P and M gene junction is the optimal insertion site in Newcastle disease virus vaccine vector for foreign gene expression

Wei Zhao,1,2† Zhenyu Zhang,1,3† Laszlo Zsak1 and Qingzhong Yu1

1Southeast Poultry Research Laboratory, Agricultural Research Services, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605, USA
2Beijing Centre for Disease Control and Prevention, Beijing 100013, PR China
3College of Life Sciences, Northeast Agricultural University, Harbin, Heilongjiang 150030, PR China

Newcastle disease virus (NDV) has been developed as a vector for vaccine and gene therapy purposes. However, the optimal insertion site for foreign gene expression remained to be determined. In the present study, we inserted the green fluorescence protein (GFP) gene into five different intergenic regions of the enterotropic NDV VG/GA vaccine strain using reverse genetics technology. The rescued recombinant viruses retained lentogenic pathotype and displayed delayed growth dynamics, particularly when the GFP gene was inserted between the NP and P genes of the virus. The GFP mRNA level was most abundant when the gene was inserted closer to the 3′ end and gradually decreased as the gene was inserted closer to the 5′ end. Measurement of the GFP fluorescence intensity in recombinant virus-infected cells demonstrated that the non-coding region between the P and M genes is the optimal insertion site for foreign gene expression in the VG/GA vaccine vector.

The Villegas-Glisson/University of Georgia (VG/GA) strain of Newcastle disease virus (NDV) is a commonly used vaccine to protect chickens from Newcastle disease (ND), one of the most important infectious diseases of poultry due to the potential for devastating losses (Miller & Guus, 2013). The VG/GA strain preferentially replicates in the intestinal tract of chickens and induces local mucosal immunoresponses (Perozo et al., 2008a). Vaccination of chickens with the VG/GA vaccine provided 100% protection of mortality to chickens against a velogenic viscerotropic NDV challenge (Beard et al., 1993; Perozo et al., 2008b). Therefore, the VG/GA strain is considered as a potential enterotropic vaccine vector to deliver antigens of poultry enteric viruses as bivalent vaccines.

NDV is a non-segmented, single-stranded negative sense RNA virus, belonging to the genus Avulavirus within the subfamily Paramyxovirinae of the family Paramyxoviridae (Lamb et al., 2005). The NDV genome is approximately 15.2 kb in length and consists of six genes flanked by a 3′ leader and 5′ trailer in the order 3′-NP (nucleocapsid protein)-P (phosphoprotein)-M (matrix)-F (fusion)-HN (haemagglutinin-neuraminidase)-L (large polymerase)-5′ (de Leeuw & Peeters, 1999; Peeters et al., 2000). The RNA genome together with NP, P and L proteins forms the ribonucleoprotein complex (RNP), which serves as the active template for transcription and replication of the viral genome (Peeters et al., 1999).

During the past decade, several strains of NDV have been developed as vectors using reverse genetics technology to express foreign antigens for vaccine or gene therapy purposes (Bukreyev & Collins, 2008; Huang et al., 2003; Schirrmacher & Fournier, 2009; Vigil et al., 2008; Zhao & Peeters, 2003). The foreign genes are usually inserted into a non-coding region at different intergenic regions of the NDV genome, and evaluation of these vaccine candidates in clinical trials revealed different levels of protection against targeted pathogen challenge (Bukreyev et al., 2005; DiNapoli et al., 2007; Hu et al., 2011; Huang et al., 2004; Park et al., 2006; Yu et al., 2013; Zhao et al., 2014). Although the immune response to vaccination is influenced by many factors, the expression level of foreign genes is undoubtedly the most important one. However, for NDV, the optimal insertion site for foreign gene expression is still unknown.

In the present study, we generated an infectious clone of the enterotropic NDV VG/GA strain as a vaccine vector and inserted the green fluorescence protein (GFP) gene into five different intergenic regions of the infectious clone. Evaluation of the pathogenicity, growth kinetics, and GFP expression of these rescued recombinant viruses allowed us to better understand the NDV transcription mechanism.
and to determine the optimal insertion site for foreign gene expression by the NDV VG/GA vaccine vector.

A full-length cDNA clone (FLC), pFLC-VG/GA, encoding the complete antisense genome of the NDV VG/GA strain was generated through three steps of cloning using an In-Fusion PCR Cloning kit (Clontech) and a similar cloning approach as described previously (Hu et al., 2011; Zhao et al., 2014). The GFP gene ORF together with the NDV transcriptional signals derived from the P-M gene junction region was successfully amplified, and inserted into the NP/P, P/M, M/F, F/HN or HN/L non-coding region in the VG/GA FLC respectively, resulting in five VG/GA-GFP recombinant cDNA clones (Fig. 1a). After co-transfection of an FLC and the supporting plasmids in HEP-2 cells and subsequent amplification in SPF chicken embryonated eggs as described previously (Zhao et al., 2014), a NDV VG/GA virus and five VG/GA-GFP recombinant viruses were rescued and propagated. Sequencing of the RT-PCR products of the viral genomes verified the GFP insertions in the VG/GA genome, and confirmed the nucleotide sequence fidelity of the rescued viruses.

