by shift to restrictive temperature, there should be no change in cRNA level produced from parental vRNA, and any decrease is likely due to progeny vRNA replicated cRNA. Also in same *ts* mutants the synthesis of mRNA was similarly unaffected at restrictive temperature, suggesting that most of mRNA is produced from parental vRNA and arguing against secondary transcription of progeny vRNA. This contradicts our results with vLuc/cLuc reporters and previous publications indicating secondary transcription (Neumann et al., 2004). Further studies would be needed to study the differences between parental and progeny vRNA and their ability to replicate. The vRNA and cRNA reporters will be useful for such testing.

5. Conclusion

In conclusion, the developed cRNA and vRNA based reporters could be utilized for effective and early detection of influenza replication (RdRp activity). At 25 h there were no significant differences between vLuc and cLuc reporters and both reporters can be used for detection of influenza replication. Also high level of unencapsidated (−) vRNA from strong ribosomal promoter may lead to the annealing with the (+) mRNAs inducing the dsRNA mediated IFN response, apoptosis and cell death. The expression of (+) cRNAs using cRNA promoter might decrease such possibility. The vRNA and cRNA promoters are highly conserved among different influenza strains, which should allow broad based detection system even for novel influenza strains. In the future development of an automated real-time influenza infection detection system might be feasible. There is also the potential to develop such reporters to monitor vaccine production in cell culture using reverse genetics. Higher sensitivity of influenza detection could be achieved by increasing the amount of transfected reporter, utilizing different cell line such as MDCK with higher level of influenza replication, producing cells stably transfected with the desired reporter, or using *Gaussia* luciferase that is 1000-fold brighter than firefly and *Renilla* luciferases (Lutz et al., 2005; Zhu et al., 2011). One disadvantage with using ribosomal promoters is that they are species specific and the application of developed reporters in the cells of other species would require the appropriate ribosomal promoter. Ribosomal promoters for dog, mice and chicken were recently isolated and novel reporters can be easily constructed (Massin et al., 2005; Murakami et al., 2008; Neumann et al., 1994).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.01.008.

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