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Protocols

Partial deletion in the spike endodomain of mouse hepatitis virus decreases the cytopathic effect but maintains foreign protein expression in infected cells

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ABSTRACT

Mouse hepatitis virus (MHV) produces a series of subgenomic RNAs for viral protein expression. As a prototype coronavirus, MHV has been explored extensively and is often used to express foreign proteins. Previously, a 13-residue deletion in the MHV spike (S) protein endodomain was found to reduce syncytium formation dramatically while inhibiting virus replication slightly. In this study, the effects of the S mutation on MHV infectivity and foreign protein expression were further examined in rat or mouse L2, NIH/3T3 and Neuro-2a cells. The replacement of the MHV 2a/haemagglutinin-esterase gene with a membrane-anchored protein hook (HK) and replacement of gene 4 with EGFP did not change the adaptability and cytopathology of recombinant viruses in these cells. However, the cytopathic effect of the recombinants with the partial S deletion was reduced significantly in these cells. The replication and foreign protein expression of the S-mutated recombinants were found to be more efficient in L2 cells than in Neuro-2a and NIH/3T3 cells. Meanwhile, the distribution patterns of HK and EGFP expressed by the recombinant viruses were similar to those in cells transfected with a eukaryotic expression vector. These results suggest that the partial deletion in the S endodomain may increase the usefulness of MHV as a viral vector by attenuation and maintaining foreign protein expression.

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1. Introduction

Coronaviruses are enveloped, positive-strand RNA viruses that cause respiratory, enteric and neurological diseases in mammalian and avian hosts (Masters, 2006; Siddell, 1995). Mouse hepatitis virus (MHV), a prototype coronavirus, is an important pathogen in laboratory animals (Baker, 2003), and the genetics and pathogenesis of this virus have been studied extensively (Masters, 2006; Siddell, 1995). The genomic RNA of MHV is about 30 kb long with a 5'-cap and a 3'-poly A tail. MHV produces seven subgenomic (sg) RNAs in infected cells (Goebel et al., 2004; Spaan et al., 2005). sgRNA1 is equivalent to the genomic RNA and encodes the replicase. sgRNA2 is responsible for the expression of open reading frame (ORF) 2a and haemagglutinin-esterase (HE), sgRNA3 for the spike (S) protein, sgRNA4 for ORFs 4a and 4b, sgRNA5 for ORF 5a and the envelope (E) protein, sgRNA6 for the membrane (M) protein and sgRNA7 for the nucleocapsid (N) protein (Masters, 2006; Siddell, 1995; Spaan et al., 2005).

It is known that genes 2a/HE and 4 of MHV are nonessential for viral replication in cultured cells. Studies show that deletion of MHV gene 4 has no effect on viral replication in cells or on viral pathogenesis in mice (de Haan et al., 2002; Ontiveros et al., 2001). Gene 2a is believed to have been acquired horizontally from sequences encoding cellular or viral 2',3'-cyclic phosphodiesterases (Mazumder et al., 2002). HE is a typical transmembrane glycoprotein related to the haemagglutinin of influenza C virus (Snijder et al., 2003) and acts as a cofactor of the S protein in the binding of virus to cells and in the release of virus from cells (Lissenberg et al., 2005). Thus, replacement of MHV genes 2/HE and 4 with foreign genes allows the possibility of using MHV as an expression vector (de Haan et al., 2003; Enjuanes et al., 2001).

Targeted RNA recombination, a viral reverse genetics technique, has been used to generate recombinant MHVs with mutations in the structural genes or the 3'-UTR (Kuo et al., 2000; Masters and Rottier, 2005). Recombinant MHVs with enhanced green fluorescent protein (EGFP) at the gene 4 site were generated to analyse viral pathogenesis in the mouse central nervous system and the interaction of the N protein with structural proteins during virion assembly (Das Sarma et al., 2002; Hurst et al., 2005). HE and the 2a gene have been replaced with a series of constructed membrane-anchored fusion proteins (HK), and functional virus particles could be assembled when a homologous S protein membrane-integrating region was introduced (Ye et al., 2004).

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The tissue specificity and pathogenicity of MHVs are different among laboratory isolates, and these differences are attributed to the S protein (Perlman and Butler, 2008; Masters, 2006; Siddell, 1995; Stohlman et al., 1998; Weiss and Navas-Martin, 2005). For example, the A59 strain causes primarily moderate hepatitis and can persistently infect the central nervous system when inoculation occurs via the nasal or brain routes (Perlman and Butler, 2008; Stohlman et al., 1998). The JHM strain, on the other hand, is associated with severe fatal encephalitis and severe neurovirulence (Perlman and Butler, 2008; Stohlman et al., 1998; Weiss and Navas-Martin, 2005). It was found that the partial deletion of the endodomain of the MHV S protein decreased syncytium formation dramatically, but there was no significant reduction in the level of viral replication in L2 cells (Ye et al., 2004). Thus, the S mutation might attenuate the toxicity of MHV when it is used as a vector for foreign protein expression. In this study, some features of recombinant MHVs with the S mutation were examined in cultured cells, including replication phenotypes, tropism for murine cell lines and expression of foreign proteins. The eukaryotic DNA expression vector pIRES2–EGFP was used as a control for the distribution of foreign proteins.

