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Short communication

Application of green fluorescent protein-labeled assay for the study of subcellular localization of Newcastle disease virus matrix protein

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A B S T R A C T

Green fluorescent protein (GFP) used as a powerful marker of gene expression *in vivo* has so far been applied widely in studying the localizations and functions of protein in living cells. In this study, GFP-labeled assay was used to investigate the subcellular localization of matrix (M) protein of different virulence and genotype Newcastle disease virus (NDV) strains. The M protein of ten NDV strains fused with GFP (GFP-M) all showed nuclear-and-nucleolar localization throughout transfection, whereas that of the other two strains were observed in the nucleus and nucleolus early in transfection but in the cytoplasm late in transfection. In addition, mutations to the previously defined nuclear localization signal in the GFP-M fusion protein were studied as well. Single changes at positions 262 and 263 did not affect nuclear localization of M, while changing both of these arginine residues to asparagine caused re-localization of M mainly to the cytoplasm. The GFP-M was validated as a suitable system for studying the subcellular localization of M protein and could be used to assist us in further identifying the signal sequences responsible for the nucleolar localization and cytoplasmic localization of M protein.

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Newcastle disease virus (NDV), a member of the *Avulavirus* genus of the family *Paramyxoviridae*, is an enveloped virus with a non-segmented, single-stranded, negative-sense RNA genome of approximately 15 kb, which contains six genes encoding the nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion protein (F), hemagglutinin-neuraminidase (HN) and large polymerase protein (L) (Alexander, 2000; Mayo, 2002; Miller et al., 2010). Of all these structural proteins, the M protein is the smallest protein and forms an outer protein shell around the nucleocapsid, constituting the bridge between the viral envelope and the nucleocapsid (Li et al., 1980; Peeples, 1991; Yusoff and Tan, 2001). Recent studies also reveal the remarkable genetic stability of M among isolates of different NDV genotypes (Panshin et al., 1997; Seal et al., 2000; Zanetti et al., 2003). In addition, the NDV M protein traffics to the cell nucleus, and by analogy with the vesicular stomatitis virus M protein could potentially function as an inhibitor of host cell nuclear functions (Ahmed et al., 2003; Yuan et al., 2001), in addition to functioning for assembly and budding of viral particles in the cytoplasm (Harrison et al., 2010; Pantua et al., 2006).

Like other paramyxoviruses, although NDV replication takes place in the cytoplasm, the M protein is localized primarily in the nucleus of infected cells in the absence of other viral proteins (Peeples et al., 1992). Previous studies have used immunofluorescence assay to examine the localization of M in NDV-infected cells (Peeples, 1988; Peeples et al., 1992). However, it has been demonstrated recently that green fluorescent protein (GFP) can be used as a powerful marker of gene expression *in vivo* without affecting the actual localization of the fused proteins (Phillips, 2001; Portugal et al., 2012; Rizzuto et al., 1995). More importantly, the localization of GFP-labeled proteins can be observed directly by fluorescence microscope and monitored in real time, thus providing a sensitive and new approach for the study of protein localization when expressed in living cells (Chalfie et al., 1994; Chudakov et al., 2010; Phillips, 2001). In this study, 12 different virulence and genotype NDV strains were selected (Table 1) and the M gene open reading frame was amplified from NDV genomic cDNA by PCR amplification using the specific primers. The PCR products were digested with *Eco*RI/*Sal*I restriction enzymes and then inserted into *Eco*RI/*Sal*I-digested green fluorescent protein mammalian expression vector pEGFP-C1 (Clontech) to create pEGFP-M. The subcellular localizations of these NDV M proteins fused to the C-terminal end of GFP

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Table 1
NDV strains used in this study.

Virus	Genotype	F cleavage site	Nuclear localization signal[a]	Accession number[b]	Pathotype
Herts/33	IV	^{112}R-R-Q-R-R↓F^{117}	**KKGKK**VTFDKLEKKI**RR**	AY741404	Velogenic
JS/5/05/Go	VIId	R-R-Q-K-R↓F	**KRGKK**VTFDKIEEKI**RR**	JN631747	Velogenic
QH-4/85	VIII	R-R-Q-K-R↓F	**KRGKK**VTFDKLEEKI**RR**	–	Velogenic
F48E8	IX	R-R-Q-R-R↓F	**KRGRK**VTFDKLERKI**RR**	FJ436302	Velogenic
Mukteswar	III	R-R-Q-R-R↓F	**KRGKK**VTFDQLERKI**RR**	EF201805	Mesogenic
JS/07/04/Pi	VIb	R-R-R-K-R↓F	**KKGKK**VTFDKIEEKI**RR**	FJ766530	Mesogenic
JS/07/22/Pi	VIb	R-R-Q-K-R↓F	**KRGKK**VTFDKIEEKI**RR**	FJ766526	Mesogenic
YZ/21/07/Pi	VIb	R-R-R-K-R↓F	**KKGKK**VTFDKIEEKI**RR**	–	Mesogenic
LX	I	G-K-Q-G-R↓L	**KKGKK**VTFDKIEEKI**RR**	–	Lentogenic
V4	I	G-K-Q-G-R↓L	**KKGKK**VTFDKLERKI**RR**	JX524203	Lentogenic
LaSota	II	G-R-Q-G-R↓L	**RKGKK**VTFDKLEKKI**RS**	JF950510	Lentogenic
XZ/7/07/Ch	II	G-R-Q-G-R↓L	**KKGKK**VTFDKIEEKI**RR**	–	Lentogenic

[a] The nuclear localization signal of NDV M proteins is located at position 247–263, with the key basic amino acids highlighted in bold.
[b] The accession number of complete genome sequences of 12 NDV strains is given if available.

were examined in living cells by fluorescence microscopy at 24 h post-transfection (hpt).

