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Genetically modified VSV_{NJ} vector is capable of accommodating a large foreign gene insert and allows high level gene expression

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ABSTRACT

It is desirable to develop a RNA virus vector capable of accommodating large foreign genes for high level gene expression. Vesicular stomatitis virus (VSV) has been used as a gene expression vector, especially Indiana serotype (VSV_{Ind}), but less with New Jersey serotype (VSV_{NJ}). Here, we report constructions of genetically modified $rVSV_{NJ}$ vector carrying various lengths of human hepatitis C virus (HCV) non-structural (NS) protein genes, level of inserted gene expression and characterization of $rVSV_{NJ}$. We modified the M gene of VSV_{NJ} by changing methionine to arginine at positions 48 and 51 ($rVSV_{NJ}$-M) (Kim and Kang, 2007) for construction of $rVSV_{NJ}$ with various lengths of HCV non-structural genes. The NS polyprotein genes of HCV were inserted between the *G* and *L* genes of the $rVSV_{NJ}$-M vector, and recombinant VSV_{NJ}-M viruses with HCV gene inserts were recovered by the reverse genetics. The recombinant VSV_{NJ}-M vector with the HCV NS genes express high levels of all different forms of the NS proteins. The electron microscopic examination showed that lengths of recombinant VSV_{NJ}-M without gene of interests, VSV_{NJ}-M with a gene of HCV NS3 and NS4A (VSV_{NJ}-M-NS3/4A), VSV_{NJ}-M with a gene of HCV NS4AB plus NS5AB (VSV_{NJ}-M-NS4AB/5AB), and VSV_{NJ}-M carrying a gene of HCV NS3, NS4AB, and NS5AB (VSV_{NJ}-M-NS3/4AB/5AB) were 172 ± 10.5 nm, 201 ± 12.5 nm, 226 ± 12.9 nm, and 247 ± 18.2 nm, respectively. The lengths of recombinant VSVs increased approximately 10 nm by insertion of 1 kb of foreign genes. The diameter of these recombinant viruses also increased slightly by longer HCV gene inserts. Our results showed that the recombinant VSV_{NJ}-M vector can accommodate as much as 6000 bases of the foreign gene. We compared the magnitude of the IFN induction in mouse fibroblast L(Y) cells infected with $rVSV_{NJ}$ wild type and $rVSV_{NJ}$ M mutant viruses and show that the $rVSV_{NJ}$ M mutant virus infection induced a higher level of the IFN-β compare to the wild type virus. In addition, we showed that the NS protein expression level in IFN-incompetent cells (Mouse-L) infected with $rVSV_{NJ}$-M viruses was higher than in IFN-competent L(Y) cells. In addition, we confirmed that HCV NS protein genes were expressed and properly processed. We also confirmed that NS3 protein expressed from the $rVSV_{NJ}$-M cleaves NS polyprotein at junctions and that NS4A plays an important role as a co-factor for NS3 protease to cleave at the NS4B/5A site and at the NS5A/5B site.

1. Introduction

The vesicular stomatitis virus (VSV) is a non-segmented negative-sense RNA virus, which belongs to the *Rhabdoviridae*. The wild type VSV virion is bullet-shaped with 170 nm in length and 65 nm in diameter. The VSV virion is comprised of a helical ribonucleoprotein core and surrounding lipid envelope bilayer that is derived from the host cell. The VSV has two major serotypes, VSV Indiana (VSV_{Ind}) and VSV New Jersey (VSV_{NJ}). The VSV_{Ind} genome contains 11,161 nucleotides, which encodes five major viral structural proteins; nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and viral RNA-dependent RNA polymerase (L) (Abraham and Banerjee, 1976; Clarke et al., 2006; Haglund et al., 2000). The Hezelhurst subtype of VSV_{NJ} genome is 11,142 nucleotides and also codes for five structural proteins. Each viral protein is translated from its own subgenomic monocistronic mRNA. There are conserved, untranslated, intergenic junctions between the genes, which contain an 11 nucleotides transcription termination and polyadenylation signals and the transcription reinitiation signals for the subsequent downstream gene (Rose, 1980; Rose and Schubert, 1987).