2. Materials and methods

2.1. Plasmid construction

The donor RNA transcription vectors pMH54, pHKP1 and pMH54GFP were kindly provided by Dr. Paul S. Masters (Wadsworth Center, New York State Department of Health, Albany, NY). pMH54GFP was developed by Susan Weiss' Laboratory and was modified by Paul S. Masters' Laboratory (Das Sarma et al., 2002; Hurst et al., 2005). A fragment with a 14-aa deletion (Fig. 1A) of a cysteine-rich motif in the S endodomain of MHV was amplified by splicing overlap extension PCR (SOE-PCR) (Aiyar et al., 1996; Ye et al., 2004). The fragment was inserted into pMH54 between the *Mlu* I and *Sbf* I sites to produce pMH-SΔCR (Fig. 1B). This fragment was also inserted into pMH54GFP with the same restriction enzymes to construct an intermediate clone. Then, a restriction fragment from pHKP1 between *Rsr* II and *Avr* II was inserted into the intermediate clone to construct the triple mutant pMH-H/S/G (Fig. 1B). In pHKP1, the 2a and HE genes of MHV were replaced with a membrane-anchored fusion protein (HK) in which the signal peptide and the ectodomain were from the pHook1 vector (Invitrogen) and the transmembrane domain and the endodomain were from the S protein of MHV A59 (Ye et al., 2004). To construct a eukaryotic expression vector, an HK fragment from pHKP1 was cut out by *Eco*R V and *Asc* I. The fragment was blunted with Klenow and inserted into the *Sma* I site of the pIRES2–EGFP vector (Clontech, BD Bioscience).

2.2. Cells and viruses

Rat lung epithelial cells (L2), *Felis catus* whole fetus (FCWF) cells and MHV stocks were kindly provided by Dr. Paul S. Masters. NIH/3T3 and Neuro-2a cells were purchased from the Institute of Cellular Biology, China Academy of Sciences. Cells were grown in minimum essential medium (MEM) or Dulbecco's modified Eagle medium (DMEM) (GIBCO, Invitrogen) with 5–10% fetal bovine serum (FBS) (HyClone) and 0.20–0.37% sodium bicarbonate (Sigma–Aldrich) in a humidified 5% CO₂ atmosphere at 37 °C. Generally, MHV recombinants infected monolayers of cells at 60–80% confluence with a multiplicity of infection (MOI) of 0.1–1.0 for 2 h at 37 °C. Further treatments were performed at 8–72 h post-infection (hpi) according to a variety of protocols. MHV recombinants were produced following the procedure of targeted RNA recombination

(Kuo et al., 2000; Masters and Rottier, 2005). Briefly, donor RNA transcription vectors were digested by *Pac* I and transcribed into mRNAs using the mMESSAGE mMACHINE T7 Ultra kit (Ambion). The mRNAs were then transfected into FCWF cells that had been infected previously (2 h) with an engineered fMHV. The transfection was conducted using one pulse at a setting of 975 μF and 0.3 kV with a Gene Pulser Xcell electroporation system (Bio-Rad). The infected/transfected FCWF cells were overlaid onto L2 monolayers. Culture supernatants containing recombinant viruses were collected from 24 to 72 hpi. Recombinant viruses were purified by two rounds of single-plaque-picking on L2 cells. The identities of the recombinant viruses were verified by RT-PCR and sequencing.

2.3. Plaque assay

L2 cells were grown in 60-mm dishes and were infected with 1 ml of media containing viruses at dilutions ranging from 10⁻³ to 10⁻⁶. Seven millilitres of 0.95% agar (Amresco) in DMEM with 5% FBS was overlaid onto cells 2 hpi. Plaques were picked between 48 and 72 hpi. For plaque staining, 3 ml of agar with 0.02% neutral red (Sigma–Aldrich) was overlaid to a dish. Six to eight hours later, the stained plaques were counted or photographed using a white light transilluminator (Upland).

2.4. Preparation of cell membrane fractions

The infected or transfected cells were washed twice with ice-cold phosphate-buffered saline (PBS), and the membrane fraction was then prepared with two protocols. The cellular proteins were separated into cytoplasmic and membrane fractions using the Membrane Extraction Kit (membrane I) following the manufacturer's protocol (Bio-Rad). Additionally, the membrane fraction was prepared by ultracentrifugation as described previously (Cushman and Wardzala, 1980). Briefly, cells were gently scraped into 1 ml of ice-cold HES homogenisation buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Hepes, pH 7.4) containing protease inhibitors. Cells were homogenised by repeated passing through a 25-G syringe needle 30 times and were incubated on ice for 20 min. Nuclei and unbroken cells were removed by centrifuging at 1000 × g for 10 min at 4 °C. The resulting supernatant was separated into the membrane fraction (pellet) and the cytosol fraction (supernatant) by centrifuging at 100,000 × g for 1 h at 4 °C. The pellet was suspended completely in 1 ml of HES buffer with 1% Triton X-100 or 1% SDS and was incubated for 10 min. The insoluble material was removed by centrifuging at 10,000 × g for 5 min.

