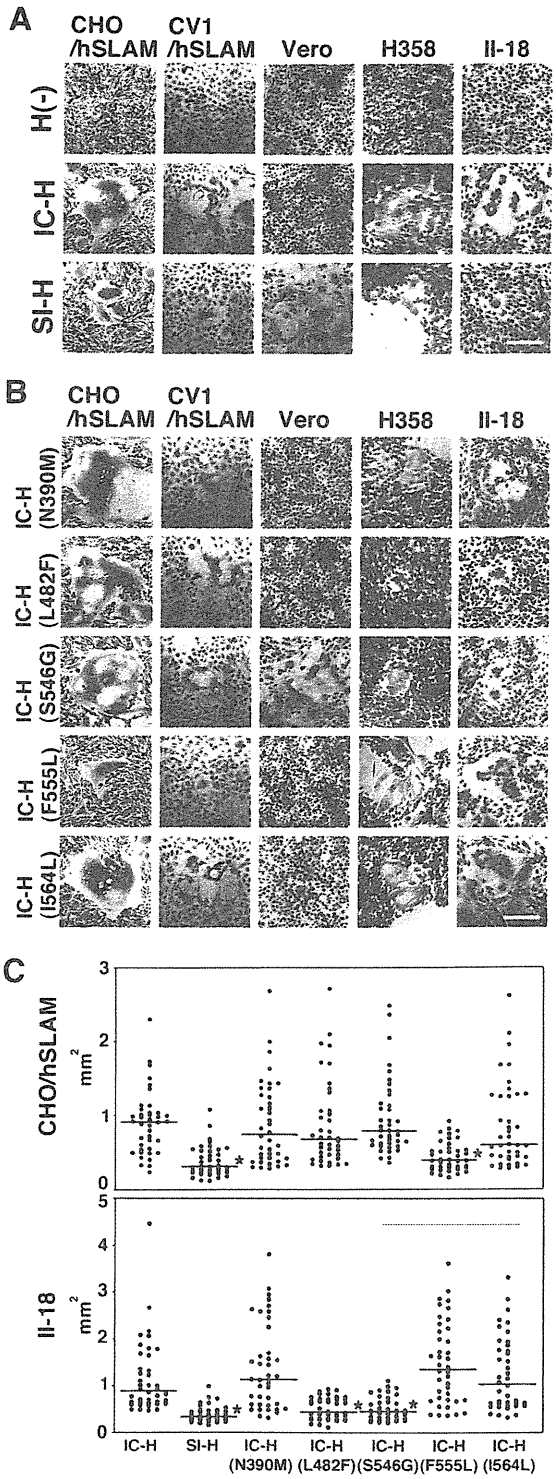


FIG. 7. Characterization of the amino acid residues in the SI-H protein that support cell-to-cell fusion in cells expressing SLAM, CD46, or ECR. (A and B) CHO/hSLAM, CV1/hSLAM, Vero, H358, and II-18 cells were transfected with plasmids expressing the H protein of the IC-B or SI strain (IC-H or SI-H, respectively) or no H protein [H(−)] (A) or mutant IC-H protein (N390M, L482F, S546G, F555L, or I564L) (B) together with a plasmid expressing the F protein of the IC-B strain. The CV1/hSLAM, H358, and II-18 cells were then incubated with an anti-CD46 MAb (M75). At 1 (CHO/hSLAM and CV1/hSLAM), 2 (Vero and II-18), or 3 (H358) days posttransfection, the cells were observed under a phase-contrast imaging microscope after Giemsa staining. Bars, 0.2 mm. (C) Quantification of syncytium formation. CHO/hSLAM and II-18 cells were transfected with IC-F-expressing plasmids and IC-H-, SI-H-, or mutant H-expressing plasmids together with an mCherry-expressing plasmid. At 48 h posttransfection, areas of each syncytium with mCherry autoflurescence were measured using an Axio Observer.D1 microscope and ImageJ software. Forty syncytia were measured for each H protein. Asterisks indicate that the area of syncytia induced by SI-H or mutant H was significantly smaller than that induced by the IC-H, based on the results of a $t$ test ($P < 0.001$). The horizontal bars indicate the median values of the areas of syncytia.

was coexpressed with SI-H, many syncytia, albeit smaller in size than the syncytia induced by IC-F and IC-H, were detected (Fig. 6A; SI-H/IC-F). These data indicated that both the SI-F and SI-H proteins exhibited lower activities than the IC-F and IC-H proteins in inducing syncytia in CHO/hSLAM cells. To identify the mutation(s) that impaired the syncytium-forming activity of SI-F, four chimeric F proteins (IC/SI-F-1, -F-2, -F-3, and -F-4) were generated using the SI and IC-B strains (Fig. 6C). These chimeric F proteins were coexpressed with IC-H. Two chimeric F proteins, IC/SI-F-2 and IC/SI-F-4, failed to produce syncytia (Fig. 6D and E). These data showed that a region between the SalI and KpnI recognition sites (amino acid positions 271 and 324) in SI-F severely restricted its membrane fusion activity (Fig. 6C). In this region, only a single amino acid substitution, E300G, was found in comparisons of SI-F and IC-F (Fig. 6C and Table 2). A glycine residue at amino acid position 300 in SI-F was replaced with a glutamic acid. The mutant F protein [Fig. 6C; SI-F(G300E)] was expressed with IC-H. The data indicated that SI-F(G300E) caused membrane fusion as well as IC-F did ($P < 0.01$) [Fig. 6D and E; SI-F(G300E)]. These findings indicated that the SI-F protein exhibited a restricted membrane fusion activity that was mainly caused by the E300G substitution.

**S546G, L482F, and F555L substitutions affected the fusion-helper function of the H protein.** To analyze the fusion-helper function of SI-H in different cell types, the protein was expressed in CHO/hSLAM ($SLAM^+$), CV1/hSLAM ($SLAM^+$, $CD46^+$), Vero ($CD46^+$), H358 ($ECR^+$, $CD46^+$), and II-18 ($ECR^+$, $CD46^+$) cells together with IC-F. CD46-dependent infection was blocked by an anti-CD46 antibody (M75) when CV1/hSLAM, H358, and II-18 cells were used for the assessment of SLAM- and ECR-dependent infection. IC-H was used as a control. When IC-F was expressed alone, no syncytia were observed in either cell line [Fig. 7A; H(−)]. As reported previously, IC-H supported cell-to-cell fusion efficiently in SLAM-positive (CHO/hSLAM) and ECR-positive (H358 and II-18) cells but not in Vero cells (Fig. 7A; IC-H) (45, 49, 59). SI-H exhibited a fusion-helper function in Vero cells (Fig. 7A; SI-H), probably because of the S546G substitution. However, SI-H supported cell-to-cell fusion less efficiently than IC-H in CHO/hSLAM, CV1/hSLAM, H358, and II-18 cells (Fig. 7A; SI-H). To identify the substitution(s) responsible for the altered fusion-helper function of SI-H, five substitutions were individually introduced into IC-H and the mutated proteins were expressed in cells together with IC-F. The five selected substitutions were N390M, L482F, S546G, F555L, and 564L, since these substitutions were unique to the SI strain and

located in the receptor-binding globular head domain (Table 4). As expected, IC-H with S546G, but not the other mutant H proteins, supported cell-to-cell fusion in Vero cells (Fig. 7B). Instead, IC-H with S546G showed a reduced fusion-helper function in H358 and II-18 cells (Fig. 7B). No significant changes were observed in CHO/hSLAM and CV1/hSLAM cells after the introduction of the S546G substitution (Fig. 7B). Similarly, IC-H with L482F showed a reduced fusion-helper function in H358 and II-18 cells but showed activities similar to those seen with IC-H in CHO/hSLAM and CV1/hSLAM cells (Fig. 7B). Quantified and statistical analyses of cell-to-cell fusion in II-18 cells indicated that the areas of syncytia produced by IC-H(S546G) and IC-H(L482F) were significantly smaller than those produced by IC-H ($P < 0.01$) (Fig. 7C). None of the N390M, F555L, and I564L substitutions significantly affected the fusion-helper function in H358 and II-18 cells (Fig. 7B and C). These findings suggested that the L482F and S546G substitutions compromised the ability of the H protein to interact with ECR. It was also noted that the H protein with F555L showed a reduction in the fusion-helper function in CHO/hSLAM and CV1/hSLAM cells (Fig. 7B and C).

## DISCUSSION

SLAM is expressed on cells of the immune system and functions as the principal receptor for MV infection (69). However, this molecule probably plays a minor role in MV growth in the CNS, because neural cells in the brain do not express SLAM (28). Indeed, the ability of the SI strain to use SLAM was compromised by the F555L substitution. We and another group recently demonstrated that certain epithelial cells that form tight junctions are highly susceptible to MV infection (25, 50, 59). These data demonstrated the existence of ECR on some epithelial cells (25, 50, 59). ECR probably contributes to the efficient transmission of MV from a patient to other individuals (53), but its roles in persistent infection of the brain with MV remain to be elucidated. ECR is a candidate for an MV receptor in the brain. However, our data indicated that the SI strain had mutated via the S546G and L482F substitutions to use ECR inefficiently. With these data, the idea that ECR functions as a receptor for MV in the brain seemed unconvincing. Instead, the SI-H protein had adapted to use CD46 via the S546G substitution. Woelk et al. identified several positive-selection amino acid sites in the SSPE strain (67), but S546G was absent from the list. It is possible that the S546G substitution was introduced into the SI strain genome during the propagation in Vero cells but not in the brain, since the SI strain was isolated using Vero cells (29). Vero cells are 100 to 1,000 times less sensitive than SLAM-positive B95a cells for the isolation of wt MV strains (22, 34), and wt MV strains readily adapt to use CD46 after several passages in Vero cells (69). However, Ogura et al. (34) indicated that Vero cells were more sensitive than B95a cells for the isolation of SSPE strains. Although their data demonstrated that SSPE strains show cell specificities different from those of wt MV strains, some SSPE strains were shown not to use CD46 as a receptor (47). Nevertheless, it is still possible that the acquisition of the ability to use CD46 contributes to the growth of some SSPE-derived strains in the brain, since various SSPE strains may employ different strategies to acquire the ability to spread in the brain. The SI strain used only CD46 efficiently. Much evidence obtained using CD46-transgenic mice has shown the contributions of CD46 in establishing MV infection of the brain. Analyses using human brain samples also showed that CD46 is a candidate molecule that contributes to the growth of some SSPE strains in the brain (5, 28, 33).

