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Short communication

Generation of a recombinant Newcastle disease virus expressing two foreign genes for use as a multivalent vaccine and gene therapy vector

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ABSTRACT

Newcastle disease virus (NDV) has been used as a vector in the development of vaccines and gene therapy. A majority of these NDV vectors express only a single foreign gene through either an independent transcription unit (ITU) or an internal ribosomal entry site (IRES). In the present study, we combined the ITU and IRES methods to generate a novel NDV LaSota strain-based recombinant virus vectoring the red fluorescence protein (RFP) and the green fluorescence protein (GFP) genes. Biological assessments of the recombinant virus, rLS/IRES-RFP/GFP, showed that it was slightly attenuated *in vivo*, yet maintained similar growth dynamics and viral yields *in vitro* when compared to the parental LaSota virus. Expression of both the RFP and GFP was detected from the rLS/IRES-RFP/GFP virus-infected DF-1 cells by fluorescence microscopy. These data suggest that the rLS/IRES-RFP/GFP virus may be used as a multivalent vector for the development of vaccines and gene therapy agents.

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Newcastle disease virus (NDV) is an enveloped, non-segmented, negative-stranded RNA virus and has been classified as avian paramyxovirus serotype 1 (APMV-1) in the genus *Avulavirus* of the family *Paramyxoviridae* [1]. NDV is an avian pathogen that can cause local infections in humans, however, there is no current data to suggest that NDV can be transmitted human-to-human [2,3]. The NDV genome consists of approximately 15.2 kb and contains six transcriptional units, encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L), in that order [4]. The genomic RNA, together with the NP, P, and L proteins, form the ribonucleoprotein complex (RNP), which serves as the active template for transcription and replication of the viral genome [5,6].

Since reverse genetics technology was first used to rescue infectious NDV from recombinant cDNA in 1999 [7,8], many NDV clones have been developed and used as vectors to express foreign genes for vaccine or gene therapy purposes [9–15]. Most of these NDV vectors express only a single foreign gene from an additional independent transcription unit (ITU) that is inserted between other native viral transcription units in the NDV genome [15]. Recently we developed a novel approach to express a foreign gene from within native viral transcription units using an internal ribosomal entry site (IRES). In this case the red fluorescence protein (RFP) gene was expressed from a second open reading frame (ORF) located in all six native transcriptional units, separately, using an IRES [16]. The addition of the second ORF did not significantly affect viral replication, and the level of foreign gene expression could be regulated by inserting the second ORF into different native viral transcription units.

In this study, we combined the ITU and IRES approaches to develop a multi-foreign gene expression vector. The NDV LaSota (LS) strain-based infectious clone containing the G gene of avian metapneumovirus subtype, pLS/aMPV-C G [17], was used as a backbone to construct a recombinant cDNA clone containing both RFP and GFP genes (Fig. 1). First, the ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies, Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), which resulted in the pLS/GFP subclone. Second, the RFP gene, amplified from the pCMV-Ds-Red-Express plasmid (Clontech), was cloned downstream of the IRES sequence in pIRES-hrGFP-2a vector (Clontech). Finally, the IRES and the RFP ORF sequences were amplified and cloned into the non-coding region of the NDV F gene in the pLS/GFP vector as a 2nd ORF in the NDV F transcription unit using the In-Fusion® PCR Cloning Kit (Clontech). The resulting

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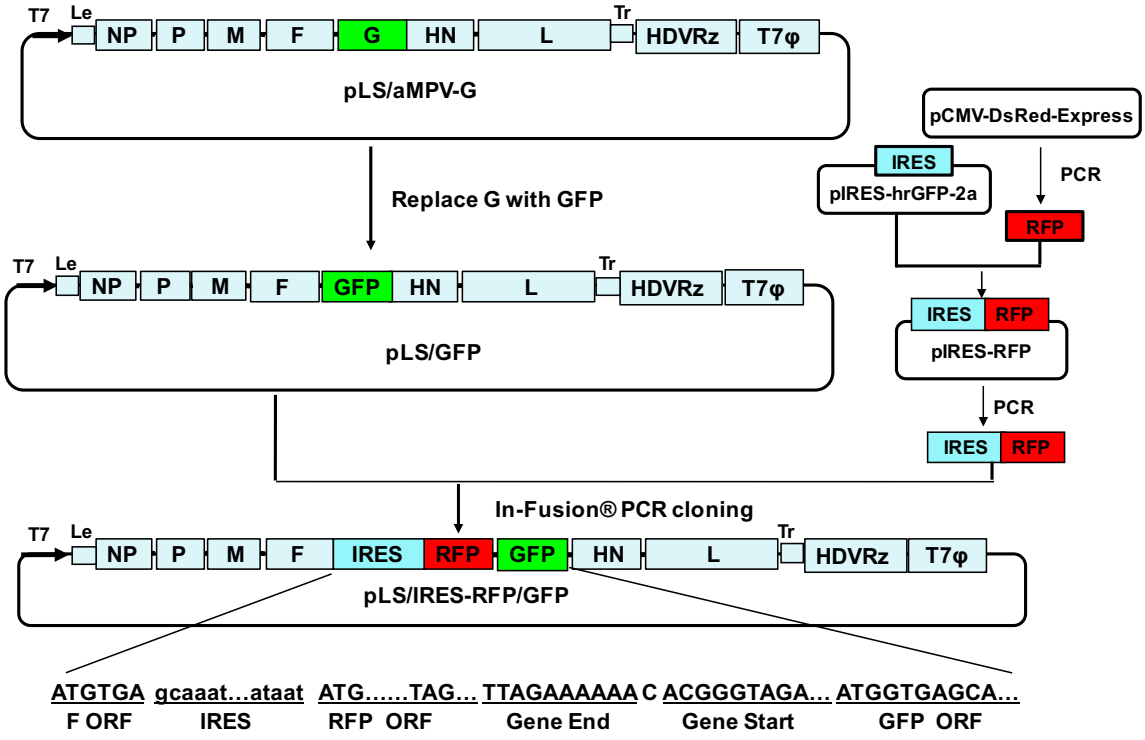