2.5. Fluorescence and confocal microscopy

The expression of HK and EGFP was detected using fluorescence and immunofluorescence. Neuro-2a and L2 cells were grown on glass coverslips to approximately 60% confluence. The cytopathic effect (CPE) was observed and photographed under an inverted microscope between 8 and 48 hpi. EGFP fluorescence was observed and photographed using a Zeiss Axiovert 200 fluorescence microscope. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 30 min and permeabilised in 0.1% Triton X-100 for 5 min. Fixed cells were blocked with 3% BSA for 30 min and were incubated with 1.0 μg/ml monoclonal antibody (Roche) to the haemagglutinin (HA) tag overnight at 4 °C, followed by incubation with TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. After the nuclei were stained with DAPI, slides were mounted with Mowiol antifade reagent (Sigma–Aldrich). Confocal images were captured using a Leica TCS-SP5 laser-scanning confocal microscope. Digital images were analysed with LAS AF software.

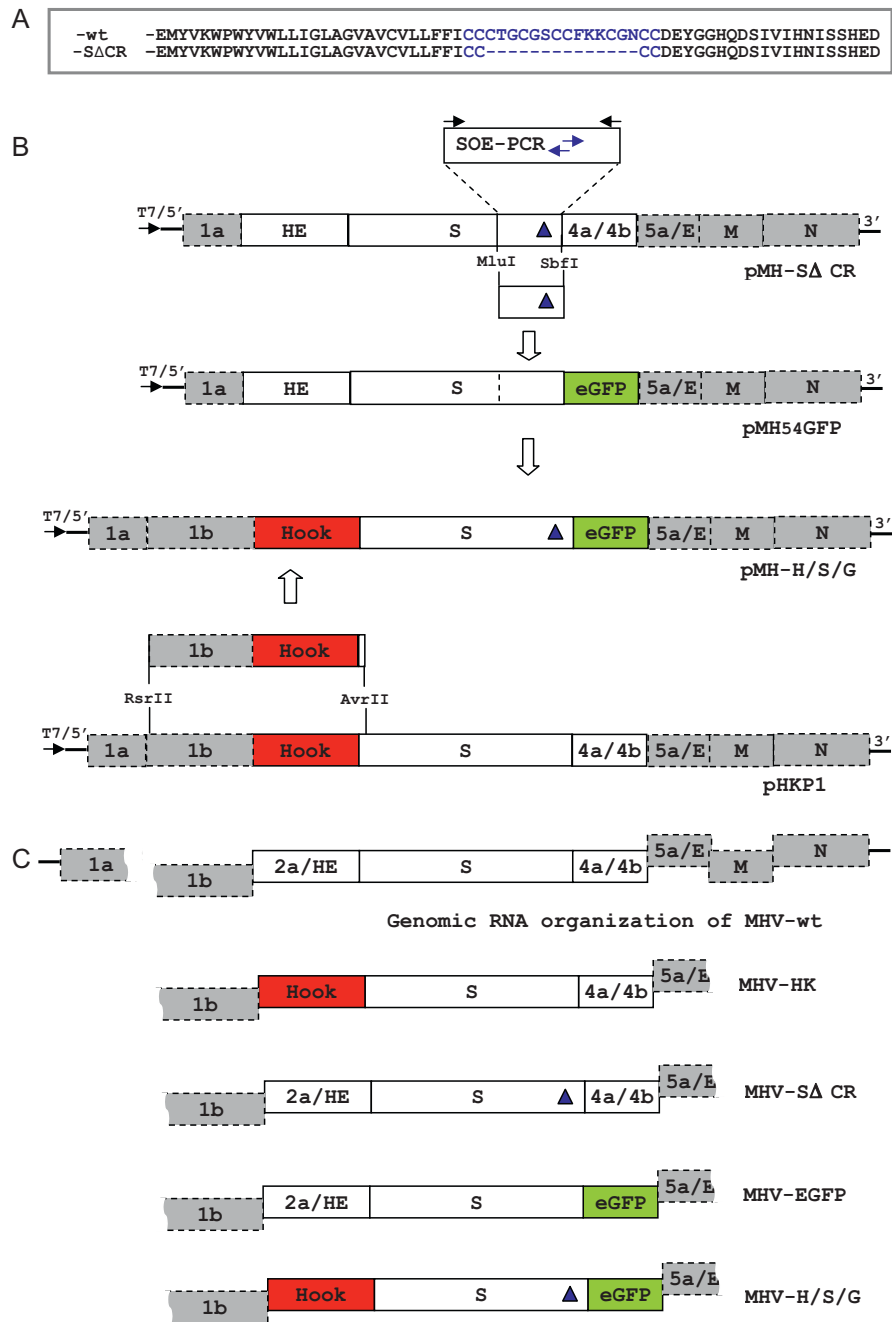


Fig. 1. Construction of donor RNA transcription vectors and generation of recombinant MHVs. (A) Deleted amino acid sequence encoding a cysteine-rich motif in the S endodomain of MHV A59. (B) Partial deletion of the S endodomain was achieved by SOE-PCR, and this deletion was introduced into pMH54 to create the donor RNA transcription vector pMH-SΔCR. In pMH-H/S/G, foreign gene replacements were performed using a multi-step removal and insertion of restriction fragments from pMH54GFP and pHPK1. (C) Genomic organization of wild-type MHV and four recombinant MHVs with replacements of and/or deletions in three adjacent genes, 2a/HE, S and 4.