Analyses using animal models have demonstrated that MV uses a transsynaptic route to spread between neurons (24, 27, 35, 40). The data indicated that receptors for the H protein are not required for the transsynaptic transmission (27, 70). It has been suggested that the F protein causes microfusion between neurons without the support of the H protein (27, 70). Ayata et al. (1) demonstrated that the F proteins of some SSPE strains contribute to the exhibition of neurovirulence in animals by showing a hyperfusion activity. Cattaneo et al. (4, 8) also demonstrated that the F proteins of SSPE strains exhibit higher levels of fusion activities than the standard F protein. These data suggest an important role for the F protein in the propagation of SSPE strains in the brain. However, our data indicated that the F protein of the SI strain showed limited membrane fusion activity because of the E300G substitution. It is unlikely that the F protein of the SI strain had acquired the E300G substitution during the propagation in cultured cells, since viruses usually acquire mutations that confer better fitness. Consequently, our data suggest that a high level of membrane fusion activity of the F protein was not a prerequisite for this SSPE strain to spread in the brain. Watanabe et al. (65) suggested that a reduction in cell-to-cell fusion mediated by amino acid changes in the F protein contributes to the persistence of MV in the brain. Their observations are consistent with our data for the SI strain. Thus, the data obtained in the present study provide a clear example of an SSPE-derived strain that exhibits limited fusion activity.

In the present study, we also established a reverse genetics system for the SI strain. Although we previously reported very efficient reverse genetics systems for MV, as shown using recombinant vaccinia viruses encoding T7 RNA polymerase (VV-T7) (30, 55, 56), they were not applicable for rescue of the SI strain from cloned cDNAs. When the previous systems were used (30, 56), infectious cycles of rSI-AcGFP were efficiently initiated in CHO/hSLAM cells by the use of the full-length genome plasmid (data not shown). However, since rSI-AcGFP did not produce cell-free virus particles and replicated poorly, it was impossible to isolate rSI-AcGFP from VV-T7. We tried to use a VV-T7-free system reported by Radecke et al. (39), but neither syncytia nor AcGFP fluorescence was detected. Therefore, a new, efficient VV-T7-free system was required for the rescue of rSI-AcGFP from cloned cDNAs. We are convinced that this new system used for the SI strain would be applicable for other SSPE strains. The success in establishing a reverse genetics system for an SSPE strain is a significant step toward the elucidation of the molecular bases and pathogenesis of SSPE.

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# Amino Acid Substitutions in Matrix, Fusion and Hemagglutinin Proteins of Wild Measles Virus for Adaptation to Vero Cells

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**Key Words**
CD46 · Fusion protein · Hemagglutinin protein · Matrix protein · Measles virus · SLAM

**Abstract**
***Background:*** Wild-type measles virus (MV) is isolated in B95a but not in Vero cells. Through an adaptation process of wild-type MV to Vero cells, several amino acid substitutions were reported. ***Methods:*** Six strains were adapted to Vero cells and membrane (M), fusion (F) and hemagglutinin (H) genes were sequenced. Cell fusion was assessed and recombinant MVs were constructed, having wild-type H or M gene with or without mutations. ***Results:*** No F gene substitution was noted. Amino-acid substitutions at positions 481 from Asn to Tyr (N481Y) and 546 from Ser to Gly (S546G) were observed in the H protein. Glu at position 89 of the M protein was substituted for Gly (E89G) and two mutations were noted at positions 62 (S62R) and 83 (S83P) in M protein. Recombinant viruses with mutation(s) detected in Vero-adapted strains induced a cytopathic effect and grew well in Vero cells, but those with the wild type did not. Recombinant viruses with mutation(s) demonstrated lower viral growth in B95a cells. ***Conclusions:*** Substitutions of E89G, S62R and S83P of the M protein were newly observed through adaptation to Vero cells, besides the mutations described in previous reports, with varying adaptation for each strain.



## Introduction

Measles virus (MV) is a member of the genus *Morbillivirus*, family Paramyxoviridae, order Mononegavirales, characterized by non-segmented, negative-strand RNA virus. The genome consists of 15,894 nucleotides, coding six structural proteins: nucleoprotein (N), phospho (P), membrane (M), fusion (F), hemagglutinin (H) and large (L) proteins. The genomic RNA is encapsidated with N protein and consisted of ribonucleoprotein, together with RNA-dependent RNA polymerase complex of L and P proteins [1, 2]. MV has two envelop glycoproteins, F and H, which play an important role in virus attachment and subsequent virus cell fusion as well as cell-to-cell fusion [3–5]. The Edmonston strain was isolated in 1954 using a primary culture of human kidney cells and, thereafter, MV was isolated using Vero cells, but the sensitivity of Vero cells was poor, and two or three blind passages were required. MV was isolated more efficiently in B95a cells, marmoset lymphoblastic cell lines transformed by Epstein-Barr virus [6]. CD46 was initially reported to act as a cellular receptor for laboratory-adapted, vaccine strains of MV. CD46 is a member of the regulators of the complement activation gene cluster and is widely expressed on epithelial cells, but not on lymphocytic cells [7, 8]. In 2000, human signaling lymphocyte activation molecule (SLAM; CD150) was reported as the receptor of wild-type MV and it was expressed on lymphocytic cells [9, 10]. The

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Edmonston strain and relevant vaccine strains were found to use both SLAM and CD46 as receptors and circulating wild-type strains utilize SLAM as a receptor, but not CD46 [9, 10].

Several amino-acid changes in H, M, L and P proteins and/or accessory V and C proteins were responsible for attenuation through adaptation to Vero cells. Some authors reported that the majority of MV strains using CD46 as a receptor have tyrosine at position 481, whereas wild-type strains have asparagine at this position [11–17]. When the wild-type MV strains became adapted to grow in Vero cells, the substitution at position 481 of H protein from asparagine to tyrosine (N481Y) was often observed after several passages [16, 18]. In some Vero cell-adapted strains, a substitution at position 546 of the H protein from serine to glycine (S546G) was observed instead of the N481Y substitution [19–22]. A single substitution of N481Y or S546G enabled the H protein of wild-type MV strains to utilize CD46, without influencing their ability to use SLAM. In addition, two amino-acid differences were observed in Edmonston-derived strains in comparison with wild-type strains at positions 64 and 89 of M protein (P64S and E89K), which allowed an interaction of M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and assembly of infectious particles in Vero cells [23–25].

In this report, amino-acid substitutions were investigated in M, F and H proteins of Vero-adapted strains from six wild-type MV strains isolated in B95a cells, in comparison with the original wild types. Mutations of N481Y or S546G of the H protein region were observed as previously reported. In addition, mutations in S62R, S83P and E89G of the M protein region were noted, being different from the mutations described in previous reports. Recombinant MV strains with mutations in the H and M genes were constructed to conduct a functional analysis of the mutations.

## Materials and Methods

*Cells and Viruses*

Six strains of the wild type were used in this study.

MVi/Tokyo.JPN/17.07-AN/B4, MVi/Mie.JPN/19.07-OY/B4, MVi/Mie.JPN/23.07-TY/B3, MVi/Mie.JPN/41.07-MA/B3 and MVi/Mie.JPN/03.08-KU/B4 were genotype D5 isolated in 2007/2008 outbreaks in Japan, using B95a cells after three or four passages. MVi/Aichi.JPN/44.06/B3 was genotype D9 [26]. Through several passages in Vero cells, eight Vero-adapted strains were obtained. AN-V4 was obtained after four passages of MVi/Tokyo.JPN/17.07-AN/B4 in Vero cells. OY-V4 and OY-V22 were obtained after four and 22 passages of MVi/Mie.JPN/19.07-OY/B4 in Vero cells, respectively. TY-V4, TY-V22, MA-V15, KU-V4 and D9-V4 strains were obtained after passages of respective strains in Vero cells. MVAT7 pol., non-replicative vaccinia virus expressing T7 RNA polymerase (a kind gift from Dr. G. Sutter), was used for fusion analysis and the recovery of infectious viruses.

B95a cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), and Vero cells in MEM supplemented with 5% FCS. 293 T cells were cultured in MEM supplemented with 10% FCS.

*Construction of F and H Expression Plasmids and Fusion Experiment*

The H protein expression plasmids were constructed from Vero-adapted strains (AN-V4, OY-V4, OY-V22, TY-V22, MA-V15, KU-V4 and D9-V4 strains) and their original wild-type isolates and AIK-C strain. The H gene was amplified by RT-PCR using the set of primers H-ATG (5′-GTTGAATTCATGTCACCAC-AACGAGACCGGA-3′) and H-TAG (5′-AATGCGGCCGCCT-ATCTGCGATTGGTTCCA-3′), containing restriction enzyme sites of *Eco*RI and *Not*I (underlined). The amplified DNA fragments were cloned into multicloning sites located downstream of the T7 promoter of pBluescript SK II–. Several clones were sequenced to analyze the frequency of mutations. Constructed H expression plasmid (0.2 μg) was co-transfected together with the AIK-C F expression plasmid into a monolayer of B95a or Vero cells infected with recombinant vaccinia virus, MVAT7 pol., using Mirus Superfect III (Invitrogen Life Technologies, Carlsbad, Calif., USA) [27]. Cells were fixed with cooled acetone and further subjected to indirect immunofluorescent (IF) staining.

*Construction of Recombinant MV Strains with Mutations*

The infectious recombinant MV strains were recovered from the infectious cDNA clone based on the AIK-C measles vaccine strain, expressing wild-type measles H protein cloned from the wild-type MV [27, 28], and the recombinant infectious cDNA constructions are shown in figure 1. Briefly, full-length cDNA of the AIK-C strain was divided into two parts: the first half consisted of AIK-C cDNA from the leader to the *Pac*I site at nucleotide position 7238 of the AIK-C genome, and the second half of the AIK-C cDNA consisted of H and L regions from the 7239 *Pac*I site to the trailer sequence. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The green fluorescent protein (GFP) sequence was inserted using *Nco*I and *Not*I restriction enzyme sites in accordance with the rule of six of the genome length [29, 30], designated as pMVAIK P/M-GFP. The H gene of the wild-type or Vero-adapted strains was cloned at *Nhe*I (genome position 7426) and *Pvu*II (genome position 9082) of the second half of the AIK-C cDNA and combined with the first half of the cDNA. The M gene of the wild-type or Vero-adapted strains was cloned at *Bgl*II (genome position 3445) and *Ban*III (genome position 4312) sites of the first half of the AIK-C cDNA clone and combined with the second half to construct full-length cDNA.