The procedure for generating replication-competent VSV entirely from cDNA clone has been established using the reverse genetics (Lawson et al., 1995; Whelan et al., 1995). The genetic malleability of VSV has allowed the development of recombinant VSVs (rVSVs) that express foreign proteins in high levels.

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Those foreign genes were functional influenza virus glycoproteins (Kretzschmar et al., 1997) and bacterial enzyme chloramphenicol acetyltransferase (Schnell et al., 1996). The rVSV became attractive as a host vector for recombinant vaccine development because of its wide host range, rapid replication, and mild pathogenicity in humans.

Until now, most of the experiments of foreign gene expression using VSV vector have been carried out by using VSV_{Ind} serotype. Recently, we developed a recombinant VSV_{NJ} serotype vector system as an expression vector in our laboratory (Kim and Kang, 2007). The matrix protein of VSV induces cytopathic effect (CPE) by disorganizing cytoskeletal elements such as actin or tubulin (Blondel et al., 1990; Melki et al., 1994). The general inhibition of host cell gene expression (Black and Lyles, 1992) can result in the systemic breakdown of the cell by apoptosis by VSV-M (Kopecky and Lyles, 2003). We showed that VSV_{NJ} carrying methionine to arginine mutation in amino acid positions 48 and 51 in M protein (M48R–M51R) loses its inhibitory effect on the host cell gene expression (Kim and Kang, 2007). We also reported expression and processing of human immunodeficiency virus type 1 (HIV-1) gp160 using the VSV_{NJ} vector system (Wu et al., 2009). Here we report expression and processing of non-structural genes of hepatitis C virus using the $rVSV_{NJ}$ vector.

Hepatitis C virus belongs to the *Hepacivirus* genus of the family *Flaviviridae*. The HCV genome is a positive sense RNA with 9600 nucleotides (nt), which has a single open-reading frame (ORF) encoding for a large polyprotein precursor of approximately 3010 amino acids. The polyprotein precursor is cleaved into ten different proteins by both host peptidase and viral serine protease (Blight et al., 2002; Paredes and Blight, 2008; Pavio and Lai, 2003). The polyprotein of the HCV cleaves to the structural proteins Core protein (C), E1 envelope glycoprotein (E1) and E2 envelope glycoprotein (E2), and a small hydrophobic polypeptide named p7. In addition, the HCV genome codes for the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Yi and Lemon, 2003; Friebe and Bartenschlager, 2002; Paredes and Blight, 2008; Shimakami et al., 2004; Suzuki et al., 2007; Lin et al., 1994). NS3 (69 kDa) is a multifunctional protein that carries a serine protease activity in the N-terminal domain and a RNA helicase/NTPase activity in the remaining C-terminal portion of the protein. NS3 protease cleaves at all downstream NS protein junctions with NS4A (54 aa) as a co-factor (Konan et al., 2003; Paredes and Blight, 2008). The NS4A interacts with the N terminus of the NS3 through its central hydrophobic domain (Bartenschlager et al., 1994; Failla et al., 1995; Satoh et al., 1995). The NS4A is absolutely required for cleavage at the 4B/5A junction, but is not absolutely required for cleavage at 5A/5B junction. Several studies have demonstrated formation of a stable NS3/4A complex (Failla et al., 1994; Koch et al., 1996; Lin et al., 1995; Tanji et al., 1995).

In this study, we describe cDNA clones of recombinant VSV_{NJ}-M vector containing various lengths of the HCV NS polyprotein genes up to 5955 nucleotides, show that the replication of the recombinant viruses is not affected by the size of inserted exogenous genes. The morphology of all recombinant VSV_{NJ}-M viruses was characterized by electron microscopy with negative staining. In addition, we confirmed that NS3 protein and NS4A protein expressed from $rVSV_{NJ}$ are functional as a protease and as a co-factor of NS3 protease.

2. Materials and methods

2.1. Cell culture

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Gibco BRL), 100 U/ml of penicillin, 100 μg/ml of streptomycin and kanamycin, and 2 mM L-glutamine (5% FBS C-DMEM). BHK-T7 (Buchholz et al., 1999) cells were cultured in 5% FBS C-DMEM containing antibiotic 500 μg/ml G418 and BHK-T7 cells for transfection were grown in DMEM containing 5% FBS and L-glutamine without antibiotics. IFN-incompetent mouse-L and IFN-competent IFN-L(Y) mouse fibroblasts, were grown in C-DMEM containing 10% FBS and in MEM containing 5% FBS, plus 100 U/ml of penicillin, 100 μg/ml of streptomycin and kanamycin, 5 ml sodium pyruvate, and 2 mM L-glutamine, respectively.