2.6. Western blotting

The infected or transfected cells were washed twice with ice-cold PBS and collected in IPP buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% Nonidet P-40 and 0.16% protease inhibitor cocktail (Roche). Cell lysates were incubated on ice for 5 min and were clarified by centrifuging at $12,000 \times g$ for 5 min at 4°C . Protein samples were mixed with an equal volume of $2 \times$ sample buffer and were heated at 95°C for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h in 5% non-fat milk and were incubated with $0.4 \mu\text{g/ml}$ monoclonal antibodies to the HA tag or GFP

(Roche) overnight at 4°C , followed by incubation with horseradish peroxidase-conjugated secondary antibody. The signal was developed using Amersham ECL (GE).

3. Results

3.1. Generation of recombinant MHVs

MHV A59-derived mutants were generated by targeted RNA recombination and L2 cell selection. Fig. 1A shows the deleted amino acid sequences encoding a cysteine-rich motif in the S endodomain of MHV A59. Four donor RNA transcription vectors derived from pMH54 were used to generate recombinants with the

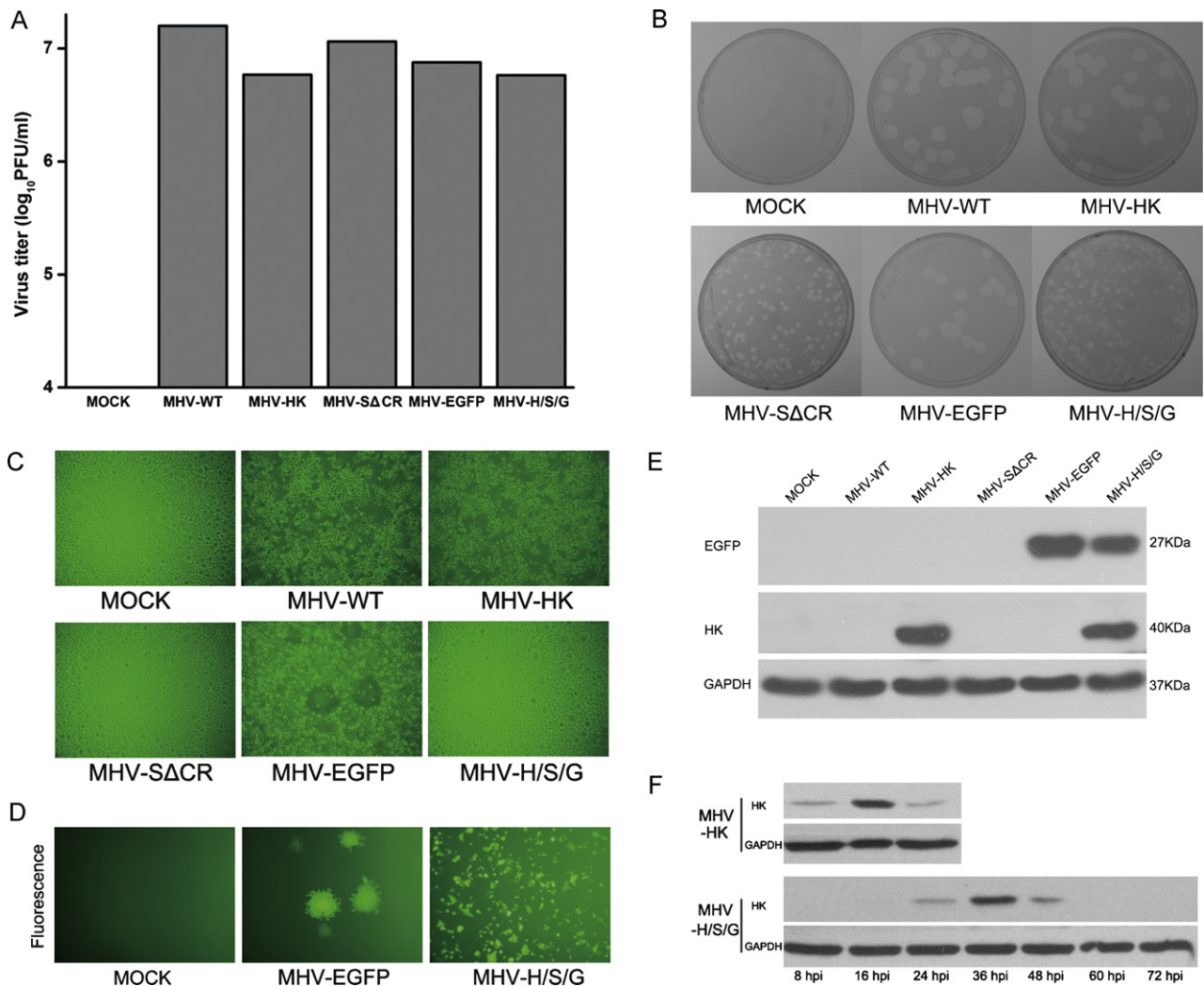


Fig. 2. Phenotype and foreign protein expression of recombinant MHVs in L2 cells. (A) The peak titres of MHV-WT and recombinant viruses (MHV-HK, MHV- Δ S Δ CR, MHV-EGFP and MHV-H/S/G). Mock infection indicates cells cultured in a medium without virus. The titre is the logarithmic value of PFU per millilitre of supernatant collected at peak points of viral production. (B) The plaque morphology of the viruses at 24 h (MHV-WT, MHV-HK and MHV-EGFP) and 48 h (MHV- Δ S Δ CR and MHV-H/S/G) post-infection. (C) CPE of the viruses at 12 h (MHV-WT, MHV-HK and MHV-EGFP) and 24 h (MHV- Δ S Δ CR and MHV-H/S/G) post-infection. (D) Fluorescence of EGFP was observed by fluorescence microscopy. (E) EGFP and HK in cellular lysates at peak infection stages were detected by Western blotting. (F) HK in cellular lysates at different infection stages was detected by Western blotting.