For the recovery of the recombinant MV, 293 T cells were infected with MVAT7 pol., and then 0.5 μg of pAIK-N, 0.25 μg of pAIK-P, 0.1 μg of pAIK-L and 1.5 μg of full-length recombinant cDNA, using Mirus Superfect III (Invitrogen Life Technologies) modified from previous reports [27, 28]. After 2 days of culture,

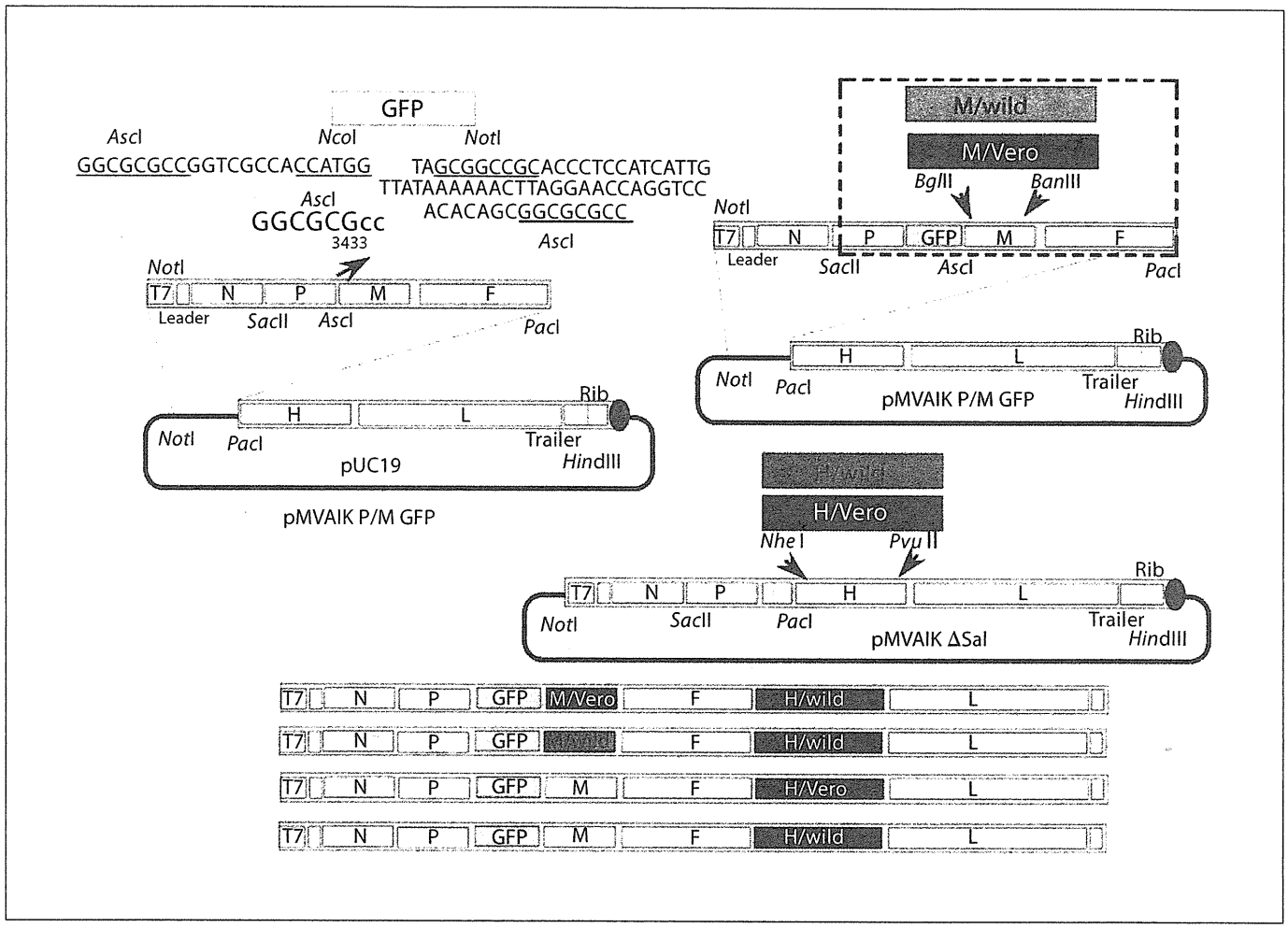


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**Fig. 1.** Construction of recombinant MV. Full-length AIK-C cDNA was divided into two parts at the *Pac*I restriction enzyme site. The H genes of the wild-type and Vero-adapted strains were amplified and cloned using *Nhe*I and *Pvu*II restriction enzyme sites. The M gene was inserted at *Bgl*II and *Ban*III restriction enzyme sites. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The GFP sequence was inserted at the P/M junction.

293 T cells were co-cultured with B95a cells. Infectious virus particles were rescued through two blind passages in B95a cells at 32.5° in 5% $CO_2$.

*Sequence Analysis*

The M, F and H genes of the wild-type isolates and Vero-adapted strains were amplified by RT-PCR and sequenced by dye terminator methods using ABI 3130 (Applied Bio Systems Japan, Tokyo). Consensus sequence was defined as the dominant sequence determined by direct sequence and respective gene cloning.

*Indirect IF Staining and GFP Expression*

B95a or Vero cells were cultured in 8-well LabTek Glass slides (Nalge Nunc International, Rochester, N.Y., USA) and infected with recombinant MV strains. GFP expression was confirmed and cells were fixed with cooled acetone and further subjected to indirect IF staining using 1:100 dilution of monoclonal antibody against measles H protein (kindly supplied by Dr. T. A. Sato, National Institutes for Infectious Diseases) and that against N protein (Chemicon, Temecula, Calif., USA). They were stained with 1:100 dilution of anti-mouse IgG monoclonal antibody labeled with FITC (Sigma-Aldrich, St. Louis, Mo., USA).

*Virus Growth and GFP Expression*

B95a or Vero cells were infected with recombinant MV strains at MOI = 0.01 and culture supernatant was obtained on days 1, 3, 5 and 7 of culture. Virus infectivity was calculated by the Reed-Muench method using B95a cells, and GFP expression was monitored with a microplate fluorescent reader, FLx 800 (Bio-Tek Instruments, Winooski, Vt., USA).

**Table 1.** Amino-acid substitutions of wild-type isolates after adaptation to Vero cells

| MV strains | Genotype | Passage in Vero cells | H original | | M original | | |
|---|---|---|---|---|---|---|---|
| | | | 481Asn | 546Ser | 62Ser | 83Ser | 89Glu |
| MVi/Tokyo.JPN/17.07-AN/B4 | D5 | AN-V4 | –[1] | Gly | – | – | – |
| MVi/Mie.JPN/19.07-OY/B4 | D5 | OY-V4 | Tyr | – | – | – | – |
| MVi/Mie.JPN/19.07-OY/B4 | D5 | OY-V22 | Tyr | Gly | Arg | Pro | – |
| MVi/Mie.JPN/23.07-TY/B3 | D5 | TY-V4 | – | – | – | – | – |
| MVi/Mie.JPN/23.07-TY/B3 | D5 | TY-V22 | – | Gly | – | – | – |
| MVi/Mie.JPN/41.07-MA/B3 | D5 | MA-V15 | Tyr | – | – | – | – |
| MVi/Mie.JPN/03.08-KU/B4 | D5 | KU-V4 | – | Gly | – | – | – |
| MVi/Aichi.JPN/44.06/B3 | D9 | D9-V4 | – | – | – | – | Gly |

AN-V4 was obtained after four passages of MV/Tokyo.JPN/17.07-AN/B4 in Vero cells. H original/M original = Amino acids of the original strain.

[1] No amino-acid substitution after adaptation to Vero cells.

**Table 2.** Sequence diversity of M and H protein-encoding plasmids derived from Vero-adapted virus strains

| | H gene | | | | M gene | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | N481Y | S546G | 481/546 | H-wt | S62R | S83P | 62/83 | E89G | M-wt |
| OY-V4 | 4 | 0 | 0 | 6 | | | | | |
| OY-V22 | 3 | 6 | 1 | 2 | 2 | 0 | 22 | | 0 |
| TY-V22 | 0 | 6 | 0 | 1 | | | | | |
| MA-V15 | 6 | 0 | 0 | 0 | | | | | |
| KU-V14 | 0 | 7 | 0 | 0 | | | | | |
| D9-V4 | | | | | | | | 4 | 2 |

## Results

*Amino-Acid Substitutions of MV for Adaptation to Vero Cells*

Eight Vero-adapted strains belonging to the D5 and D9 genotypes were obtained after 4–22 passages: AN-V4, OY-V4, OY-V22, TY-V4, TY-V22, MA-V15 and KU-V4 from five wild-type D5 strains and D9-V4 after four passages of wild-type D9 strain. Original wild-type strains did not show any cell fusion in Vero cells and eight Vero-adapted strains were obtained, demonstrating syncytia formation. No mutation was noted in the F gene in the strains studied and amino-acid substitutions in the M and H proteins are shown in table 1. Regarding the H gene of the Vero-adapted strains, an amino-acid substitution at position 481 from Asn to Tyr (N481Y) was noted in OY-V4 and MA-V15. An amino-acid substitution at position 546 from Ser to Gly (S546G) was observed in AN-V4, TY-V22 and KU-V4. OY-V22 showed substitutions at positions 481 and 546. No mutation was observed for TY-V4 and D9-V4. With respect to the M gene, substitutions were observed at position 62 of the M protein from Ser to Arg (S62R) and at position 83 from Ser to Pro (S83P) of OY-V22, and at position 89 from Glu to Gly (E89G) of D9-V4. The mutation site(s) for adaptation to Vero cells was different from strain to strain.