To evaluate the influence of the inserted GFP gene on NDV pathogenicity and replication, the rescued viruses were examined in vitro and in vivo by conducting virus titration, mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays (Alexander, 1998). As shown in Table 1, the rescued viruses appeared to be slightly attenuated in embryonated eggs and day-old chickens, with a longer MDT (>120 h) and a lower ICPI (0.0) than the parental VG/GA strain. The titres of the rescued viruses grown in embryonated eggs or in DF-1 cells, measured by EID₅₀, TCID₅₀ and HA, were one or two logs lower but were comparable to that of the parental VG/GA strain (Table 1). As shown in Fig. 1b, the growth kinetics of rVG/GA were slightly delayed when compared with the parental virus VG/GA; however, after 48 h, the rescued virus rVG/GA displayed similar kinetics and magnitude of the replication. In contrast, the kinetics of replication of the five recombinant viruses showed that the insertion of the GFP gene resulted in a delay in the onset of replication, and their titres were about one log lower than that of the rVG/GA virus even after 48 h of infection (Fig. 1b). Also, it is interesting to note that the recombinant viruses rVG/GA-GFP-NP/P, rVG/GA-GFP-P/M, rVG/GA-GFP-M/F, rVG/GA-GFP-F/HN and rVG/GA-GFP-HN/L were in an ascending order of titres at most of the time points, which indicated that the delayed growth kinetics that resulted from the GFP gene insertion was most prominent when it was located at intergenic regions closer to the 3’ end of the viral genome.

The cytopathic effects (CPE) and expression of the GFP in DF-1 cells infected with the rescued viruses were examined by fluorescence microscopy as described previously (Hu et al., 2011). After 48 h of infection, green fluorescence was observed in the recombinant virus-infected DF-1 cells (Fig. 1c). Interestingly, the levels of CPEs induced by the recombinant viruses appeared to correlate with the position of GFP insertion in the viral genome. There was a trend of increasing CPEs in DF-1 cells induced by the recombinant viruses in the order from rVG/GA-GFP-NP/P, rVG/GA-GFP-P/M, rVG/GA-GFP-M/F, rVG/GA-GFP-F/HN to rVG/GA-GFP-HN/L. This finding is in accordance with the result of the growth kinetics of the recombinant viruses (Fig. 1b).

To determine the effect of different insertion sites on GFP gene transcription, the GFP mRNAs in the virus infected DF-1 cells were measured by reverse transcription and quantitative real-time PCR (qPCR) using a Power SYBR Green PCR Master Mix kit (Applied Biosystems). Comparison of the GFP mRNAs in virus-infected cells over a non-infected cell control was normalized to the NP gene and calculated by the comparative threshold cycle (ΔΔCt) method (Le Nouën et al., 2009). As shown in Fig. 2a, an apparent correlation between the GFP transcription level and the location of the GFP gene in the viral genome was observed, such that the GFP mRNA abundance was gradually decreased regarding to the insertion position toward the 5’ end of the viral genome. Compared with the GFP mRNA abundance at NP-P junction, the GFP gene transcription was decreased by 30 % at the P-M junction, 28 % at the M-F junction, 8 % at the F-HN junction and 24 % at the HN-L junction. This transcription pattern proved the theoretical hypothesis that there is a gradient of decreasing mRNA abundance according to the position of the gene relative to the 3’ end of the NDV genome.

The levels of GFP production of recombinant viruses were quantified by measuring the green fluorescence intensity in the virus infected DF-1 cells at every 12 h post-infection using Fluorescence Microplate Reader (BioTek, FLx800). It appeared that during the first 36 h of infection there was little GFP expression from the cells infected with the recombinant viruses as their fluorescence intensities were virtually the same as that from the parental rVG/GA virus-infected cells. However, beyond 48 h post-infection, the GFP fluorescence intensity in rVG/GA-GFP-P/M infected cells increased rapidly as the infection progressed, and reached a highest level at 84 h post-infection among the different recombinant virus-infected cells (Fig. 2b). The increase rates of GFP fluorescence intensity in other recombinant virus-infected cells were somewhat lower than that in rVG/GA-GFP-P/M infected cells, with 83.1 %, 75.5 % and 48.4 % relative GFP fluorescence intensity for rVG/GA-GFP-M/F, rVG/GA-GFP-NP/P, and rVG/GA-GFP-F/HN infected cells at 84 h post-infection, respectively.