S deletion mutation and with foreign gene replacements (Fig. 1B). Mutations were focused on three adjacent structural genes: genes 2 (2a/HE), 3 (S) and 4 (ORF: 4a/4b) (Fig. 1C). Mutants with the partial S deletion (designated MHV- Δ S Δ CR), HK replacement (designated MHV-HK) or EGFP replacement (designated MHV-EGFP) and a combined mutant (designated to MHV-H/S/G) in which the S mutation and the two replacements were all present were constructed using a strategy similar to that used in previous work (Hurst et al., 2005; Ye et al., 2004).

3.2. Cytopathology and foreign protein expression of recombinant MHVs in L2 cells

Plaque assays in L2 cells were carried out for titre determination and biological purification of the recombinant MHVs. Fig. 2A shows that the peak titre of wild-type MHV (MHV-WT) was 1.58×10^7 plaque-forming units (PFU)/ml. The titres of the four mutants (MHV-HK, MHV- Δ S Δ CR, MHV-EGFP and MHV-H/S/G) were between 0.5×10^7 and 1.2×10^7 PFU/ml, slightly lower than that of MHV-WT. Moreover, MHV-EGFP and MHV-HK were very similar to MHV-WT in terms of the size and morphology of the plaques (Fig. 2B).

MHV- Δ S Δ CR and MHV-H/S/G formed small plaques compared with MHV-WT. Similarly, the CPE of the recombinant viruses was used to divide the viruses into two groups: MHV-EGFP and MHV-HK formed typical syncytia similar to MHV-WT, and MHV- Δ S Δ CR and MHV-H/S/G did not produce significant CPE (Fig. 2C). The expressed EGFP was directly examined by fluorescence microscopy (Fig. 2D). The immunoblot showed that the two foreign genes, HK and EGFP, were expressed efficiently in L2 cells infected with either wild-type or mutant S viruses (Fig. 2E). The time-course experiments showed that the peak expression of HK proteins in L2 cells by mutant S viruses (MHV-H/S/G) was delayed about 20 h. Peak expression occurred at 36 hpi for the mutant S viruses, while the peak expression of the wild-type S virus (MHV-HK) occurred at 16 hpi; the amount of protein expressed at the time of peak expression was not significantly different between the two mutants (Fig. 2F).

3.3. Cytopathology and foreign protein expression of recombinant MHVs in NIH/3T3 and Neuro-2a cells

In the NIH/3T3 and Neuro-2a cell lines, MHV-WT, MHV-HK and MHV-EGFP produced obvious CPE within 24 hpi (Fig. 3A), corre-

