Amino acid substitution at position 464 in the haemagglutinin-neuraminidase protein of a mumps virus Urabe strain enhanced the virus growth in neuroblastoma SH-SY5Y cells.
Amino acid substitution at position 464 in the haemagglutinin-neuraminidase protein of a mumps virus Urabe strain enhanced the virus growth in neuroblastoma SH-SY5Y cells

Kengo Ninomiya\textsuperscript{a}, Tetsuya Kanayama\textsuperscript{a}, Nao Fujieda\textsuperscript{a}, Tetsuo Nakayama\textsuperscript{b}, Katsuhiro Komase\textsuperscript{c}, Kyosuke Nagata\textsuperscript{a}, Kaoru Takeuchi\textsuperscript{a,*}

\textsuperscript{a}Department of Infection Biology, Graduate School of Comprehensive Human Sciences and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan, \textsuperscript{b}Laboratory of Viral Infection, Kitasato Institute for Life Sciences, Minato-ku, Tokyo 108-8641, Japan, \textsuperscript{c}Department of Virology 3, National Institute of Infectious Diseases, Musashi-murayama, Tokyo 208-0011, Japan

Key words: Mumps virus; Reverse genetics; SH-SY5Y cells

Running title: neural cell specificity of mumps virus

Word count for abstract: 154, Word count for main text: 2797

*Corresponding author. Department of Infection Biology, Graduate School of Comprehensive Human Sciences and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.

Phone: +81-29-853-3472 Fax: +81-29-853-3472

e-mail: ktakeuch@md.tsukuba.ac.jp
Abstract

Mumps virus (MuV) infects various organs including central nervous system. However, the molecular basis of the neural cell specificity of MuV is not well understood. We found that the Hoshino vaccine strain rescued from cDNA replicated moderately in neuroblastoma SH-SY5Y cell line, while an Urabe strain (Ur89-250) isolated from a post-vaccination aseptic meningitis case replicated efficiently in the same cells. In order to examine the contribution of individual genes of Ur89-250 to the growth in SH-SY5Y cells, recombinant Hoshino vaccine strains in which each gene(s) was replaced with corresponding gene(s) of Ur89-250 were generated. A recombinant virus possessing the small hydrophobic and haemagglutinin-neuraminidase (HN) genes of Ur89-250 grew as efficiently in SH-SY5Y cells as Ur89-250. Further analysis indicated that an amino acid substitution at position 464 in the HN protein was most important for efficient growth. Thus, single amino acid substitution in the HN protein could affect neural cell specificity of mumps virus.
1. Introduction

Mumps virus (MuV) is a member of the *Rubulavirus* genus of the *Paramyxoviridae* family [1]. MuV virion has a single-stranded negative-sense RNA genome of 15,384 nucleotide that encodes seven genes: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN) and large polymerase protein (L). Of these, the P gene encodes three proteins, the P, V and I proteins by an RNA editing mechanism. MuV has two glycoproteins, the F and HN proteins, the former mediates virus-cell fusion, and the latter mediates receptor binding. The SH protein is a membrane protein and not essential for virus growth in vitro. Cellular receptor for MuV is sialic acid on cellular glycoprotein and lipid. MuV infects various organs including central nervous system (CNS). Aseptic meningitis is the most common CNS complication in MuV infection, and encephalitis, hydrocephalus and deafness are also associated in rare cases.

The MuV vaccines were highly effective, and the introduction of routine MuV or measles-mumps-rubella (MMR) vaccination programs resulted in a reduction in the number of mumps cases. However, some MuV vaccines including the Urabe AM9 strain were reported to cause post-vaccination aseptic meningitis at unacceptably high rate (about 1/1,000-10,000 recipients) and were withdrawn from the market [2].

At present, the molecular basis of neural cell specificity of MuV is not well understood. Rubin et al. indicated that the hamser-neuroadapted Kilham strain replicated more efficiently in rat astrocytoma cell line (C₆) and human neuroblastoma
cell line (SH-SY5Y) than the Jeryl-Lynn vaccine strain [3]. Subsequently, Rubin et al. generated neuroadapted MuV Jeryl-Lynn vaccine strain by passaging this strain in SH-SY5Y cells and found amino acid substitutions in the NP, M, and L proteins [4]. On the other hand, Santos-Lopez et al. indicated that an Urabe AM9 strain having the HN protein with 335K replicated more efficiently in SH-SY5Y cells than an Urabe AM9 strain having the HN protein with 335E [5]. However, the contribution of other proteins could not be ruled out [6]. Thus, genes or amino acid substitutions important for efficient MuV growth in neural cells are poorly understood.