It is widely accepted that paramyxoviruses synthesize and transcribe the genes into mRNAs in a sequential and polar manner by a stop-and-restart mechanism at each gene junction; therefore, the promoter-proximal genes are expressed more efficiently than promoter-distal ones (Lamb & Parks, 2007). However, this assumption has so far been neither proved nor disproved for NDV. In this study, we
quantitatively measured the levels of GFP mRNAs from the cells infected with the recombinant NDVs vectoring the GFP gene at different intergenic regions. Our results demonstrated that there was a gradient of GFP mRNA expression from the 3' end to 5' end of the viral genome with the transcription level decreased by about 8% to 30% at each subsequent gene junction compared to the GFP mRNA abundance at NP-P junction. To the best of our knowledge, it is the first time that this sequential and polar transcription hypothesis has been experimentally proved on NDV.

According to the sequential transcription theory, the best position for foreign gene expression would be the closest to the 3' end of NDV genome. On the other hand, the insertion of a foreign gene into a promoter-proximal position might interfere with NDV replication more seriously than a promoter-distal position, resulting in lower levels of foreign gene expression.

---

**Fig. 1.** (a) Schematic representation of NDV VG/GA strain full-length cDNA clones containing the GFP gene at different intergenic regions. The GFP transcription cassette which contains the NDV trans-acting signal sequences (gene end, intergenic region and gene start), Kozak sequence and GFP open reading frame, was amplified from the subclone pVG/GFP, and cloned into the different intergenic regions (NP/P, P/M, M/F, F/HN and HN/L) of the VG/GA full-length cDNA clone using an In-Fusion PCR Cloning kit (Clontech). The sequences of all primers used in construction of the full-length cDNA clones are available upon request. The NDV gene end and gene start signal sequences, the Kozak sequence and GFP sequences are boxed or underlined. The direction of the T7 promoter is indicated by a bold black arrow. (b) Growth dynamics of the recombinant viruses. DF-1 cells were infected with the indicated NDV viruses at 0.01 m.o.i. Every 12 h post-infection, the infected cells were harvested. Virus titres were measured by TCID_{50} titration on DF-1 cells for each time point in triplicates from two independent experiments, and expressed in mean log_{10} TCID_{50} ml^{-1} with standard deviation (error bars). (c) The cytopathic effects and expression of GFP by the rescued viruses. DF-1 cells in a 12-well plate were infected with the recombinant viruses at 0.1 m.o.i. At 48 h post-infection, the cytopathic effects and the fluorescence of the infected cells were examined and digitally photographed by fluorescence microscopy at \times 100 magnification (Nikon, Eclipse Ti).
gene expression (Carnero et al., 2009). Therefore, the genomic location of foreign genes, replication of the virus, and the abundance of foreign gene expression were all kept in a subtle balance.

Previously, Zhao & Peeters (2003) and Ramp et al. (2011) inserted foreign genes into different positions of NDV genome, and showed that the foreign gene expression levels differed only moderately in various positions. However, it is important to note that the positions of each insertion in the above studies varied relative to the gene start of the downstream genes of NDV genome, which may influence the insertion transcription efficiency caused by the variation of virus genome lengths and sequences (Skiadopoulos et al., 2000). To avoid any potential effects on transcription efficiency caused by the variation of virus genome lengths and sequences, we inserted the GFP gene cassette at 40 nt upstream of the GE sequences of the viral genes. Thus, all of the recombinant viruses possess an identical independent GFP transcription unit at the exact location relative to the gene start of the downstream genes.

Quantitative measurements of the GFP fluorescence intensity in recombinant virus-infected cells demonstrated a gradient abundance of expressed GFP in the following order: rVG/GA-GFP-NP/P, rVG/GA-GFP-P/M, rVG/GA-GFP-M/F, rVG/GA-GFP-F/HN, rVG/GA-GFP-HN/L. Clearly, the level of GFP expression strongly correlated with the gene order of the NDV genome, except the insertion between NP and P genes. This disproportionate effect occurred presumably due to the GFP insertion that altered the ratio between the NP and P proteins. It has been demonstrated in vesicular stomatitis virus that a specific

