Short Communication

Development of a Newcastle disease virus vector expressing a foreign gene through an internal ribosomal entry site provides direct proof for a sequential transcription mechanism

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In the present study, we developed a novel approach for foreign gene expression by Newcastle disease virus (NDV) from a second ORF through an internal ribosomal entry site (IRES). Six NDV LaSota strain-based recombinant viruses vectoring the IRES and a red fluorescence protein (RFP) gene behind the nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) or large polymerase (L) gene ORF were generated using reverse genetics technology. The insertion of the second ORF slightly attenuated virus pathogenicity, but did not affect ability of the virus to grow. Quantitative measurements of RFP expression in virus-infected DF-1 cells revealed that the abundance of viral mRNAs and red fluorescence intensity were positively correlated with the gene order of NDV, 3′-NP-P-M-F-HN-L-5′, proving the sequential transcription mechanism for NDV. The results herein suggest that the level of foreign gene expression could be regulated by selecting the second ORF insertion site to maximize the efficacy of vaccine and gene therapy.

Newcastle disease virus (NDV) is a member of the genus Avulavirus within the subfamily Paramyxovirinae of the family Paramyxoviridae (Lamb et al., 2005). The genome of NDV is a non-segmented, negative-sense ssRNA of 15.2 kb that contains six genes, in the order 3′-nucleocapsid (NP)-phosphoprotein (P)-matrix (M)-fusion (F)-haemagglutinin-neuraminidase (HN)-large polymerase (L)-5′, flanked by a 3′ UTR leader and 5′ trailer (de Leeuw & Peeters, 1999; Peeters et al., 2000). Two additional proteins (V and W) are produced through editing of the phosphoprotein mRNA (Steward et al., 1993). The genomic RNA is encapsidated with the NP protein and associated with the P and L proteins to form the ribonucleoprotein complex (RNP), which is an active template for viral RNA transcription and replication (Peeters et al., 1999). Based on their pathogenicity for chickens, NDV strains have been classified into lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence) pathotypes (Miller & Koch, 2013). Virulent strains of NDV are the causative agents of Newcastle disease (ND), affecting a wide variety of birds and causing significant economic losses to the poultry industry worldwide (Alexander, 2001). Some lentogenic NDV strains, such as LaSota and B1, have been used as vaccines to protect poultry against ND worldwide.

During the late 1990s, reverse genetics technology was developed to rescue infectious NDV from cloned cDNAs (Peeters et al., 1999; Römer-Oberdörfer et al., 1999). Since then, many strains of NDV have been developed as vectors to express foreign genes for vaccine or gene therapy purposes (Bai et al., 2014; Bukreyev et al., 2005; DiNapoli et al., 2007, 2010; Ge et al., 2007, 2010; Hu et al., 2011; Kong et al., 2012; Kortekaas et al., 2010; Niu et al., 2014; Yu et al., 2013; Zhao & Peeters, 2003; Zhao et al., 2014). Usually, the foreign genes are inserted into a non-coding region in the NDV genome as an additional independent transcription unit (ITU) that consists of NDV gene start, the foreign gene and NDV gene end sequences. Based on the well-accepted ‘stop and start’ transcription mechanism for non-segmented, negative stranded RNA viruses, NDV is thought to transcribe its genes into mRNAs in a gradient of decreasing mRNA abundance according to the position of the gene relative to the 3′ end of the genome (Lamb & Parks, 2013; Samal, 2013).
NDV particles contain a single genome (Goff et al., 2000; Skiadopoulos et al., 2000; Wertz et al., 2002; Zhao et al., 2015). Therefore, the addition of an ITU in the viral genome would attenuate its downstream gene transcription, subsequently may decrease virus replication and the level of foreign gene expression (Krishnamurthy et al., 2000; Skiadopoulos et al., 2000; Wertz et al., 2002; Zhao et al., 2015).

To overcome the drawback of an additional ITU on downstream gene transcription and virus replication, different approaches for expression of a foreign gene by NDV have been explored. Gao et al. (2008) expressed foreign genes from a two-segmented NDV genome to increase the capacity of expressing larger size or more foreign genes. However, there is concern about the stability of the engineered segmented NDV because the majority of infectious NDV particles contain a single genome (Goff et al., 2012). In 2015, Wen et al. (2015) developed a new approach to express a foreign gene by NDV as a fusion protein with the M protein, followed by self-cleavage of foot-and-mouth disease 2A peptide and ubiquitin coding sequences. After cleavage, the foreign protein contains an extra 20 aa at the amino-terminus and 17 aa at the carboxyl-terminus derived from the M protein of NDV and the 2A peptide and ubiquitin, respectively. Thus, there is a possibility that these extra amino acids may alter the antigenicity or biological functions of the foreign protein.