2.2. Cloning of HCV NS genes into $prVSV_{NJ}$-(M48R+M51R)

For the expression of HCV non-structural (NS) polyproteins, we used pBluescript II KS (pBKS) subcloning vector, recombinant VSV_{NJ}-M (mutated M protein, M48R+M51R; Kim and Kang, 2007) (Fig. 1A) as expression vector, and HCV (H77 1a) (Blight et al., 2003) genome as PCR template. To get the HCV protein genes (Fig. 1B), the first, we performed PCR. The primers for amplification of HCV polyprotein genes are shown in Table 1. The forward primers for PCR contain intergenic (IG) junction sequences of recombinant VSV_{NJ}-M. The purified cDNAs of HCV protein genes, which are NS3, NS4A, NS3/4A, NS3/4A/4B, NS4A/4B, NS4B/5A, NS5A, NS5B, NS5A/5B, NS4B/5A/5B, NS4A/4B/5A/5B, and NS3/4A/4B/5A/5B, amplified by PCR were purified by using purification kit (Promega) and digested by *Kpn*I. And then the processed HCV genes were cloned into pBKS treated with same enzyme. And sequences of these clones were analyzed by sequencing using the primers in Table 1. HCV NS genes were cut from the recombinant pBKS plasmids, pBKS-NS3, pBKS-NS4A, pBKS-NS3/4A, pBKS-NS3/4A/4B, pBKS-NS4A/4B, pBKS-NS4B/5A, pBKS-NS5A/5B, pBKS-NS4B/5A/5B, pBKS-NS4A/4B/5A/5B, and pBKS-NS3/4A/4B/5A/5B with *Kpn*I, and then the HCV genes were again cloned into $rVSV_{NJ}$-M at the *Kpn*I site, which is between *G* and *L* gene (Fig. 1A). The $rVSV_{NJ}$-M plasmids with HCV NS protein genes were designated as $prVSV_{NJ}$-M-NS3, $prVSV_{NJ}$-M-NS4A, $prVSV_{NJ}$-M-NS3/4A, $prVSV_{NJ}$-M-NS3/4A/4B, $prVSV_{NJ}$-M-NS4A/4B, $prVSV_{NJ}$-M-NS4B/5A, $prVSV_{NJ}$-M-NS5A/5B, $prVSV_{NJ}$-M-NS4B/5A/5B, $prVSV_{NJ}$-M-NS4A/4B/5A/5B, and $prVSV_{NJ}$-M-NS3/4A/4B/5A/5B, respectively. The constructed $rVSV_{NJ}$-M plasmids were used to get the virus.

2.3. Plasmid DNA Transfection

BHK-T7 cell line, which expresses bacteriophage T7 RNA polymerase, was used for the recovery of virus. Transfection was carried out using Lipofectamine 2000 (Gibco-BRL/Invitrogen) according to the manufacturer's protocol. The cells were split to be 70–90% confluent at the time of transfection using the media, DMEM containing 5% FBS and L-glutamine, without antibiotics, about 20 h before the transfection, and were changed the media 2 h before the transfection with 7 ml same media. And briefly, the BHK-T7 cells were co-transfected with 10 μg of pBKS-IRES/N_{NJ}, 10 μg of pBKS-IRES/P_{NJ}, 5 μg of pBKS-IRES/L_{NJ} (Kim and Kang, 2007) and 1.6 pmol of the recombinant VSV_{NJ}-M plasmids, and 60 μl Lipofectamine 2000 was added. The culture media was harvested when more than 70% of cells showed cytopathic effects (CPE) at approximately 4–6 days of incubation after transfection. The recovered viruses were purified by three consecution plaque picking and viral stocks were prepared in BHK-21 cells. To get the titer of the recombinant viruses, BHK-21 cells were infected with the viruses using M.O.I. of 0.1, and then the culture media was harvested at 16–20 h postinfection. Viral titers of the amplified viruses were determined by the plaque assay.