For each Vero-adapted MV strain, H and M expression plasmids were constructed, and the results of sequence analyses are shown in table 2. Among ten H expression plasmids derived from the OY-V4 strain, four had N481Y and the remaining six were wild type. Among 12 H expression plasmids of OY-V22, three had N481Y substitution, six had S546G, one had both N481Y and S546G, and the remaining two were the original wild type. Six H expression plasmids of MA-V15 showed N481Y substitution. Six of seven plasmids derived from

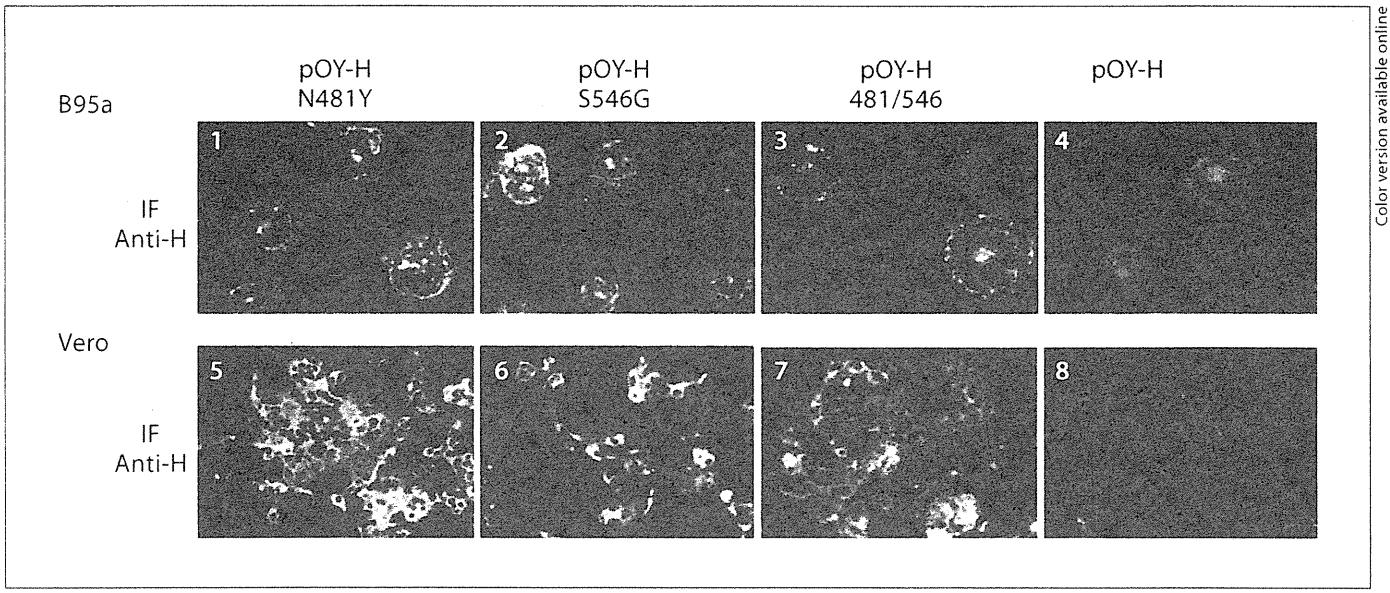


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**Fig. 2.** Fusion experiment involving H protein expression plasmids. The H protein expression plasmids were constructed: pOY-H was constructed from MVi/Mie.JPN/19.07, pOY-H N481Y, pOY-H S546G and pOY-H 481/546 from OY-V22 (Vero-adapted strain from MVi/Mie.JPN/19.07-OY). They were co-transfected with the F expression plasmid of the AIK-C in B95a cells (upper panels) and Vero cells (lower panels). The appearance of cell fusion and the results of IF using monoclonal antibody against H protein.

TY-V22 and all seven from KU-V4 showed S546G substitution. For the M gene, substitutions of S62R and S83P were observed in OY-V22 and that of E89G in D9-V4. Among 24 M gene clones derived from OY-V22, there were two clones with S62R and the remaining 22 had both S62R and S83P substitutions. As for the D9-V4 strain, four plasmids out of six M gene clones showed E89G substitution.

*Expression Experiments Involving F and H Proteins*

Through cloning experiments, the H protein expression plasmid was constructed from the original MVi/Mie.JPN/19.07-OY strain (pOY-H), and three expression plasmids with mutation(s) derived from OY-V22 (Vero-adapted strain of MVi/Mie.JPN/19.07-OY strain) were constructed: pOY-H N481Y (with an amino-acid substitution at position 481 of H protein), pOY-H S546G (with an amino-acid substitution at position 546) and pOY-H 481/546 (with two substitutions). pAIK-F was used as an F expression partner constructed from the AIK-C vaccine strain. The H expression plasmids were co-transfected with pAIK-F as the F expression partner in B95a or Vero cells, and the results of indirect IF staining are shown in figure 2. They induced a similar level of cell fusion in B95a cells, but the original wild-type pOY-H did not induce cell fusion in Vero cells (panel 8). The plasmids with an amino-acid substitution of N481Y (pOY-H N481Y) or S546G (pOY-H S546G) induced cell fusion in both B95a and Vero cells, and plasmid with both N481Y and S546G substitutions (pOY-H 481/546) induced more prominent cell fusion in Vero cells (panel 7).

*Construction of Recombinant MV Strains with H Mutations*

Recombinant cDNAs having wild-type original H gene of MVi/Mie.JPN/19.07-OY and mutated H gene from the Vero-adapted strains (OY-V22) were constructed based upon AIK-C cDNA, and infectious viruses were recovered: rMV-OY H without amino-acid substitution, rMV-OY H N481Y with amino-acid substitution of N481Y, rMV-OY H S546G with amino-acid substitution of S546G and rMV-OY H 481/546 with both substitutions. These recombinant MV strains were designed to express GFP, and the expression of GFP and IF staining against N protein are shown in figure 3. rMV-OY H did not show cell fusion in Vero cells similar to the original wild-type MVi/Mie.JPN/19.07-OY (panels 13, 18), but the Vero-adapted strain OY-V22 induced cell fusion in Vero as well as B95a cells (panels 5, 14). rMV-OY H N481Y and rMV-OY H S546G induced cell fusion in Vero cells to a

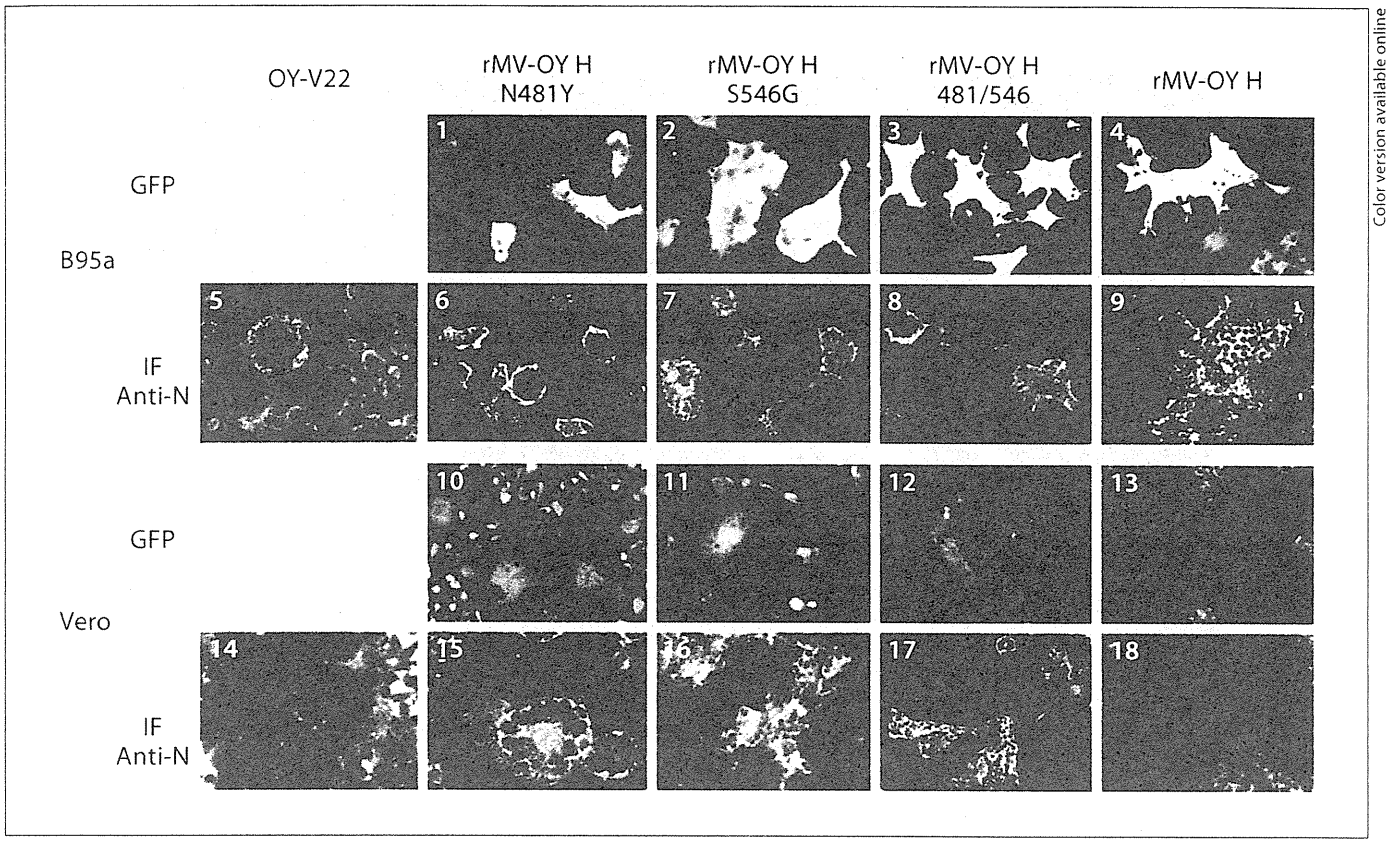


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**Fig. 3.** GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with substitutions of H protein. OY-V22 was a Vero-adapted strain after 22 passages in Vero cells. Four recombinant MV strains were constructed: rMV-OY H had no mutation, and rMV-OY H N481Y, rMV-OY H S546G and rMV-OY H 481/546 had mutations at the respective positions. They were used to infect B95a and Vero cells, and the results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

similar extent, and rMV-OY H 481/546 led to slightly more extensive fusion in Vero cells.

*Construction of Recombinant MV Strains with M Gene Mutations of OY-V22*

Two amino-acid substitutions were noted in the M gene of the OY-V22 strains, S62R and S83P. The AIK-C vaccine strain has Tyr (Y) at position 481 of the H region, and mutation was introduced to generate Asn (N) at 481 (rMVAIK H481N). The M gene of the AIK-C was replaced by that of OY-V22 in the pMVAIK H481N cDNA, and two recombinant MV strains were recovered: rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. These M gene mutations were introduced into the cDNA of rMV-OY H and rMV-OY H/M S62R and rMV-OY H/M 62/83 were recovered. GFP expression and expression of measles N protein are shown in figure 4. rMVAIK H481N did not induce cell fusion in Vero cells (panels 11, 16), but rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83 strains induced cell fusion (panels 12, 13, 17, 18). rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells (panels 14, 15, 19, 20).