In the present study, we aimed to express an authentic foreign protein by NDV through an internal ribosomal entry site (IRES). IRES is an RNA element that has been found in viral RNAs as well as a variety of cellular mRNAs with long 5' UTR regions (Jang et al., 1988; Pelletier & Sonenberg, 1988). IRES can promote internal initiation of translation of RNA to facilitate the expression of two or more proteins from a polycistronic transcription unit in eukaryotic cells (Mountford & Smith, 1995). IRES has been used in rabies virus, a non-segmented negative strand RNA virus, to investigate the role of the phosphoprotein isoforms in virus replication and virulence (Marschalek et al., 2009, 2012). Here, we utilized IRES to promote expression of a foreign gene from a second ORF of NDV genes and the result provided direct proof for a sequential transcription mechanism for NDV.

Six NDV LaSota strain-based recombinant cDNA clones were constructed by inserting the IRES sequence and a red fluorescence protein (RFP) gene immediately downstream from the translation stop codon of the NP, P, M, F, HN and L genes as a second ORF in the pFLC-LaSota infectious clone (Hu et al., 2011), as illustrated in Fig. 1(a). The sequences of all primers used in the construction of full-length cDNA clones are available upon request. The length of all six recombinant cDNA clones was identical (16482 nt) and divisible by six, abiding by the ‘rule of six’ (Kolakofsky et al., 2005). After co-transfection of an rLS-IRE-RFP cDNA clone and the supporting plasmids in HEP-2 cells and subsequent amplification in SPF chicken embryonated eggs (Estevez et al., 2007; Hu et al., 2011), six recombinant viruses, designated rLS-NP-IRE-RFP, rLS-P-IRE-RFP, rLS-M-IRE-RFP, rLS-F-IRE-RFP, rLS-HN-IRE-RFP and rLS-L-IRE-RFP, respectively, were rescued and propagated. Sequencing analysis of the reverse transcription polymerase chain reaction (RT-PCR) products of the viral genomes verified the RFP insertions in the LaSota genome and confirmed the nucleotide sequence fidelity of the rescued viruses.

To evaluate the effects of the IRES-RFP insertion on NDV pathogenicity and growth ability, the rescued viruses were examined by conducting virus titration and the 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 and a lower ICPI than the parental LaSota strain. However, the titres of the rescued viruses measured by the TCID50 assay on DF-1 cells, the 50 % egg infective dose assay in 9 day-old SPF chicken embryos and haemagglutination (HA) test were similar to that of the parental LaSota strain (Table 1). There was no significant difference in the growth kinetics between the recombinant viruses and their parental virus (Fig. 1b).

The cytopathic effects (CPEs) and expression of the RFP in DF-1 cells infected with the rescued viruses were examined by using an inverted fluorescence microscope (AMG; EVOS). As shown in Fig. 2, after 24 h of infection, the red fluorescence was observed in the rLS-NP-IRE-RFP virus-infected cells. As infection progressed, the red fluorescence was observed in all other recombinant virus-infected cells at 72 h post-infection. As expected, LaSota virus-infected cells did not show any red fluorescence. It is interesting to note that the levels of RFP expressed by the recombinant viruses appeared to positively correlate with the RFP gene position relative to the 3' end of the viral genome. After 24 h of infection, the infected cells started to develop visible CPEs with minor cell fusion to extensive cells fusion after 72 h of infection (Fig. 2). There was little difference of CPEs among the different virus-infected cells at the same time point of examination.