The subcellular localization of GFP-M of NDV strain JS/5/05/Go was first examined in transfected COS-1 cells. As shown in Fig. 1A, the GFP-M fusion protein was found primarily in the nucleus and nucleolus (as indicated by the white arrows), with less fluorescence in the cytoplasm. This result behaved in an identical manner to that of untagged M expressed by a eukaryotic vector pBC-M (Coleman

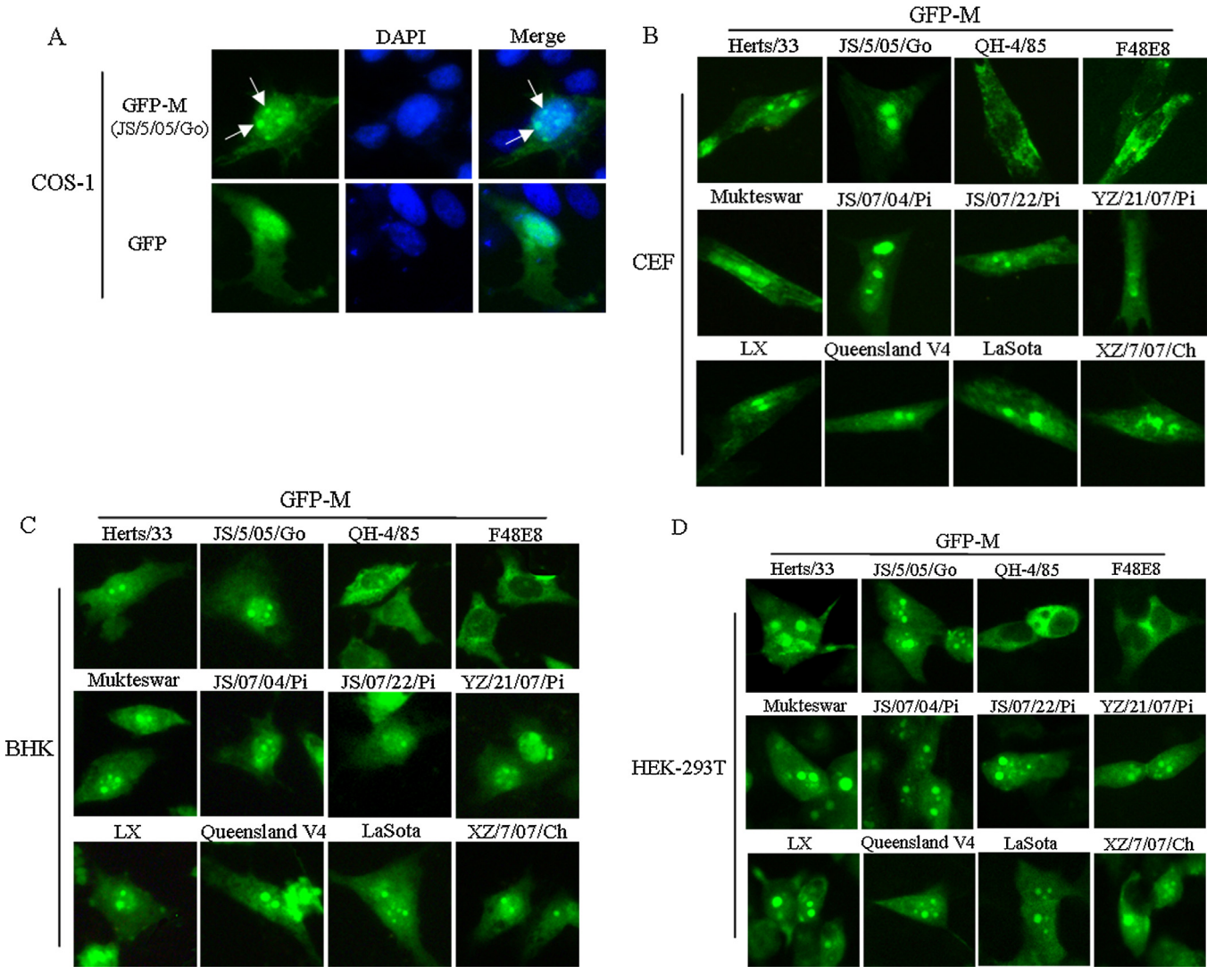

Fig. 1. The subcellular localization of NDV M proteins fused with GFP in living transfected cells. The M gene open reading frame was amplified from NDV genomic cDNA by PCR amplification using Pfx50^{TM} DNA polymerase (Invitrogen) and the primers used were 5′ TCGGAATTCAATGGACTCATCCAG 3′ and 5′ GGCAGTCGACTTATTTCCTGAAAGG 3′. The products were digested with *Eco*RI/*Sal*I and inserted into pEGFP-C1 vector (Clontech) cut with *Eco*RI/*Sal*I to create pEGFP-M. The recombinant expression plasmids were purified using a Plasmid Mini Kit (Qiagen) and then transfected into COS-1, CEF, BHK and HEK-293T cells, respectively. At 24 h post-transfection, cells were washed with fresh growth medium and observed by fluorescence microscope. Representative images are shown in A, B, C and D, respectively.

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