In this study, we generated a series of chimeric viruses between the Hoshino vaccine strain and a particular Urabe strain isolated from a post-vaccination aseptic meningitis case and found that N464S substitution in the HN gene of an Urabe strain is important for the efficient MuV growth in SH-SY5Y cells.

2. Materials and methods

2.1. Cells and viruses

Vero (African green monkey kidney) cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum. SH-SY5Y (human neuroblastoma) cells were obtained from European Collection of Cell Cultures and were grown in MEM supplemented with 10% fetal calf serum. BSR T7/5 cells expressing the bacteriophage T7 RNA polymerase were generous gift from K.-K. Conzelmann [7] and were grown in MEM supplemented with 10% fetal calf serum and 1 mg/ml G418 every other passage.
The Hoshino vaccine strain (Lot. KO5) was obtained from the Kitasato Institute, Tokyo, Japan. An Urabe AM9 strain (Ur89-250) isolated from a post-vaccination aseptic meningitis case was obtained from the Saitama institute of public health in Japan and propagated once in Vero cells.

2.2. The Hoshino vaccine strain genome sequencing

The genomic RNA of the Hoshino vaccine strain was extracted directly from a bulk vaccine with High Pure Viral RNA Kit (Roche Diagnostics, Indianapolis, IN). Then, seven overlapping cDNA fragments spanning the entire length of the genome were synthesized by RT-PCR using RT/PLUTINUM Taq Kit (Invitrogen, Carlsbad, CA) according to the instruction of the manufacture. 5’ termini of negative- and positive-sense genomes were reverse transcribed and amplified using 5’ Full RACE Core Set (TaKaRa, Kyoto, Japan). Nucleotide sequences of cDNA fragments were determined using ABI PRIZM3100-AVANT Genetic Analyzer (Applied Biosystems, Carlsbad, CA) and MuV-specific primers. MuV-specific primers were deduced on the basis of the published sequence for the Miyahara and Urabe AM9 strains.

2.3. Construction of a complete cDNA plasmid of the Hoshino vaccine strain genome

Five cDNA fragments of the Hoshino vaccine strain synthesized from the genomic RNA by RT-PCR were introduced into pBluescript II KS(+) plasmid whose multiple cloning site was replaced with BssHII, BamHI, PacI, HindIII, and NotI sites [8] or pCAGGS-P7 [9], and the complete cDNA clone was assembled by successive cloning...
of five cDNA fragment (Fig. 1). Briefly, the MP2 fragment was released from
pCA-MP2 with BstEII/NotI and introduced into pBS-MP1 digested with BstEII/NotI,
resulting pBS-MP1-MP2. The MP5 fragment containing ribozyme was released from
pCA-MP5 with HindIII/NotI and introduced into pCA-MP4 digested with HindIII/NotI,
resulting pCA-MP4-MP5. The fragment MP4-MP5 was released from
pCA-MP4-MP5 with PacI/NotI digestion and introduced into pBS-MP1-MP2 digested
with PacI/NotI, resulting pBS-MP1-MP2-MP4-MP5. Finally, the fragment MP3
containing Q383L substitution in the F protein synthesized by PCR-based mutagenesis
was released from pBS-MP3 with PacI and introduced into pBS-MP1-MP2-MP4-MP5
digested with PacI, resulting pMuVHoF-383L.

2.4. Introduction of EGFP gene into the Hoshino vaccine strain genome cDNA clone

To introduce an additional transcription unit of the enhanced green fluorescent
protein (EGFP), an original NotI site in the vector sequence of pMuVHoF-383L was
deleted, and a new NotI site was created by inserting 12 bp nucleotides
(TTCGCCGCGCA) in the non-coding region of P/V gene (between nucleotide
positions 3182 and 3183 of the genome of the Hoshino vaccine strain) resulting
pMuVHoF-383L(NotI). The EGFP gene containing the P/V gene end sequence and
the M gene start sequence was synthesized by PCR and introduced into NotI site of
pMuVHoF-383L(NotI) after NotI digestion, resulting pMuVHoF-383L-EGFP.