To further compare the levels of RFP expressed by the different recombinant viruses, we quantitatively measured the abundance of RFP-containing mRNAs by reverse transcription and quantitative real-time PCR (qPCR) using a Power SYBR Green PCR Master Mix kit (Applied Biosystems). As shown in Fig. 3(a), there was a distinctly positive correlation between the abundance of RFP mRNAs and the location of the RFP gene relative to the 3' end of the viral genome. The RFP mRNA abundance was in the order: rLS-NP-IRE-RFP >> rLS-P-IRE-RFP >> rLS-M-IRE-RFP >> rLS-F-IRE-RFP >> rLS-HN-IRE-RFP >> rLS-L-IRE-RFP. Quantitative measurement of the red fluorescence intensity in the virus-infected DF-1 cells (Fig. 3b) by using a fluorescence microplate reader (FLX800; BioTek) showed that during the first 36 h of infection there was no significant difference in RFP fluorescence intensities among the cells infected with different recombinant viruses.
(a) Schematic representation of NDV LaSota strain-based recombinant cDNA clones containing IRES-RFP. The subclone pTOPO-IRES-RFP was constructed by cloning the IRES from pIRES plasmid (Clontech) and RFP gene from pCMV-DsRed-Express plasmid (Clontech) into the TOPO TA vector (Life Technologies) using the In-Fusion PCR Cloning kit (Clontech). The IRES-RFP cassette was amplified from the pTOPO-IRES-RFP subclone and cloned behind the NP, P, M, F, HN or L gene in the LaSota full-length cDNA clone (Hu et al., 2011) as a second ORF. The sequences of all primers used in construction of the full-length cDNA clones are available upon request. The NDV NP gene, the IRES and RFP sequences are 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 strains 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\(^{-1}\). Error bars indicate the SD of virus titres at different time points post-infection.
virus. However, after 48 h of infection, the RFP fluorescence intensities increased at a considerably different rate among the different recombinant virus-infected cells. RFP fluorescence intensity in rLS-NP-IRES-RFP-infected cells started to increase rapidly after 48 h of infection and reached the highest level (deemed 100 %) at 84 h post-infection among all recombinant virus-infected cells. The fluorescence intensity in rLS-P-IRES-RFP-, rLS-M-IRES-RFP- and rLS-F-IRES-RFP-infected cells increased slowly and reached approximately 65, 52 and 40 % of fluorescence intensity, respectively, compared to the level observed in rLS-NP-IRES-RFP-infected cells at 84 h post-infection. The rLS-HN-IRES-RFP- and rLS-L-IRES-RFP-infected cells expressed a low level of RFP, slightly above the base level of fluorescence.

Since the first reverse genetics system established for NDV (Peeters et al., 1999; Römer-Oberdörfer et al., 1999), many strains of NDV have been developed as vectors to express a foreign gene for vaccine and gene therapy purposes. In most cases, the foreign gene was inserted into the NDV genome as an ITU and evaluation of these vaccine candidates in clinical trials revealed different levels of protection against targeted pathogen challenge (Bukreyev et al., 2005; Cardenas-García et al., 2015; 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. To optimize foreign gene expression, previously we and other researchers have investigated the effect of genomic location of the foreign genes on the level of foreign gene expression (Carnero et al., 2009; Zhao & Peeters, 2003; Zhao et al., 2015). The results have shown that the P and M gene junction is the optimal insertion site in NDV vaccine vector for foreign gene expression from an ITU. According to the sequential transcription hypothesis, the best position for foreign gene expression would be the closest to the 3′ end of NDV genome, i.e. the NP gene. However, the insertion of a foreign gene transcription unit into a promoter-proximal position interfered with NDV replication more seriously than a promoter-distal position, resulting in lower levels of foreign gene expression (Zhao et al., 2015). Besides the insertion site, the size and specific properties of the foreign gene and/or its gene product may also affect NDV replication (Al-Garib et al., 2003; Dinapoli et al., 2007; Gao et al., 2008). The data presented in this study showed the insertion of the foreign gene cassette, IRES-RFP, as a second ORF in NDV genes did not overtly affect virus growth ability when compared with the parental virus and that the level of RFP expressed by the rLS-NP-IRES-RFP virus was the highest among these recombinant viruses. These results suggest that the 3′ proximal NP gene of NDV is the optimal insertion site in NDV vaccine vector for foreign gene expression through an internal initiation of translation. It would be interesting to compare the IRES and classic ITU approaches for levels of foreign gene expression from their optimal insertion sites, i.e. the NP gene for the IRES and the P/M gene junction region for the ITU, in the future work.

The transcription process of NDV is thought to be similar to that of other non-segmented and negative stranded RNA viruses (Lamb & Parks, 2013; Samal, 2011). Transcription begins at a single promoter that is present in the 3′ leader region (Marcos et al., 2005). At each gene junction region, the RNA dependent RNA polymerase of NDV may undergo two different transcription processes: (1) terminate transcription at the gene end (GE) region, scan through the intergenic region and initiate transcription of the downstream gene at the gene start region; (2) terminate transcription at the GE region and detach from the RNP template. According to this ‘stop–start’ transcription theory, NDV would synthesize and transcribe its genes into mRNAs in a sequential and polar manner and the promoter-proximal genes would be expressed more efficiently than promoter-distal ones. However, until now this sequential transcription hypothesis remained to be proved for NDV. In this study, we inserted the IRES-RFP gene sequences immediately downstream from the translation stop codon of six viral genes, respectively, as a second ORF of the viral genes. Thus, the recombinant viruses retained the same numbers of transcription units as the wild-type NDV. Quantitative measures of viral mRNAs containing the RFP from the recombinant virus-infected DF-1 cells revealed that the abundance of viral transcripts was positively correlated with the gene order of NDV, 3′-NP-P-M-F-HN-L-5′. The transcription level decreased at each subsequent gene junction and hence demonstrated a gradient mRNA expression from 3′ end to 5′ end of the viral genome. To the best of our knowledge, this is the first time that this sequential and polar transcription hypothesis has been experimentally proved for NDV.