*Virus Growth of Recombinant MV Strains with H and M Gene Mutations*

All recombinant MV strains were designed to express GFP and virus growth was monitored by GFP expression, measured as fluorescence units (FU). The cell-free infectious virus titer was examined on days 1, 3, 5 and 7 after infection. The results of virus growth of recombinant MV strains with mutations in the H gene are shown in figure 5. Baseline GFP expression was <400 FU. rMV-OY H (Hwt) failed to produce syncytia in Vero cells without GFP expression, similar to the fusion experiment using

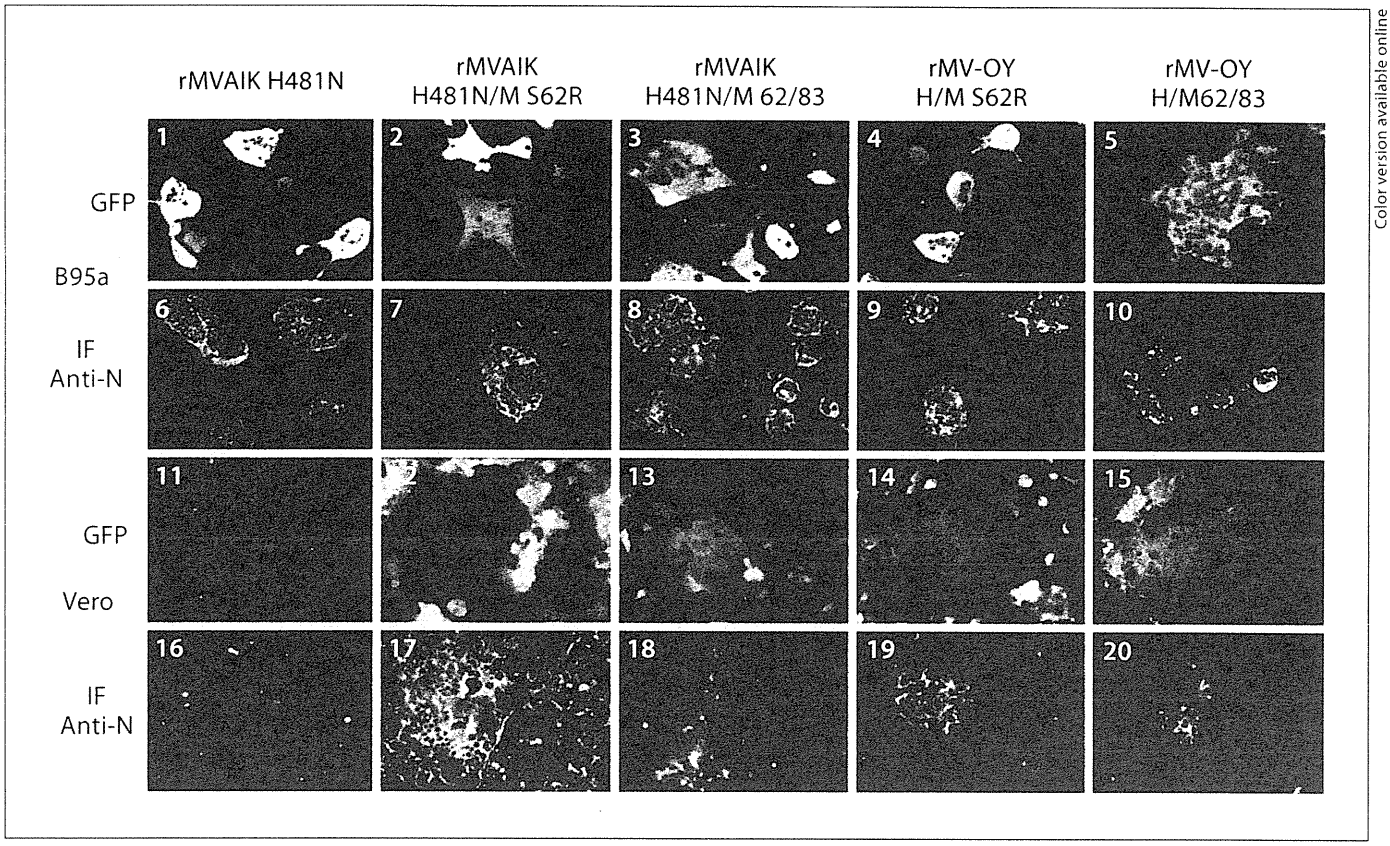


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**Fig. 4.** GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with M protein substitutions. The M gene of AIK-C was replaced with that obtained from OY-V22 with mutation of S62R, S62R/S83P, and the H gene was also replaced by OY-H wild-type (rMV-OY H/M S62R, rMV-OY H/M 62/83). The M gene mutations were also introduced into rMVAIK H481N, rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. The results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

the H expression plasmids, and no infectious virus was observed in the supernatants. rMV-OY H 481/546 (H481/546) grew better on day 5 or 7 after infection with a high infectious titer of $10^5$ $TCD_{50}$ and high GFP expression over 2,500 FU in Vero cells, and induced more marked fusion in Vero cells than in the other recombinant MV strains with N481Y or S546G substitution. rMV-OY H S546G (H546) induced higher GFP expression than rMV-OY H N481Y (H481), but there were no significant differences in the production of infectious virus particles. In B95a cells, four recombinant MV strains demonstrated similar infectious virus production, but the rMV-OY H S546G strain led to a lower expression of GFP than rMV-OY H, rMV-OY H481N and rMV-OY H 481/546 (fig. 5).

GFP expression and the production of infectious virus in Vero and B95a cells infected with recombinant MV strains with M gene mutation(s) are shown in figure 6. M gene mutation(s) was introduced into rMVAIK-H481N or rMV OY-H. rMV OY-H (Hwt) did not express GFP and no infectious virus particle was produced in Vero cells. rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt) induced GFP expression, and an infectious virus titer of $10^{2.0-3.0}$ $TCD_{50}$ was obtained in the supernatants on day 7 of Vero cell culture. rMV-OY H induced extensive cell fusion in B95a cells with higher level of GFP expression and particle formation, but four other recombinant MV strains with M gene mutation(s) induced lower GFP expression with a lower production of infectious particles. Although recombinant MV stains with mutated M protein produced cell fusion in Vero cells, they induced lower cell fusion with lower numbers of fusion foci in comparison with those produced in B95a cells.

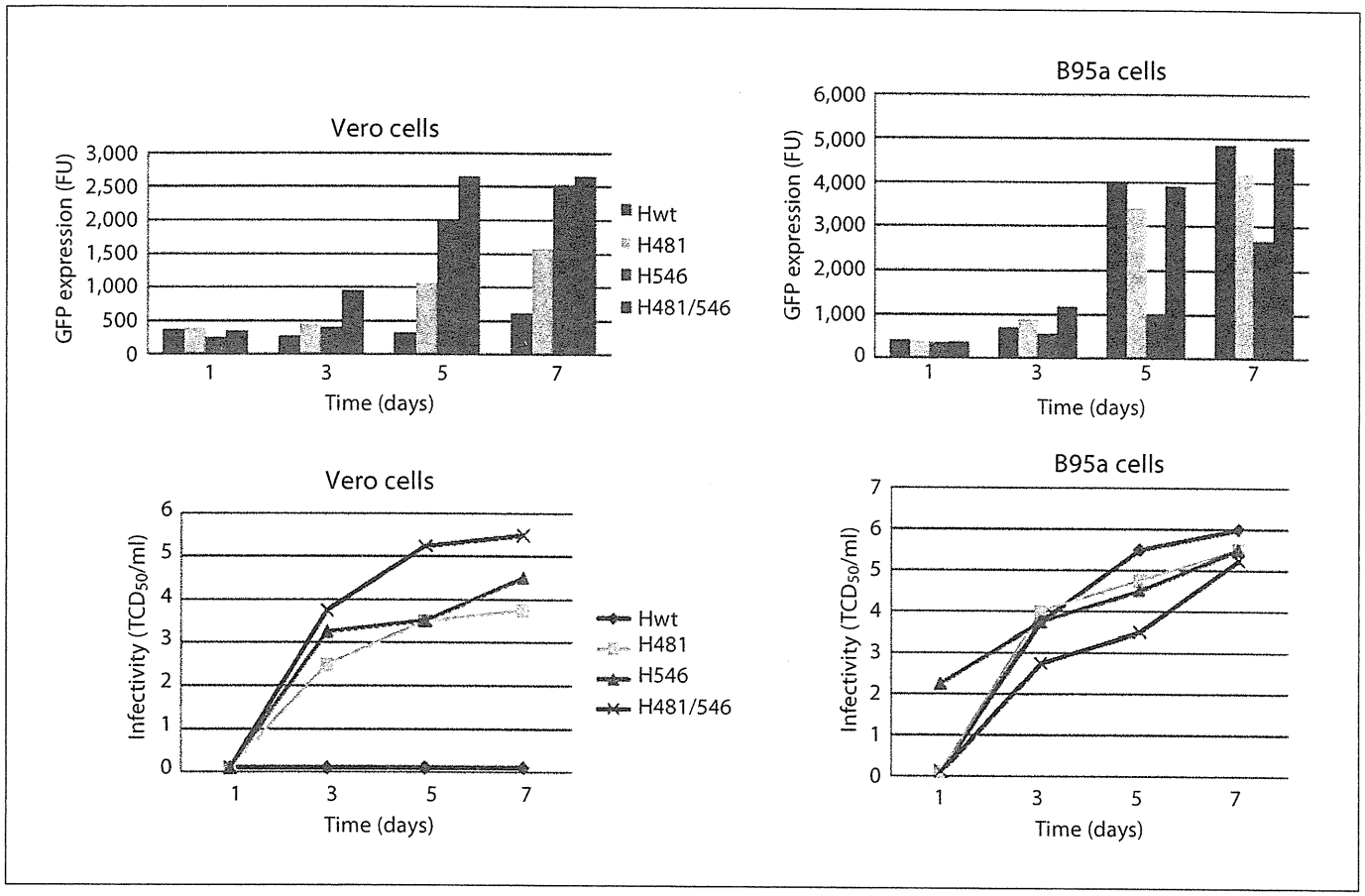


**Fig. 5.** GFP expression and virus growth of recombinant MV strains with substitution of H protein. Vero and B95a cells were infected with rMV-OY H (Hwt), pOY-H N481Y (H481), pOY-H S546G (H546) and pOY-H 481/546 (H481/546) strains at MOI = 0.01. GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

*Recombinant MV Strains Derived from D9 Strain*

D9-V4 was a mixture of M gene mutation. Four clones showed mutation at position 89 of the M gene from Glu to Gly, and the remaining two clones showed no mutation. The H and M genes of AIK-C cDNA were replaced with those amplified from the D9-V4 strain. rMV-D9 H/M has the original wild-type H and M genes, and rMV-D9 H/M E89G has wild-type H gene and E89G mutation of the M gene, similar to the D9-V4 strain. The results of cell fusion, GFP expression and infectivity in culture fluids are shown in figure 7. Two recombinant MV strains showed similar cell fusion in B95a cells, with similar infectious virus production. Whereas rMV-D9 H/M E89G induced cell fusion and produced infectious virus particles ($10^{3.1}$ $TCD_{50}$/ml) in Vero cells, rMV-D9 H/M did not induce cell fusion and showed no virus growth in Vero cells.