Table 1. Biological assessments of the NDV recombinant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>MDT* (h)</th>
<th>ICPI†</th>
<th>HA‡</th>
<th>EID₅₀§</th>
<th>TCID₅₀¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>VG/GA</td>
<td>110</td>
<td>0.08</td>
<td>2¹²</td>
<td>1.47 × 10⁸</td>
<td>1.76 × 10⁸</td>
</tr>
<tr>
<td>rVG/GA</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹²</td>
<td>1.76 × 10⁷</td>
<td>1.76 × 10⁷</td>
</tr>
<tr>
<td>rVG/GA-GFP-NP/P</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹¹</td>
<td>3.12 × 10⁸</td>
<td>3.12 × 10⁷</td>
</tr>
<tr>
<td>rVG/GA-GFP-P/M</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹²</td>
<td>1.32 × 10⁸</td>
<td>1.76 × 10⁷</td>
</tr>
<tr>
<td>rVG/GA-GFP-M/F</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹⁰</td>
<td>3.78 × 10⁸</td>
<td>9.88 × 10⁷</td>
</tr>
<tr>
<td>rVG/GA-GFP-F/HN</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹⁰</td>
<td>2.32 × 10⁸</td>
<td>3.12 × 10⁷</td>
</tr>
<tr>
<td>rVG/GA-GFP-HN/L</td>
<td>&gt;120</td>
<td>0</td>
<td>2¹⁰</td>
<td>3.78 × 10⁸</td>
<td>1.76 × 10⁷</td>
</tr>
</tbody>
</table>

*Mean death time assay in embryonating eggs.
†Intracerebral pathogenicity index assay in day-old chickens.
‡Haemagglutination assay.
§The 50 % egg infective dose assay in embryonated eggs.
¶The 50 % tissue infectious dose assay on DF-1 cells.

Fig. 2. (a) Quantification of the GFP mRNA transcription. DF-1 cells in 6-well plate were infected with the indicated NDV viruses at 0.1 m.o.i. At 48 h post-infection, the total RNAs were extracted by KingFisher automatic purification instrument (Ambion) and MagMAX-96 Total RNA Isolation kit (Ambion). The RNAs were treated with DNase I to remove residual genomic DNA and reverse transcribed with Oligo (dT) primer. The prepared cDNAs were used in quantitative real-time PCR (qPCR) for quantification of the GFP mRNA transcription. The NP gene transcripts from the same samples were quantified and used for internal normalization. qPCR results were expressed as fold difference with SD (error bars) relative to that of cell control using the comparative threshold cycle (ΔΔCt) method. (b) Measurement of GFP fluorescence intensity. DF-1 cells were grown in a 96-well plate and infected with the indicated NDV viruses at 0.1 m.o.i. Every 12 h post-infection, GFP fluorescence intensities were measured by using Fluorescence Microplate Reader (BioTek, FLx800) in triplicate wells from two independent experiments. The results were expressed as percentages of the mean GFP fluorescence intensities with SD (error bars) relative to the highest intensity, which was set as 100 %.
ratio of the NP to P protein is optimal for supporting efficient replication and encapsidation (Pattnaik & Wertz, 1990; Wertz et al., 2002). This disproportionate effect was also observed on NDV by other researchers (Carnero et al., 2009; Zhao & Peeters, 2003), suggesting that the non-coding region between the NP and P proteins is not an optimal insertion site for foreign gene expression.

Despite the fact that foreign gene insertion between P and M genes affects several downstream virus genes preceded only by the NP and P junction, the replication of rVG/GA-GFP-P/M virus was not affected as much as the rVG/GA-GFP-NP/P virus. In addition, the foreign gene inserted in the non-coding region between the P and M genes expressed the highest level of the foreign gene product among the different recombinant virus infected cells. Thus, it is reasonable to conclude that the junction between P and M genes contains an optimal insertion site for foreign gene expression in the VG/GA NDV vaccine vector.

In summary, in the present study we have successfully constructed an infectious clone of the enterotropic NDV VG/GA vaccine strain and engineered five recombinant viruses vectoring GFP at different intergenic regions (NP/P, P/M, M/F, F/HN and HN/L). Quantification of GFP mRNAs expressed from different recombinant viruses confirmed the theoretical hypothesis of the gradient mRNA expression of NDV. Measurement of the GFP fluorescence intensity in recombinant virus-infected cells demonstrated that the non-coding region between the P and M genes was the optimal insertion site for foreign gene expression in the VG/GA vaccine vector. Overall, data suggest that the NDV VG/GA vaccine strain is a potential enterotropic live vaccine vector that can be used to deliver antigens of poultry enteric viruses as bivalent vaccines.

Acknowledgements

The authors wish to thank Xiuqin Xia and Fenglan Li for excellent technical assistance, Patti Miller for critical reading of the manuscript, and the SEPRL sequencing facility personnel for nucleotide sequencing. W. Z. and Z. Z. were sponsored by a scholarship from China Scholarship Council. This research was supported by USDA, ARS CRIS project 6612-32000-067-00D.

References