Fig. 1. Schematic representation of pLS/IRES-RFP/GFP construction. The ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies, Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), resulting in a subclone pLS/GFP. The RFP ORF, amplified from the plasmid pCMV-Ds-Red-Express (Clontech), was cloned downstream of the IRES sequence in the pIRES-hrGFP-2a vector (Clontech). Subsequently, the IRES and the RFP ORF sequences were amplified and cloned into the non-coding region of the NDV F gene in the pLS/GFP vector using an In-Fusion® PCR Cloning Kit (Clontech). The NDV Gene End and Gene Start signal sequences and the RFP and GFP sequences are underlined. The direction of the T7 promoter is indicated by a bold black arrow. HDVRz and T7Φ represent the site of the Hepatitis delta virus ribozyme and the T7 terminator sequences, respectively.

recombinant clone, designated as pLS/IRES-RFP/GFP, was amplified in Stbl2 cells at 30 °C for 24 h and purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

After co-transfection of the pLS/IRES-RFP/GFP clone and the supporting plasmids, encoding for the NDV NP, P, and L proteins, into HEp-2 cells, the rescued the LaSota strain-based recombinant virus vectoring the RFP and GFP genes, designated as rLS/IRES-RFP/GFP, was subsequently amplified in SPF chicken embryonated eggs as described previously [18]. The rLS/IRES-RFP/GFP virus was purified and further propagated in SPF chicken embryonated eggs for ten times. The fidelity and stability of the rescued rLS/IRES-RFP/GFP virus were examined by sequencing the isolated viral genome from the 10th egg passaged (EP) virus stock. There was no difference in genomic sequences between the EP10 virus stock and the recombinant cDNA clone (data not shown).

To determine if the addition of the two foreign genes, RFP and GFP, affects the viral replication and pathogenicity of the rLS/IRES-RFP/GFP virus, the hemagglutination (HA), 50% tissue culture infectious dose ($TCID_{50}$), 50% egg infective dose (EID_{50}), mean death time (MDT), and intracerebral pathogenicity index (ICPI) were examined [19]. As shown in Table 1, the recombinant virus appears to be slightly attenuated with a lower ICPI (0.0) in day-old chickens, longer MDT (>150 h) in embryos, and greater than a half-log reduction in viral yield in DF-1 cells compared to the parental LaSota strain. The viral yields of the recombinant virus grown in embryonated eggs, measured by EID_{50} and HA, were comparable to the yields of the parental LaSota strain (Table 1). Overall, the replication rate of rLS/IRES-RFP/GFP appears to be similar when compared to the replication rate of LaSota in DF-1 cells (Fig. 2).

The co-expression of the RFP and GFP proteins from rLS/ IRES-RFP/GFP infected DF-1 cells at 24 h post-infection was examined by fluorescence microscopy at 100 x magnification (Nikon, Eclipse Ti, Melville, NY) [17]. As shown in Fig. 3, both GFP (Fig. 3b) and RFP (Fig. 3c) expression was observed. After merging both fluorescent images (Fig. 3d), GFP and RFP co-localized to the same infected cells as seen by NDV induced viral cytopathic effects (CPE) observed under bright field (Fig. 3a). It is notable that there are a few infected cells that express GFP but not RFP that most likely can be attributed to the different promoters that drive the expression of the reporter genes or different timings of RFP and GFP

Table 1
Biological assessments of the rLS/IRES-RFP/GFP and parental LaSota viruses.

Virus	MDT[a]	ICPI[b]	HA[c]	EID_{50}[d]	$TCID_{50}$[e]
LaSota	110 h	0.15	1024	2.37×10^9	9.88×10^8
rLS/IRES-RFP/GFP	>150 h	0	512	2.37×10^9	1.76×10^8

[a] MDT: Mean death time assay in embryonated chicken eggs.
[b] ICPI: Intracerebral pathogenicity index assay in day-old chickens.
[c] HA: Hemagglutination assay.
[d] EID_{50}: 50% egg infective dose assay in embryonated chicken eggs.
[e] $TCID_{50}$: 50% tissue infectious dose assay in DF-1 cells.