## Discussion

MV induces extensive syncytium formation with cell fusion, and the appearance of a syncytium is a positive indicator of virus isolation. Binding of the H protein to the receptor induces the conformational changes of H and F proteins required for the protrusion of the fusion domain into lipid bilayers of the cell membrane [3–5]. At present, two MV receptors have been identified: CD46 and SLAM (CD150). CD46 is expressed widely on the surface of epithelial cells, including Vero cells, whereas CD150 is a lymphocyte-stimulating factor expressed on the surface of lymphoreticular cells [10, 31]. In the past, MV was isolated after three or more blind passages in Vero cells, and the isolation rate was low. Otherwise, current wild-type MV strains were isolated in B95a cells. The

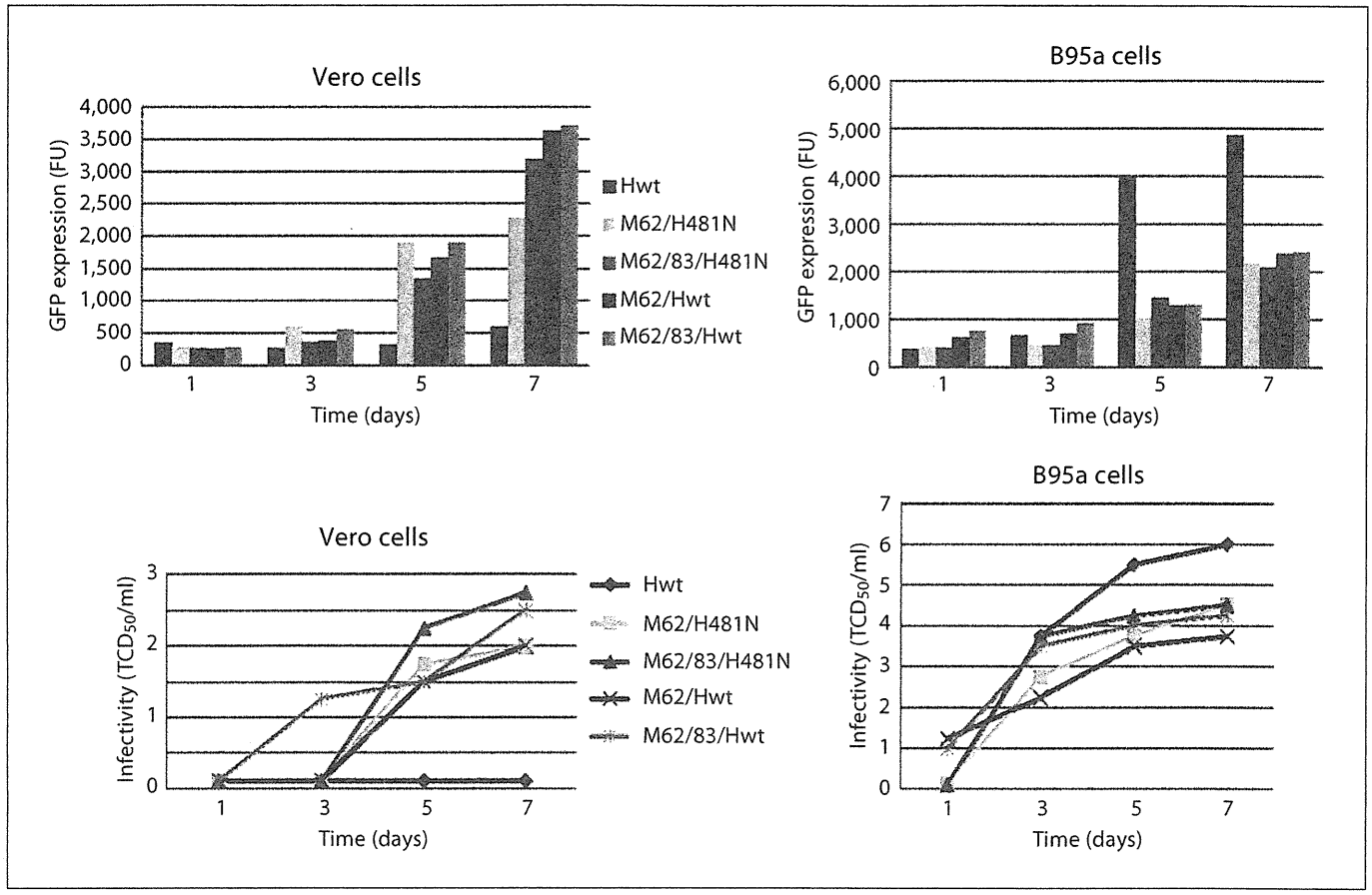


**Fig. 6.** GFP expression and virus growth of recombinant MV strains with substitution of M protein. Vero and B95a cells were infected with rMV-OY H (Hwt), rMVAIK H481N/M S62R (M62/H481), rMVAIK H481N/M 62/83 (M62/83/H481N), rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt). GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

adaptation of the current wild MV to Vero cells led to amino-acid substitution(s) in the H gene that increased the binding capacity of the measles H protein to CD46 [19]. Lecouturier et al. [12] reported that substitutions of two amino-acid positions of 451 and 481 in H protein of the Halle strain abrogated the fusion inducibility of the functional domain(s) of the measles H protein. Hsu et al. [14] reported that a single amino-acid change at position 481 determined the ability of H protein to bind CD46. Xie et al. [15] reported that Asn at position H481 of the wild-type expression plasmid was replaced by various amino acids, and the mutant plasmid with Tyr, similar to the Edmonston strain, induced cell fusion, but this substitution did not cause the down-regulation of CD46 expression, unlike the Edmonston strain. Thus, Tyr at position 481 was indispensable for measles H protein to interact with CD46, similar to the other reports [16, 21]. A single substitution of N481Y of the wild H protein was not sufficient to use CD46, suggesting that further substitutions were required for efficient virus growth in Vero cells [22]. From the comparison of the H gene of the Edmonston and current circulating strains, three substitutions (N309I and E492G, plus N416D or T446S) were necessary for efficient virus growth in Vero cells [32]. Li and Qi [20] examined the amino-acid substitutions of MV H protein when three hemadsorption-negative strains were passaged >20 times in Vero cells. They reported that amino-acid substitution at position 546 of the measles H protein from Ser to Gly was critical for hemadsorption and CD46 binding besides the amino-acid change at position 481. In the three-dimensional surface representation of the structural model, three of these residues (D505, D507 and

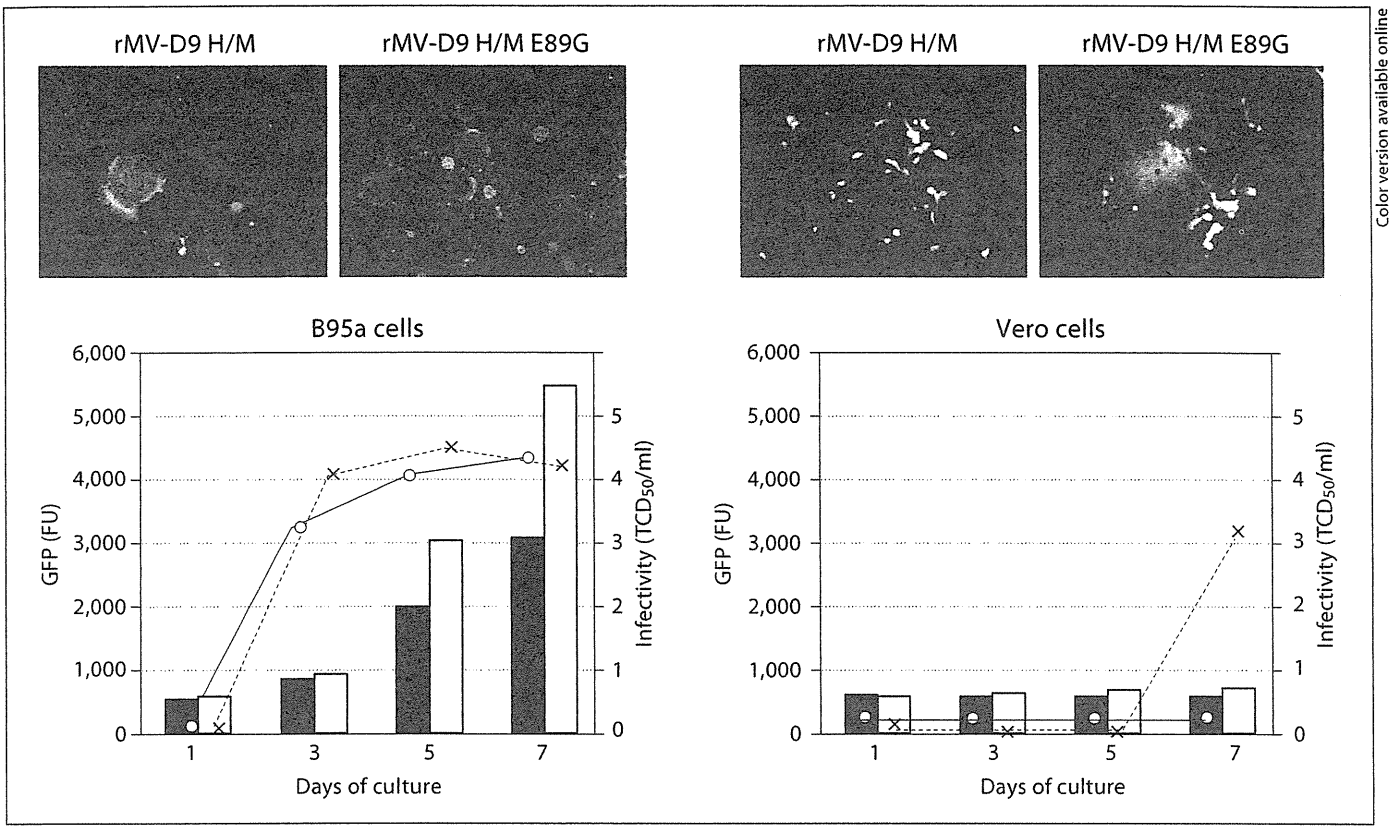


**Fig. 7.** Characteristics of recombinant MV strains with a mutation in M protein observed in the D9-V4 Vero-adapted strain. The M and H genes of AIK-C were replaced with those of MV/Aichi.JPN/44.06 (rMV-D9 H/M), and the mutated M gene was introduced (rMV-D9 H/M E89G). GFP expression is shown in the upper panels. Vero and B95a cells were infected, and GFP expression (■; □) and virus infectivity (——; - - -) were monitored. ——○——/■ = rMV-D9 H/M; - - × - -/□ = rMV-D9 H/M E89G.