2.5. Introduction of unique restriction site in the MuV cDNA clone
The full-length MuV plasmid pMuVHoF-383L was subsequently modified by PCR-based mutagenesis to include a number of unique restriction sites in the 3’ non-coding regions of each gene. A BspEI site was introduced in the 3’ non-coding region of the NP gene. Similarly, an AsiSI site was introduced in the 3’ non-coding region of the P/V gene, a RsrII site was introduced in the 3’ non-coding region of the M gene, a MluI site was introduced in the 3’ non-coding region of the F gene, a SbfI site was introduced in the 3’ non-coding region of the HN gene. The resulting plasmid was designated pMuVHo-BARMS. pMuVHo-BARMS had the F gene encoding the authentic F protein with 383Q.

2.6. Construction of a series of the Hoshi no complete cDNA containing corresponding gene(s) of Ur89-250

pMuVHo-BARMS was cleaved with the appropriate two restriction enzymes to insert Ur89-250 NP (BssHII/BspEI), Ur89-250 P/V (BspEI/AsiI), Ur89-250 M (AsiSI/RsrII), Ur89-250 F (RsrII/MluI), Ur89-250 SH and HN (MluI/SbfI) and Ur89-250 L (SbfI/NotI) genes and ligated with the equivalently restricted Ur89-250 gene fragments generated by RT-PCR from Ur89-250 genomic RNA.

2.7. Recovery of infectious MuV from cDNA

BSR T7/5 cells at 90% confluency in one well of a 6-well plate were transfected with MuV genome cDNA plasmids and supporting pGem3 plasmids encoding NP, P and L protein with Trans-IT LT1 (Mirus, Madison, WI) or GeneJuice (Novagen, Gibbstown,
The amounts of plasmid were as follows: 5 μg of MuV genome cDNA plasmid, 300 ng of pGEM-NP, 50 ng of pGEM-P and 200 ng of pGEM-L. After incubation for 48 hr transfected BSR T7/5 cells were harvested by trypsin-EDTA solution and mixed with equal amount of Vero cells and seeded in a 10-cm-diameter dish. When cytopathic effect was evident, the culture media were transferred to new Vero cells in 10-cm-diameter dishes to prepare MuV stocks. Infectivity titer of MuV stocks was determined by plaque assay using Vero cells and indicated as plaque forming units (PFU).

2.8. Viral growth in culture

Monolayer cultures of Vero or SH-SY5Y cells in 24-well plates were infected with MuV at a multiplicity of infection (MOI) of 0.01 PFU/cell. At various times, the culture media harvested and infectivity titer was determined by plaque assay using Vero cells.

3. Results

3.1. Determination of the complete sequence for the Hoshino vaccine strain

In order to study the attenuation and cell specificity of MuV vaccines and to develop new viral vectors, we tried to establish a reverse genetics system of the Hoshino vaccine strain. The nucleotide sequences for the F, SH and HN genes of the Hoshino vaccine strain were previously reported [10], but the entire nucleotide sequence of the genome
was not known. Therefore, the nucleotide sequence was first determined using viral RNA prepared from a bulk Hoshino vaccine (Lot. KO5). The complete nucleotide sequence of the genome of the Hoshino vaccine strain was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB470486. The Hoshino vaccine strain belongs to the genotype B based on the nucleotide sequence of the SH genes as reported previously [10,11], and overall genome sequences are closely related to those of the Miyahara vaccine and Urabe AM9 vaccine strains [12,13].

3.2. Establishment of reverse genetics for the Hoshino vaccine strain

The Hoshino vaccine strain induces very small syncytium in Vero cells [14], and we thought this phenotype might hamper the rescue of infectious MuV from cDNA. Therefore, Q383L substitution in the F gene was introduced in the complete cDNA. The F protein of the Hoshino vaccine strain having Q383L substitution induced larger cell-cell fusion in Vero cells (Yoshida et al, manuscript in preparation). The full-length Hoshino vaccine strain cDNA encoding the F protein with Q383L substitution was assembled from five cDNA fragments (Fig. 1), and infectious virus (rHoF-383L) was successfully recovered by using BSR T7/5 cells as described in the Materials and methods.