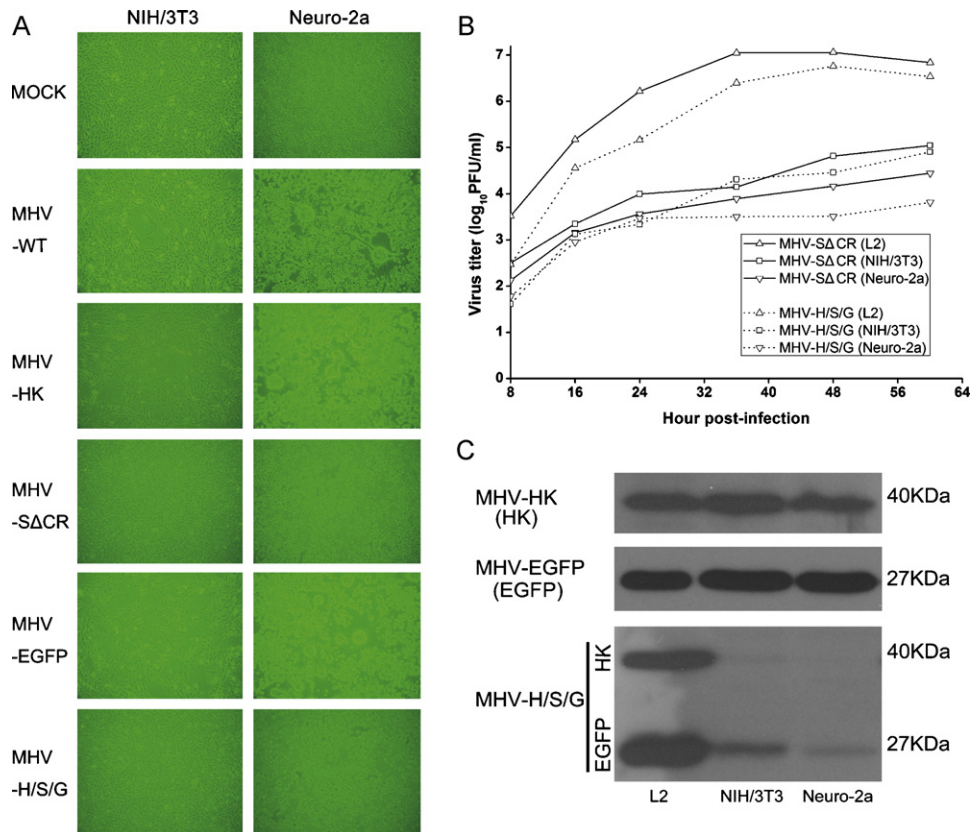


Fig. 3. Viral replication and foreign protein expression in NIH/3T3 and Neuro-2a cells. (A) CPE was demonstrated in NIH/3T3 (left column) and Neuro-2a (right column) cells 24 h post infected with mock, MHV-WT, MHV-HK, MHV-EGFP, MHV-SΔCR or MHV-H/S/G. (B) Replication of MHV-SΔCR and MHV-H/S/G in NIH/3T3 and Neuro-2a cells. The titre was determined by plaque assay as described in Section 2.3. (C) Expression of HK and EGFP in NIH/3T3, Neuro-2a and L2 cells infected with the viruses was analysed by Western blotting.

sponding to a delay of approximately 12 h relative to L2 cells. Titres and foreign protein expression of three viruses reached to peak at the same time and the data are equal to that of these viruses in L2 cells. Similar to the results for L2 cells, MHV-SΔCR and MHV-H/S/G infection did not produce observable CPE in NIH/3T3 and Neuro-2a cells until 48 hpi. The titres of MHV-SΔCR and MHV-H/S/G reached $\sim 10^7$ PFU/ml in L2 cells but only $\sim 10^4$ PFU/ml in NIH/3T3 and Neuro-2a cells, corresponding to a 3-log decrease (Fig. 3B). Fig. 3C shows that HK and EGFP were expressed in L2, NIH/3T3 and Neuro-2a cells infected with MHV-HK or MHV-EGFP. The levels of EGFP and HK expression were significantly lower in NIH/3T3 and Neuro-2a cells infected with MHV-H/S/G, indicating that the expression of foreign genes is dependent on cell type.

3.4. Localisation of foreign proteins in cells infected with recombinant MHVs

To determine if foreign proteins are distributed correctly in cells infected with the recombinant MHVs, a eukaryotic expression vector, pIRES2-EGFP, was used as a control. The HK membrane fusion protein gene was cloned upstream of EGFP so that the vector could express HK and EGFP simultaneously. The plasmid was transfected into L2 and Neuro-2a cells. The efficiencies of MHV-H/S/G infection and pIRES2-EGFP-HK transfection were different (Fig. 4A). Cells treated using the Bio-Rad membrane extraction kit were separated into soluble (cytoplasmic) and membrane (membrane and debris) components (Fig. 4B, left panel). When the cells were homogenised and separated by a series of centrifugation steps, a post-nuclear supernatant fraction (PNS) containing the whole cell components, an aqueous phase (cytosol) and a pellet containing the membrane

components (including subcellular organelles and plasma membrane) were obtained (Fig. 4B, right panel). The results showed that the HK protein was mainly located in membrane-associated fractions and that EGFP was primarily located in the cytoplasmic fractions. Similar foreign protein expression distribution patterns were observed in cells infected with MHV-H/S/G and in cells transfected with pIRES2-EGFP-HK.

Confocal microscopy was performed to confirm the subcellular localisation of the foreign proteins. The results showed that EGFP was homogeneously distributed in the infected L2 cells and the transfected Neuro-2a cells (Fig. 4C, EGFP panel). HK was membrane associated, and the distribution was different in two cell lines (Fig. 4C, HK panel). In the infected L2 cells, HK was mainly located around the nuclei and might be associated with some organelles. In the transfected Neuro-2a cells, HK was distributed primarily throughout the cells and seemed to be associated with plasma membranes.

4. Discussion

The S protein of coronaviruses can be divided into three functional parts: the N-terminal part (S1), the extracellular part of S2 and the C-terminal part of S2. S1 contains the receptor-binding domain and is responsible for viral tropism. The extracellular part of S2, which contains a fusion peptide and two heptad repeat (HR1 and HR2) domains, is involved in membrane fusion. The C-terminal part of S2, which contains the transmembrane domain and the intracellular domain (endodomain), is involved in viral assembly and membrane fusion. There is a unique cysteine-rich motif in the S endodomain of most coronaviruses. In MHV A59, this motif con-