One of the benefits of using this foreign gene expression approach through IRES from a second ORF of the viral genes is that the level of foreign gene expression could be regulated by taking advantage of the sequential

<table>
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<tr>
<th>Virus</th>
<th>MDT(h)*</th>
<th>ICPI†</th>
<th>HA</th>
<th>EID&lt;sub&gt;50&lt;/sub&gt;§</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;§</th>
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<tr>
<td>LaSota</td>
<td>110</td>
<td>0.15</td>
<td>2&lt;sup&gt;10&lt;/sup&gt;</td>
<td>6.81 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.5 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>rLS-NP-IRES-RFP</td>
<td>153</td>
<td>0.10</td>
<td>2&lt;sup&gt;11&lt;/sup&gt;</td>
<td>6.81 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.12 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>rLS-P-IRES-RFP</td>
<td>139</td>
<td>0.00</td>
<td>2&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.47 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.76 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>rLS-M-IRES-RFP</td>
<td>137</td>
<td>0.00</td>
<td>2&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.16 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.12 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>rLS-F-IRES-RFP</td>
<td>144</td>
<td>0.00</td>
<td>2&lt;sup&gt;10&lt;/sup&gt;</td>
<td>4.81 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.12 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>rLS-HN-IRES-RFP</td>
<td>132</td>
<td>0.00</td>
<td>2&lt;sup&gt;10&lt;/sup&gt;</td>
<td>4.22 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.76 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>rLS-L-IRES-RFP</td>
<td>146</td>
<td>0.00</td>
<td>2&lt;sup&gt;10&lt;/sup&gt;</td>
<td>6.81 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.12 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
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*MDT assay in embryonated eggs.
†ICPI in day-old chickens.
‡The 50 % egg infective dose assay in embryonated eggs.
§TCID<sub>50</sub> in DF-1 cells.
transcription mechanism. If a higher amount of a foreign antigen is desired to be expressed for a vaccine purpose, the NP gene downstream non-coding region would be the best choice for the foreign gene insertion. Whereas an intermediate or low level of expression of a foreign gene, for instance a cytokine, IL-2, is required for anti-cancer therapy, the M or F gene downstream non-coding regions could be chosen for the insertion site because overexpression of IL-2 may result in a toxic side-effect to animals and humans (Gaffen & Liu, 2004; Vigil et al., 2008).

In summary, in this study, we have developed a novel approach for foreign gene expression by NDV from a second ORF through an IRES. Unlike the conventional expression approach through an additional transcription unit, the insertion of the IRES-RFP as a second ORF into NDV genes did not discernibly interfere with virus growth ability. Quantitative measurements of the RFP expression from recombinant virus-infected cells proved the sequential transcription mechanism for NDV. Our results suggest that the level of foreign gene expression

**Fig. 2.** The CPEs and expression of RFP by the recombinant viruses. DF-1 cells in a 12-well plate were infected with the recombinant viruses at 0.01 m.o.i. Every 24 h post-infection, the CPEs and the fluorescence of the infected cells were examined and digitally photographed under an inverted fluorescence microscope at × 100 magnification.
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from a NDV vector could be regulated by selecting an optimal second ORF insertion site to maximize the efficacy of vaccine and gene therapy.

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References


Fig. 3. (a) Effect of different insertion sites of IRES-RFP in the recombinant viruses on RFP transcription. DF-1 cells in six-well plates were infected with the indicated NDV strains at 0.01 m.o.i. Every 24 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. Quantification of cDNAs reverse transcribed from the RFP mRNAs was carried out by RT-qPCR using a Power SYBR Green PCR Master Mix kit (Applied Biosystems). The results were expressed as percentage of the mean RFP mRNAs expressed from different recombinant virus-infected cells relative to the highest amount of RFP mRNA detected in the same experiment, which was deemed 100%. Error bars indicate the SD of RFP mRNAs. (b) Effect of different insertion sites of IRES-RFP in the recombinant viruses on RFP expression. DF-1 cells were grown in 96-well plates and infected with the indicated NDV strains at 0.01 m.o.i. Every 12 h post-infection, RFP fluorescence intensities were measured by using a fluorescence microplate reader (FLx800; BioTek) with a 540/35 excitation filter and a 600/40 emission filter in triplicate wells from two independent experiments. The results were expressed as percentage of the mean RFP fluorescence intensities relative to the highest intensity detected in the same experiment, which was set as 100%. Error bars indicate the SD of RFP fluorescence intensities.