3.3. Growth of the Hoshino vaccine and Urabe-related strains in SH-SY5Y cells

We examine the growth of rHoF-383L in various cultured cell lines and found that
rHoF-383L grew moderately in SH-SY5Y cells (Fig. 2). The Hoshino vaccine strain
did not grow efficiently in SH-SY5Y cells (data not shown). On the other hand,
Ur89-250 grew efficiently in the same cells (Fig. 2). Both rHoF-383L and Ur89-250
did not induce apparent cell-cell fusion and cytopathic effect in SH-SY5Y cells.

3.4. Dissemination of rHoF-383L in SH-SY5Y cells

To evaluate the infection and dissemination of rHoF-383L in SH-SY5Y cells, an
additional transcriptional unit of EGFP was introduced in the non-coding region in the
P/V gene (Fig. 3a), and an infectious virus (rHoF-383L-EGFP) was recovered.
rHoF-383L-EGFP grew almost the same extent as rHoF-383L in Vero cells (Fig. 3b).
When Vero cells were infected with rHoF-383L-EGFP at an MOI of 0.01, strong
fluorescent signal for EGFP was observed in the entire region of Vero cell culture after 4
days post infection, and cytopathic effect was observed in some areas (Fig. 3c). On
the other hand, when SH-SY5Y cells were infected with rHoF-383L-EGFP in the same
condition, EGFP autofluorescence was detected only in restricted areas after 4 days post
infection (Fig. 3c). No cytopathic effect of rHoF-383L-EGFP was seen in SH-SY5Y
cell culture (Fig. 3c).

3.5. Generation of a series of chimeric viruses and growth in SH-SY5Y

To exchange corresponding genes between rHoF-383L and Ur89-250 easily, we
modified pMuVHoF-383L to have unique restriction sites between each gene resulting
pMuVHo-BARMS (Fig. 4a). Then, we constructed a series of the complete cDNA
plasmids which contained individual gene(s) of Ur89-250. Those plasmids were
pMuVHoNP\textsuperscript{UR}, pMuVHoP/V\textsuperscript{UR}, pMuVHoM\textsuperscript{UR}, pMuVHoF\textsuperscript{UR}, pMuVHoSH-HN\textsuperscript{UR} and
pMuVHoL\textsuperscript{UR} containing the NP, P/V, M, F, SH-HN and L genes of Ur89-250,
respectively (Fig. 4a). Then, infectious viruses, NP\textsuperscript{UR}, P/V\textsuperscript{UR}, M\textsuperscript{UR}, F\textsuperscript{UR}, SH-HN\textsuperscript{UR}
and L\textsuperscript{UR}, were recovered as described in the Materials and methods and grown in Vero
cells to make virus stocks. As a control, infectious virus, rHo, which had the authentic
F protein with 383Q, was also recovered from pMuVHo-BARMS. To compare the
growth of recombinant viruses in SH-SY5Y cells, multistep growth analysis was
performed. As shown in Fig. 4b, SH-HN\textsuperscript{UR} grew most efficiently and reached high
titer (almost $10^7$ PFU/ml at 3 dpi) comparing to other recombinant viruses. M\textsuperscript{UR} and
F\textsuperscript{UR} grew better than rHo. This result indicated that the SH-HN genes of Ur89-250
were most important for the efficient growth in SH-SY5Y cells. All recombinant
viruses did not induce cell-cell fusion and cytopathic effect in SH-SY5Y cells. In Vero
cells, both rHo and SH-HN\textsuperscript{UR} grew efficiently and reached almost the same titer (Fig.
4c). pMuVHo-BARMS had two amino acid mutations (E379K and L1755V) in the L
protein. However, these amino acid mutations did not cause apparent effect on virus
growth in SH-SY5Y and Vero cells (Fig. 4a and b).

