Commentary

Have we found an Optimal Insertion Site in a Newcastle Disease Virus Vector to Express a Foreign Gene for Vaccine and Gene Therapy Purposes?

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Abstract | Different approaches have been used to investigate the most suitable insertion site for optimal expression of a foreign gene without compromising the replication of the Newcastle disease virus. Recently, inserting a green fluorescent protein (GFP) gene upstream from the GE sequences of the viral genes and subsequent quantitative measurements of the GFP fluorescence intensity, we have concluded that the P and M junction region is the optimal insertion site for a high level of foreign gene expression by a NDV vector.

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During late 1990s, reverse genetics technology was developed to rescue infectious Newcastle disease virus (NDV), a member of the Paramyxoviridae family, from cloned cDNAs (Peeters et al., 1999; Romer-Oberdorfer et al., 1999). This technology allows researchers to genetically manipulate the genome of the virus for study of the molecular biology of the virus (Estevez et al., 2011; Estevez et al., 2007; Kim and Samal, 2010; Marcos et al., 2005; Paldurai et al., 2014; Yan & Samal, 2008), and for development of the genetically engineered vaccines (Cardenas-Garcia et al., 2014; Miller et al., 2009). Since then, many strains of NDV have been developed as vectors to express a foreign gene for the purposes of vaccine and gene therapy using the reverse genetics technology (Bukreyev and Collins, 2008; DiNapoli et al., 2007; Huang et al., 2003; Kim et al., 2014; Nakaya et al., 2001; Schirrmacher and Fournier, 2009; Vigil et al., 2008). The evaluation of vaccine candidates in clinical trials revealed different levels of protection against targeted pathogen challenge (Bukreyev et al., 2005; Cardenas-Garcia et al., 2014; 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. Therefore, expressing the desired level of a foreign gene product from a NDV vector is a critical requisition for success of the vaccine and gene therapy candidates.

The level of foreign gene expression from a NDV vector can be affected by many factors, such as the growth ability and tissue tropism of the viral vector, the size and sequence of the foreign gene insert, and the genomic location of the foreign gene in the vector. Among these factors, the genomic location of the foreign gene is the most important one if the same insert and vector are being used. To date, most of the foreign genes are inserted into a non-coding region in

the NDV genome as an additional independent transcription unit (ITU) that consists in the order of NDV gene start (GS), the foreign gene, and NDV gene end (GE) sequences (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). According to the sequential transcription theory for negative stranded RNA viruses (Lamb and Parks, 2013), the best position for foreign gene expression would be the closest to the 3'end of NDV genome. However, the insertion of a foreign gene as an ITU into a promoter-proximal position may interfere with NDV replication more seriously than a promoter-distal position, resulting in lower levels of foreign gene expression (Carnero et al., 2009; Zhao and Peeters, 2003; Zhao et al., 2015). Therefore, a balance in virus replication and the abundance of foreign gene expression must be considered for selection of a foreign gene insertion site.

To identify an optimal insertion site for foreign gene expression, researchers have inserted a foreign gene into different gene junction regions of NDV vectors (3'-NP-P-M-F-HN-L-5') as an ITU, and evaluated the level of the foreign gene expression (Carnero et al., 2009; Ramp et al., 2011; Zhao and Peeters, 2003; Zhao et al., 2015). Zhao and Peeters (2003) found that insertion of an additional gene resulted in a delay in the onset of virus replication. This effect was most prominent when the gene was inserted between the NP and P genes. Ramp et al. (2011) reported that the expression levels of the foreign genes only differed moderately when placed in various positions. Whereas Carnero et al. (2009) showed that there was a gradient abundance of the foreign gene expression when it was inserted between the P and M genes and positions located after the M gene. Notably, the insertion of a foreign gene more proximal to the 3' end, between the NP and P gene, expressed a low level of the foreign protein. It is important to note that the positions of each insertion in the above studies are varied relative to the gene start of the downstream genes of NDV genome, which may influence the insert 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, Zhao et al. (2015) inserted a green fluorescent protein (GFP) gene as a reporter at 40 nucleotides upstream from 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 that positively correlated with the insertion site relative to the 3' end of the virus genome, except the insertion between NP and P genes that expressed a low level of GFP. Thus, they concluded that the P and M junction region is the optimal insertion site for a high level of foreign gene expression by a NDV vector.

In addition to the "conventional approach" for foreign gene expression though an ITU, different approaches for expression of a foreign gene by NDV have been explored. Wen et al. (2015) developed a new approach to express a foreign gene as a fusion protein with the NDV M protein followed by self-cleavage of FMDV 2A peptide and Ubiquitin coding sequences (Wen et al., 2015). After the cleavage, the foreign protein contains extra 20 amino acids (aa) at its 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. Gao et al. expressed foreign genes from two segmented NDV genomes (Gao et al., 2008). Basically, this approach still employed the ITU for foreign gene expression, but increased the capacity of expressing a larger gene or more than one foreign gene. However, there is a concern on the stability of the engineered segmented NDV because a majority of infectious NDV particles contain a single genome (Goff et al., 2012). Expression of a foreign gene though an internal ribosomal entry site (IRES) from a second open reading frame in a NDV vector is being investigated (Yu unpublished data), and results suggested that the NP gene downstream non-coding region is the optimal insertion site for a high level of foreign gene expression.

In summary, currently available data demonstrate that the P and M gene junction region is the optimal insertion site for a high level of foreign gene expression through an independent transcription unit. The gene junction regions between the M and F, and F and HN genes can also be used as insertion sites for a moderate level of foreign gene expression, whereas the NP and P gene junction region is not a good insertion site for foreign gene expression. The optimal insertion sites for foreign gene expression through other approaches

require further investigation.

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