R533) align along the rim on one side of the cavity on the top surface of the measles H globular head, and form the basis of a single continuous site that overlaps with the 546-548-549 CD46 binding site. Mutations at position 481 or 546 induce conformational changes in the measles hemagglutinin globular head and influence the affinity for CD46 binding [33]. The MV H protein three-dimensional approach suggested that the SLAM- and CD46-relevant residues are located in contiguous areas in propeller β sheets 5 and 4, respectively, and several CD46-relevant amino acids may be shielded from direct receptor contact [34–36]. Using the Edmonston H protein, the association rate for SLAM binding to H protein was very low; about 20 times lower than CD46. However, SLAM bound to H protein more tightly than CD46, as revealed by a 5-fold lower dissociation rate [37].

The Vero-adapted MV strains showed several amino-acid changes in the other genes: two in the P, V and C proteins, three in the H gene (Ala H14 Gly, Leu H423 Pro and Ser H546 Gly) and two in the L gene [13]. Only two nucleotide differences were reported at 2331 genome position of P/V/C and at the 3628 genome position (amino acid position 64 of M protein), and none in the H gene between the MV genome isolated in B95a cells and that isolated in Vero cells from the same patient [38]. Through comparative studies of the M gene sequence of wild-type and Edmonston strains, substitutions of P64S and E89K from wild-type M protein were reported to be responsible for the fusion inducibility and efficient virus growth in Vero cells [23]. These two substitutions (P64S and E89K) allowed the interaction of the M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and the assembly of infectious particles in Vero cells [24]. M protein also had binding activity to the cytoplasmic domain of H protein together with F protein and ribonucleoprotein complex, and was transported to the mem-

brane raft fraction [25, 39, 40]. In this report, no substitution was noted in the F protein for adaptation to Vero cells but substitutions were reported at positions 439 and 464 of the F protein, as well as those in the N, P/V/C, H and L proteins after adaptation to Vero cells [41].

In this report, cell fusion was observed after four passages of MVi/Mie.JPN/3.07-TY/B3 (TY-4 strain) in Vero cells but TY-4 had no amino-acid substitution in the M, F and H proteins. There was a possibility of mutation(s) in the P and/or L genes and, after 22 passages, substitution of H546G was detected. Amino-acid substitutions for adaptation to Vero cells were different from strain to strain. All D5 genotype strains showed a substitution at position 481 or 546 of the H protein region at an early stage of passage. These were mutated through the adaptation process, and no substitution except for those at these positions was observed on direct sequencing analysis. Thus, the substitutions of N481Y or S546G were essential for adaptation to Vero cells, and the other regions were not changeable. OY-V4 showed a mixed population of N481Y and original wild-type clones, whereas OY-V22 was a mixture of the N481Y substitution, S546G, substitutions of both N481Y and S546G, and the original sequence. Through the results of expression experiments involving H plasmids and GFP expression of recombinant MV strains, rMV-OY H 481/546 induced more extensive cell fusion in Vero cells than MV strains with either of the substitutions. Double mutants were predicted to use CD46 more efficiently, leading to efficient infectious virus production and growth in Vero cells.

Further repeated passages in Vero cells accumulated mutations in the M gene in addition to the H gene. Two strains adapted to Vero cells showed substitutions in M protein. Substitution of E89G was observed in D9-V4, and those of S62R and S83P in OY-V22. rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells. These recombinant MV strains decreased virus growth and particle formation in B95a cells and the single substitution of S62R was sufficient. These positions were different from those in previous reports, demonstrating the fusogenicity of combined substitutions of P64S and E89K in Vero cells [23, 24, 32]. The backbone of our reverse genetics is the AIK-C vaccine strain and rMV H481N and rMV-OY H have K at position 89 of M protein, but these two strains did not induce cell fusion without infectious virus production. Thus, the single mutation of E89K would not be a critical region for interaction between M and H proteins for efficient virus growth in Vero cells. Substitution of E89G of the M protein was observed in D9-V4. rMV-D9 H/M (E at position 89 of M protein) did not induce cell fusion, with no virus growth, but GFP was demonstrated in cells without fusion. rMV-D9 H/M E89G induced a small fusion with a low level of infectious virus production, even though it had wild-type H protein. This may suggest the presence of another unidentified receptor for MV and, recently, the possibility of a molecule related to tight junctions on the basolateral sides of epithelial cells was reported [42, 43].

Adaptation would occur in a different manner depending on the strains or experimental condition. Three strains showed mutations in the H gene within four passages, and repeated passages added additional mutations to the M and H genes. One strain of D9 showed a substitution in the M protein, even without H protein substitution at N481Y or S546G which enables the virus to enter cells efficiently. Thus, we supposed that the amino acids at positions 481 and 546 of H protein are critical for the different tropisms based on the results of expression experiments. From the results of recombinant MV strains with M protein mutations, substitution in the M protein promoted efficient MV growth and particle formation in Vero cells, and would influence efficient receptor usage of the wild-type H protein to induce cell fusion irrespective of H gene mutation.

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# Interruption of the Circulation of an Indigenous Measles Genotype and the Introduction of Other Genotypes After a Mass Vaccination Campaign in the Philippines

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Molecular analysis of measles viruses in the Philippines was conducted from 2000 to 2008. No confirmed measles cases were detected in the surveillance in 2005 after the mass vaccination campaign in 2004. However, a re-emergence of measles cases occurred in 2007, which was caused by other genotypes and the previous circulating genotype had disappeared. ***J. Med. Virol.***



**KEY WORDS:** measles; molecular epidemiology; genotyping

## INTRODUCTION

In 2005, the Western Pacific Regional Office of the World Health Organization (WHO) set a goal of eliminating measles by 2012 [WHO, 2007]. In the Philippines, the Department of Health set a target to eliminate measles by 2008 [Department of Health, 2006]. Nationwide mass measles vaccination campaigns were conducted in 1998 (coverage: 94%), 2004 (95%), and 2007 (95%). The national measles surveillance in the Philippines monitors measles by using a sentinel surveillance system, which includes major hospitals in each region [National Epidemiology Center, 2004]. To define the proportion of actual measles cases among suspected cases, selected serum samples from suspected cases are sent to the Research Institute for Tropical Medicine in Manila for laboratory confirmation, which consists of detecting measles IgM antibody [Sobel et al., 2009]. Genotypes of circulating measles viruses (the order Mononegavirales, the family Paramyxoviridae, the genus *Mobillivirus*, Measles virus) can provide useful information on the source of infection and transmission pathway. However, such data are limited for the Philippines. This study describes the molecular epidemiology of measles viruses in the Philippines using stored serum samples.

## MATERIALS AND METHODS

Thirty-five serum samples positive for measles IgM antibody were selected at random for each year from 2000 to 2004. In subsequent years, all IgM-positive samples with sufficient volume were included. Viral RNA was extracted using the PureLink Viral RNA/DNA MiniKit (Invitrogen, Carlsbad, CA) and subjected to reverse transcription (RT). Standard PCR and nested PCR protocols were used to amplify the C-terminal hypervariable region of the N gene (456 nt) [National Institute of Infectious Disease, 2002]. PCR products were sequenced, and phylogenetic analysis was conducted using MEGA 3.1 [Kumar et al., 2004] by including reference strains for each genotype [WHO, 1999] and other D3, D9, and G3 sequences that are available in GenBank. Sequences described in this study were submitted to GenBank (AB514002-514030).

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TABLE I. The Number of PCR Positive Cases Based on N Gene Among Sample Tested for PCR and the Results

| Year | Samples tested for ELISA | Measles IgM positive | Samples tested for PCR | Sample positive for PCR (N) | Genotypes detected |
|---|---|---|---|---|---|
| 2000 | 2,506 | 2,193 | 35 | 9 | D3 |
| 2001 | 2,665 | 2,143 | 35 | 6 | D3 |
| 2002 | 2,552 | 2,124 | 35 | 5 | D3 |
| 2003 | 1,225 | 1,081 | 35 | 4 | D3 |
| 2004 | 221 | 155 | 35 | 1 | D3 |
| 2005 | 71 | 0 | Not tested | Not tested | Not tested |
| 2006 | 114 | | 3 | 0 | |
| 2007 | 569 | 174 | 64 | 4 | D9.G3 |
| 2008* | 523 | 167 | 122 | 1 | D9 |
| Total | 10,446 | 7,836 | 364 | 30 | |

*AsofM.25th200S, RITM.

## RESULTS

Table I shows the measles surveillance data from 2000 to 2008. In 2004, the number of measles IgM-positive cases began to decline, and no positive samples were detected in 2005. However, in 2007, an increase in the number of cases was detected. From 7,836 laboratory confirmed cases, 409 samples were tested for the measles gene by RT-PCR. In total, 30 samples were positive for the N gene (Table I).

All samples collected between 2000 and 2004 from five regions [National Capital Region (NCR), Regions 3, 5, 7, and 11] were classified as genotype D3 (Fig. 1). However, samples collected in 2007 and 2008 were classified as D9 and G3 (Fig. 1). D9 was found in two regions (NCR and Region 9) (Fig. 2). G3 was also found in two regions [NCR and Autonomous Region in Muslim Mindanao (ARMM); Fig. 2]. Both D9 and G3 were detected in NCR, where only D3 had been detected previously.

One sample (Region9.PHL/2007/07-0120) was not classified as any of the known genotypes (Fig. 1) (AB514025). The sequence differences between Amsterdam.NET/49.97(G2) and Region9.PHL/2007/07-0120 were 4.4% and 4.9% in the N and H genes (partial H gene), respectively. The sequence differences between Gresik.INO/17.02(G3) and Region9.PHL/2007/07-0120 were 2.7% and 2.7% in the N and H genes (partial H gene), respectively. Sequencing of the whole H gene for Region9.PHL/2007/07-0120 was not possible due to insufficient viral RNA content in the sample.