3.6. Identification of amino acid important for efficient MuV growth in SH-SY5Y cells
Comparative sequencing analysis of the Hoshino vaccine and Ur89-250 in the
SH-HN genes indicated that there were two amino acid differences (I22V and A37V) in
the SH protein and four amino acid differences (V20F, V21I, R119C and N464S) in the
HN protein (Table 1). To further characterize the mutation important for the efficient
growth in SH-SY5Y cells, five additional complete cDNA plasmids, namely,
pMuVHoSH\text{UR}, pMuVHoHN\text{UR}, pMuVHoHN\text{V20F/V21I}, pMuVHoHN\text{R119C} and
pMuVHoHN\text{N464S} were generated (Fig. 5a), and infectious viruses, SH\text{UR}, HN\text{UR},
HN\text{V20F/V21I}, HN\text{R119C} and HN\text{N464S} were recovered from corresponding plasmids.
When SH-SY5Y cells were infected with those recombinant viruses, SH\text{UR}, HN\text{UR},
HN\text{V20F/V21I}, HN\text{R119C} replicated as moderately as rHo, on the other hand, HN\text{UR}, HN\text{N464S} replicated
as efficiently as SH-HN\text{UR} (Fig. 5b). This result indicated that N464S substitution in
the HN protein is most important for the efficient growth in SH-SY5Y cells.

4. Discussion

Wild-type MuV strains infect a number of organ systems including central nervous
system. However, the molecular basis of the neural cell specificity of MuV is not well
understood. Taking advantage of a reverse genetics of MuV, we identified that N464S
substitution in the HN protein is most important for the efficient growth in SH-SY5Y
cells. As the Urabe M9 vaccine strains do not have the HN protein with 464S, N464S
substitution could be introduced in the HN protein during virus replication in a vaccinee.
An Urabe strain having HN protein with N464S might have some advantage in
replication in a vaccinee. Interestingly, genetic heterogeneity at position 464 (464N/K)
in the HN protein was reported for Urabe AM9 vaccine strains [13,15], and adaptation
of Urabe AM9 vaccine strain to chicken embryo fibroblast cells was associated with the
heterogeneous amino acids (464N/K) to homogeneous amino acid (464K) change [16].
Adaptation of the wild-type 88-1961 strain to chicken fibroblast cells was associated with S466N substitution in the HN protein [4]. Thus, amino acid substitutions in this site were frequently observed. As amino acid position 464 is proposed to be at or near active site of the HN protein, amino acid substitution around this site might affect enzymatic activity of HN protein and might change the cell specificity of MuV [4]. On the other hand, amino acids 464 to 466 form a potential N-linked glycosylation site, and amino acid substitutions in this site were predicted to result in loss of N-linked glycosylation [4,15,16,17]. According to a 3D structure model of the MuV HN protein [18], the position 464 is on the top of the HN protein (data not shown). Loss of N-linked glycosylation at this site might affect the accessibility of the HN protein to cellular receptors and might change the cell specificity of MuV. At present we do not know which mechanism contributes to the neural cell specificity of Ur89-250. Analysis of N-linked sugars on the HN protein would be important to elucidate this point.

The Urabe AM9 vaccine is a mixture of closely related virus strains, and the compositions of virus strains are different from manufacture to manufacture [13,15,19-23]. Several reports indicated that an Urabe AM9 strain having the HN protein with 335K was associated with post-vaccination meningitis cases [19,22-24], although others did not confirm this observation [13,21]. In vitro analysis indicated that an Urabe AM9 strain having the HN protein with 335K replicated more efficiently in SH-SY5Y cells than an Urabe AM9 strain having the HN protein with 335E [5]. Thus, it was speculated that amino acid substitution at position 335 in the HN protein is
important for both pathogenicity in vivo and virus growth in vitro. However, as both
the Hoshino vaccine strain and Ur89-250 have the HN protein with 335K, this particular
amino acid substitution did not explain the difference in their growth in SH-SY5Y cells.

By using a rodent model [25,26] and a reverse genetics for the Jeryl-Lynn vaccine
strain [27], it was shown that the F gene is a major determinant of neurovirulence [28].
A single amino acid change in the F protein was shown to reduce the neurovirulence of
the wild-type 88-1961 strain [29]. Recently, it was shown that a particular Urabe AM9
strain is more attenuated than other MuV strains [30]. At present, we do not know the
effect of N464S substitution in the HN protein on neurovirulence. A rodent model
might provide clues to the effect of N464S substitution in the HN protein on
neurovirulence of MuV.