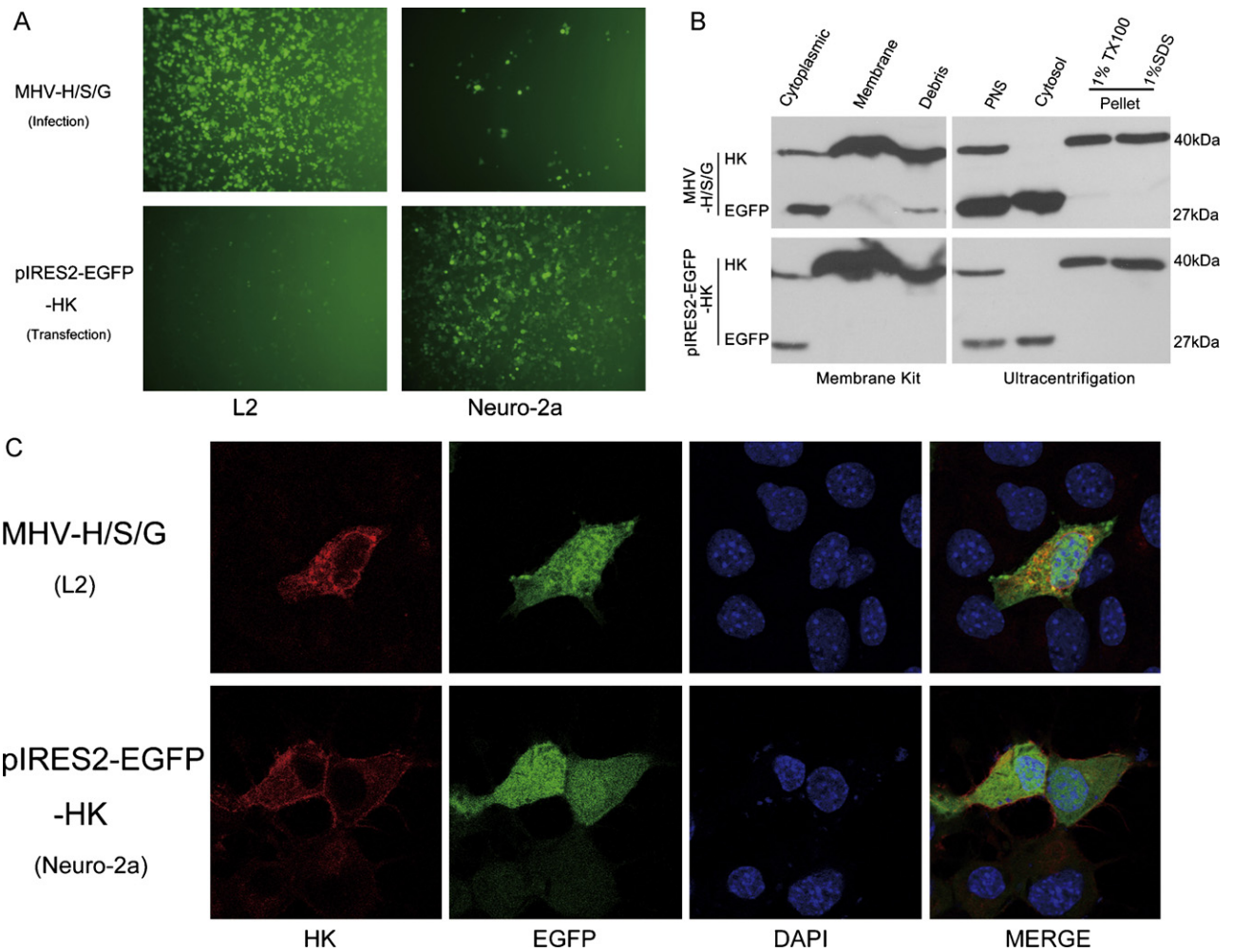


Fig. 4. Subcellular localisation of the foreign proteins HK and EGFP. (A) Fluorescence was displayed in L2 and Neuro-2a cells infected with MHV-H/S/G or transfected with pIRES2-EGFP-HK. (B) The cytoplasmic and membrane-associated fractions were separated using a membrane kit (left panel) or ultracentrifugation (right panel). HK and EGFP in fractions obtained from cells infected with MHV-H/S/G (up panel) or transfected with pIRES2-EGFP-HK (bottom panel) were analysed by Western blotting. (C) Fluorescence of HK and EGFP in L2 cells infected with MHV-H/S/G and Neuro-2a cells transfected with pIRES2-EGFP-HK. Green fluorescence indicates the localisation of EGFP, and red fluorescence indicates the distribution of HK. Nuclei were stained with DAPI (purple fluorescence).

tains nine cysteines (Fig. 1A). This motif has been demonstrated to play an important role in cell–cell fusion during syncytium formation induced by the S protein, which might be associated with the palmitoylation of these cysteine residues (Bos et al., 1995; Chang et al., 2000; Thorp et al., 2006).