## DISCUSSION

This study demonstrated that the circulating genotype in the Philippines was D3 until 2004. This result confirmed previous findings that the genotype of imported cases with epidemiological links to the Philippines was D3 [Rota et al., 2002, 2004; WHO, 2005a; Cheng et al., 2009]. All of those importations of D3 from the Philippines were reported between 1989 and 2002. The sequences of D3 detected in the Philippines were placed within at least two lineages and did not cluster with the D3 reported from Japan and Papua New Guinea (Fig. 1). The genotype change in 2007 occurred simultaneously with the increase in the number of measles cases. Before this change, the nationwide mass measles immunization campaign "Ligtas Tigdas" that targeted all children aged 9–48 months was conducted in February 2004 [Department of Health, 2006]. The data suggested that the vaccination campaign in 2004 covered a sufficient proportion of the target age group and succeeded in interrupting the chain of transmission of the endemic measles strain [Sobel et al., 2009]. After D3 disappeared in the Philippines, D3 had not been reported [WHO, 2008]. The documented importation cases from the Philippines in 2009 and 2010 were also D9 [WHO, 2010]. Those facts support the finding that D3 circulation in the Philippines, which used to be a possible major source of D3 has ended. The reported incidence of measles per 100,000 population was less than one based on the sentinel surveillance system in 2005 [WHO, 2005b, 2006]. However, the number of measles cases started to increase again in 2007. This resurgence may have been caused by newly introduced genotypes. Similar shifts in genotype have been reported in other countries such as Spain [Rima et al., 1997] and Germany [Santibanez et al., 2002]. Changes in circulating measles genotypes can occur in countries that have suboptimal control programs. In such situation, interruption of transmission for a short period may be possible, but failure to maintain sufficient herd immunity can result in the resurgence of measles due to introductions of the virus that spreads quickly among the susceptible population that has been accumulated gradually. A second follow-up measles immunization campaign in the Philippines was conducted between 15th October and 15th November, 2007 [Department of Health, 2006]. However, the number of laboratory confirmed cases continued to increase in 2008 (Fig. 1). Because of the high infectivity, 93–95% herd immunity is required to interrupt measles transmission [Moss, 2007]. A sustainable vaccination strategy that includes both routine immunization and supplemental immunization activity is needed to achieve such a high immunity level. The D9 and G3 genotypes have been circulating mainly in the Asia-Pacific Region. According to recent reports, D9 was detected in Indonesia, Hong Kong, Singapore,

and New Zealand, and G3 was detected in Indonesia, Australia, and New Zealand [WHO, 2007, 2008]. Among D9 variants, one strain (NCR.PHL/2007/07-0351) exhibited 100% homology with measles genotypes detected in Taiwan (Yilan.TWN/48.03, Hualian.YWN/01.08), Australia (Victoria.AU/14.04), and Malaysia (Sarawak.MAS/2.05). However, the

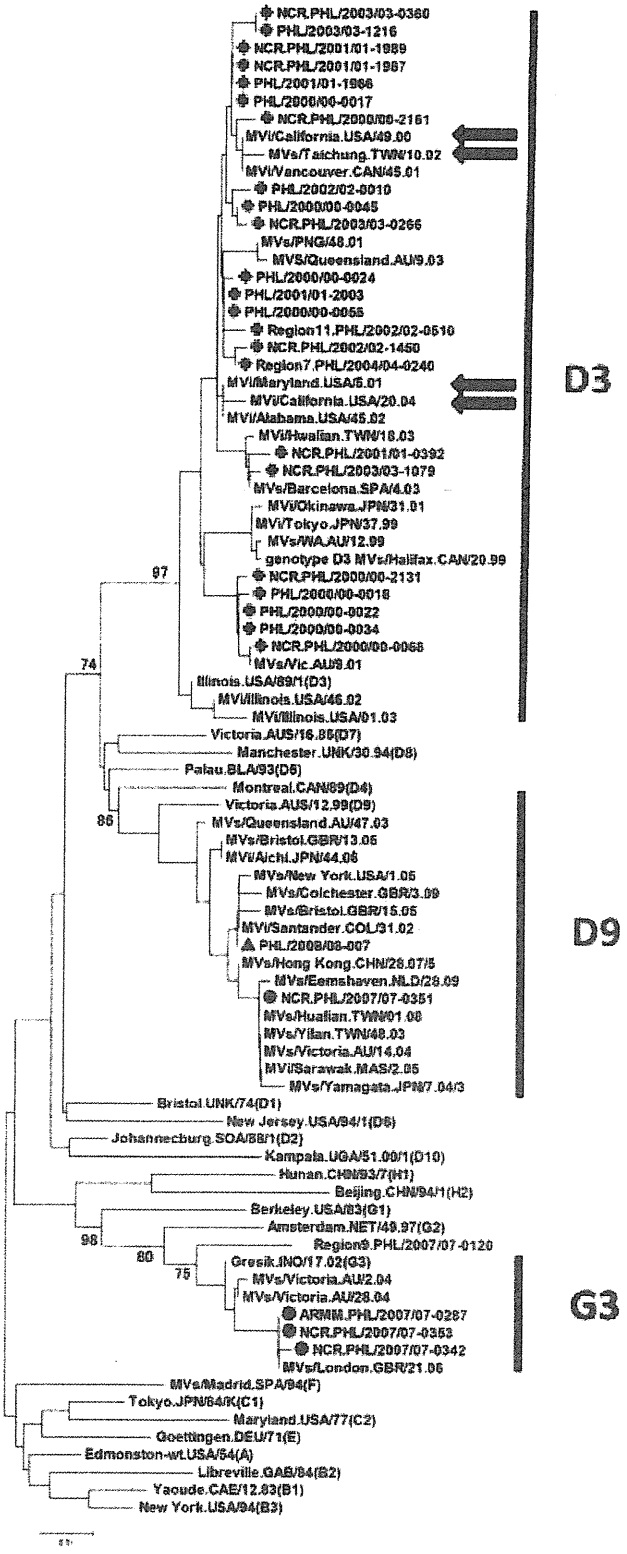


Fig. 1. Genotyping of measles virus strains from the Philippines by sequencing and phylogenetic analysis based on the 450 C-terminal nucleotides of the N gene by using the Neighbour-Joining method. A square indicates the strains that were isolated until 2004, a circle indicates the strains collected in 2007 and a triangle indicates the strains collected in 2008. Genotypes are indicated by color as follows: Red: D3, Green: D9, Yellow: unclassified and Blue: G3. The arrow indicates the sequence of imported cases from the Philippines. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

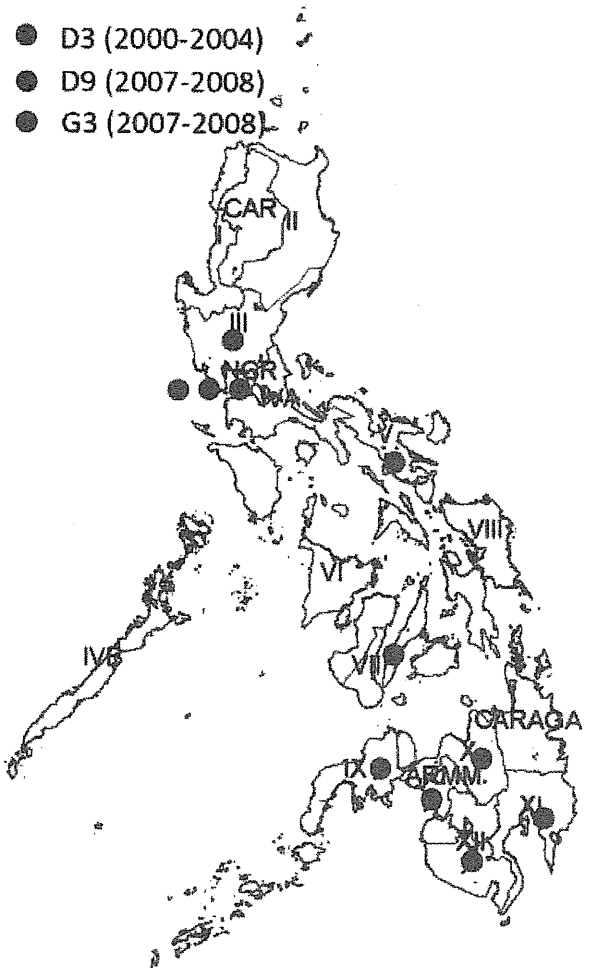


Fig. 2. Geographical distribution of detected measles genotypes in the Philippines between 2000 and 2008. Region name and number are shown. CAR, NCR, and ARMM indicate Cordillera Administrative Region, National Capital Region, and Autonomous Region in Muslim Mindanao, respectively.

other strain (PHL/2008/08-007) was identical to a strain from Colombia (Santander.COL/31.02; Fig. 2). This suggests that the importation event might have occurred more than once. G3 detected from the Philippines showed 100% homology with the genotype from England (Mvs/London.GBR/21.06). G3 strains detected in NCR (NCR.PHL/2007/07-0353) and ARMM (ARMM.PHL/2007/07-0287) were also 100% homologous despite a long distance between the two regions (Fig. 2). This may indicate that G3 was

imported into the Philippines once and subsequently spread throughout the country. The route of importation to the Philippines could not be identified because of the lack of detailed epidemiological information and incomplete genetic information for other countries. To eliminate measles worldwide, efforts in a single country are insufficient due to frequent cross-border transmissions.

The current study has several limitations. First, only serum samples were available, and the PCR-positive rate was low due to the low viral RNA content in serum and the timing of sample collection [Riddell et al., 2001]. Second, one strain could not be classified as any of the existing genotypes. The sequence of this strain is located between G2 and G3. However, it could not be identified as G2 or G3. According to the past proposal for new genotypes, new genotypes are designated if the nucleotide sequence differs from the closest reference sequence by at least more than 2.5% in hypervariable region of N and 2.0% in full length H gene [WHO, 2001]. The N gene nucleotide sequence of this strain differs enough to be classified as new genotype, but it was not possible to amplify the full H gene sequence. In addition, the detection of such unidentified strain was limited to one sample. The collection of nasopharyngeal swabs or leukocytes for isolating the virus is necessary in order to characterize fully the genotypes of circulating measles viruses.

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