Reverse genetics system established in this study for the MuV Hoshino vaccine strain
would be useful for developing safer MuV vaccines or new multivalent MuV vaccines.

Acknowledgement

We thank K. Conzelmann for providing BSR T7/5 and M. Kawano, M. Okuwaki, T.
Naito, T. Nishie, A. Yamada, K. Tanabayashi, A. Kato and M. Kidokoro for valuable
suggestions and critical comments on the manuscript. This work was supported in part
by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and
Technology of Japan.
References


Figure legends

Fig. 1. Construction of a complete cDNA plasmid of the Hoshino vaccine strain genome. Schematic representation of the method for the construction of the MuV Hoshino vaccine strain full-length genome cDNA plasmid. The T7 RNA polymerase promoter (T7pro) and the HDV ribozyme (HDV-Rbz) sequence were positioned to synthesize MuV antisense genome with the exact 5’- and 3’- ends.

Fig. 2. Replication kinetics of rHoF-383L and Ur89-250 in SH-SY5Y cells. Cells were infected with rHoF-383L and Ur89-250 at an MOI of 0.01 PFU/cells. Media were harvested at days 0, 1, 2, 3 and 4 after infection, and infectivity titers were determined by plaque assay by using Vero cells. Average PFU in triplicate experiments are shown. Standard deviations are also indicated.

Fig. 3. Dissemination of the rHoF-383L-EGFP in Vero and SH-SY5Y cells. (a) Schematic representation of the complete cDNA plasmid of rHoF-383L containing EGFP gene. (b) Replication kinetics of rHoF-383L and rHoF-383L-EGFP in Vero cells. Cells were infected with rHoF-383L and Ur89-250 at an MOI of 0.01 PFU/cells. Media were harvested at days 0, 1, 2, 3 and 4 after infection, and infectivity titers were determined by plaque assay by using Vero cells. Average PFU in triplicate experiments are shown. Standard deviations are also indicated. (c) EGFP autofluorescence in rHoF-383L-EGFP-infected cells. Vero and SH-SY5Y cells were
infected with rHoF-383L-EGFP at an MOI of 0.01. At 4 days after infection, cells were observed under light and fluorescence microscope.

Fig. 4. Contributions of individual genes of Ur89-250 to growth in cultured cells. (a) Structures of cDNA of recombinant MuV strains. Wide boxes indicate ORFs of the MuV NP, P/V, M, F, H and L proteins. Narrow boxes between the ORFs indicate the gene end and start sequences. Regions derived from Ur89-250 are shaded, and those from the Hoshino vaccine strain are white. Black boxes indicate T7 promoter and ribozyme. Restriction enzyme restriction sites used for exchanging the genes between the Hoshino vaccine strain and Ur89-250 are indicated. (b) Replication kinetics of recombinant MuV strains in SH-SY5Y cells. Cells were infected with recombinant MuV strains at an MOI of 0.01 PFU/cells. Media were harvested at days 0, 1, 2, 3 and 4 after infection, and infectivity titers were determined by plaque assay by using Vero cells. (c) Replication kinetics of recombinant MuV strains in Vero cells. Cells were infected with rHo and SH-HN^{UR} at an MOI of 0.01 PFU/cells. Media were harvested at days 0, 1, 2, 3 and 4 after infection, and infectivity titers were determined by plaque assay by using Vero cells.

Fig. 5. Contributions of individual amino acid substitutions in the SH and HN protein of Ur89-250 to growth in SH-SY5Y cells. (a) Structures of cDNA of recombinant MuV strains. Wide boxes indicate ORFs of the MuV NP, P/V, M, F, H and L proteins. Narrow boxes between the ORFs indicate the gene end and start sequences. Regions...
derived from Ur89-250 are shaded, and those from the Hoshino vaccine strain are white.

Amino acid substitutions specific for Ur89-250 are indicated. Black boxes indicate T7 promoter and ribozyme. Restriction enzyme restriction sites used for exchanging the genes between the Hoshino vaccine strain and Ur89-250 are indicated. (b) Replication kinetics of recombinant MuV strains in SH-SY5Y cells. Cells were infected with recombinant MuV strains at an MOI of 0.01 PFU/cells. Media were harvested at days 0, 1, 2, 3 and 4 after infection, and infectivity titers were determined by plaque assay by using Vero cells.