Previous experiments have shown that a 13-residue deletion in this motif of recombinant MHVs reduced syncytium formation dramatically but did not affect replication in L2 cells (Ye et al., 2004). However, alanine-scanning experiments demonstrated that a single cysteine mutation did not influence viral replication or CPE; effects were only observed when three cysteines were mutated simultaneously (data not shown). Further deletion mutations revealed that the number of cysteines present has a substantial influence on the replication of recombinant viruses: fewer than two is lethal, three allows survival and more than four allows the virus to replicate in L2 cells normally (data not shown). In MHV-SΔCR, 14 residues were deleted, and 4 cysteines remained (Fig. 1A). The changes in the CPE and plaque formation in L2 cells induced by the partial deletion of the S endodomain might be the result of a decrease in cell–cell membrane fusion. The efficient replication of recombinant viruses with the S mutation in L2 cells might result from the reduced interference in virus–cell membrane fusion during viral entry. Although the deletion delayed the expression of HK

and EGFP in L2 cells, the amounts of both foreign proteins were almost equal to those expressed by wild-type S viruses (Fig. 2F). One possible advantage of this mutated MHV as an expression vector is that the infected cells maintained normal morphology for at least 72 h. Conversely, L2 cells infected with wild-type S viruses formed significant syncytia at 8 h and became broken and detached from the culture surfaces within 24 h (data not shown). Apart from the palmitoylation of cysteine residues in the S endodomain, the deleted residues might also be involved in the trafficking of the S protein to the plasma membrane through recruiting distinct cellular cofactors.

The natural hosts of MHV are limited to rodents, including mice and rats (Masters, 2006; Perlman and Butler, 2008). Rat or mouse cell lines such as L2, 17C11 and DBT are typically used in multiplication and other experiments with MHV (Das Sarma et al., 2002; de Haan et al., 2002, 2003; Enjuanes et al., 2001; Hurst et al., 2005; Kuo et al., 2000; Masters and Rottier, 2005; Ontiveros et al., 2001; Ye et al., 2004). Here, two mouse cell lines, NIH/3T3 and Neuro-2A, were used to assess the replication and foreign protein expression of recombinant viruses. The results showed that multiplication and foreign protein expression of MHV-SΔCR and MHV-H/S/G in both NIH/3T3 and Neuro-2A cells decreased significantly (Fig. 3B). However, the multiplication and foreign protein expression of the

wild-type S viruses (MHV-HK and MHV-EGFP) in both cell lines did not show a significant decrease, but the CPE was delayed about 12 h compared to infection in L2 cells. The decreased foreign protein expression might be attributed to the low level of viral replication because the expression of HK or EGFP was efficient in cells infected with MHV-HK or MHV-EGFP. Similar results were also obtained in 17C1 and DBT cells (data not shown). However, the infectivity of these cells, even though there was a 2- to 3-log reduction, indicated that the S-mutated recombinant virus maintained the ability to infect multiple tissues including the central nervous system of mouse models. Further experiments using animal models of infection with the viruses are needed. Moreover, in both NIH/3T3 and Neuro-2A cells, expression of EGFP was found to be better than that of HK (Fig. 3). The differences in expression were related to their genomic locations in MHV. Downstream genes (EGFP) are expressed earlier than upstream genes (HK). This difference in the expression timeline might result from the transcription sequence of the subgenomic mRNAs of coronaviruses (Sawicki and Sawicki, 1998).

HK is a type I membrane protein, while EGFP is a soluble protein reporter. The correct cellular distribution of these proteins may reflect the proper modification and trafficking of the foreign proteins expressed by recombinant MHVs. To confirm the effects of MHV replication on foreign protein distribution, a eukaryotic expression DNA vector (pIRES2-EGFP) was used as a control. Because a cell line with identical virus infection and plasmid transfection efficiencies was unavailable, MHV-H/S/G-infected L2 cells and pIRES2-EGFP-HK-transfected Neuro-2a cells were selected for the comparison of foreign protein distributions (Fig. 4A).

Coronaviruses utilise a special module for gene expression. A set of sgRNAs are transcribed by a viral RNA-dependent RNA polymerase, and the first ORF of each sgRNA is translated individually (Masters, 2006; Siddell, 1995; Spaan et al., 2005). For eukaryotic expression vectors, DNA is transferred first into cellular nuclei and then initiates RNA transcription by the IE promoter of CMV. RNAs are then transferred to the cytoplasmic matrix for protein translation. In the pIRES2-EGFP-HK vector, the target HK gene was cloned upstream of EGFP, and the two genes were linked with the EMCV IRES sequence. Although the transcription and translation mechanisms are very different between the recombinant coronaviruses and eukaryotic expression vectors, the distributions of foreign proteins expressed by both viruses and plasmids were shown to be consistent and correct. The correct location of the two proteins was confirmed by confocal microscopy: HK is membrane associated, and EGFP is cytoplasmic (Fig. 4). However, the membrane-associated localisation of HK in L2 and Neuro-2a cells was different as viewed by microscopy (Fig. 4C). This difference might result from the assembly of HK proteins in virus particles in infected cells through an interaction between the HK endodomain and the viral M and N proteins (Ye et al., 2004). In transfected cells, the proteins may be transferred quickly to the plasma membrane.

In this study, mutations within three adjacent genes of MHV were analysed with recombinant viruses produced by targeted RNA recombination. Partial deletion of the S endodomain did not impede foreign gene replacement or expression of upstream gene 2 and downstream gene 4 of MHV; however, viral replication was inhibited in infected cells. Foreign proteins expressed by MHV were properly localised in cellular fractions whether the proteins are membrane anchored or not. Meanwhile, the effects of partial deletion of the S endodomain on syncytium formation were more significant than the effects on the replication of MHV, and these effects were cell-type dependent. These results provide insight into the development and use of MHV or other coronaviruses as expression vectors in cells or animal models.

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