J. Tokyo Med. Univ., **60**(1) : 9～18, 2002

An amino acid replacement at HN 521 inhibits cell fusion in a mumps vaccine strain adapted to HeLa cells

Rina UZUKA[1),3)], Katsuhiro KOMASE[2)], Masaharu NAKAGAWA[3)] and Tetsuo NAKAYAMA[3)]

[1)]Department of Pediatrics, Tokyo Medical University
[2)]Center for Biological Products, The Kitasato Institute
[3)]Department of Virology, The Kitasato Institute for Life Sciences, Kitasato University

Abstract

When the Hoshino mumps vaccine seed strain (KO-3) was adapted to HeLa cells, typical CPE was not observed. To identify the region responsible for the reduction in cell fusion, we constructed fusion (F) and hemagglutinin-neuraminidase (HN) protein expression plasmids, pKO-3-F and pKO-3-HN from the KO-3, and p18HL-F and p18HL-HN from the 18HL strain which was obtained after 18 passages in HeLa cells. No amino acid change was observed in p18HL-F but p18HL-HN had an amino acid substitution at 521 from Ala to Asp. Extensive cell fusion was observed when HeLa cells were transfected with pKO-3-F and pKO-3-HN (521 Ala) under the control of T7 RNA polymerase. When p18HL-HN (521 Asp) was used, hemadsorption and neuraminidase activities were observed but cell fusion inducibility decreased. Sequence analysis of plasmid clones constructed from virus after the first, second, third or fifth passage in HeLa cells, demonstrated that mutation at HN 521 occurred as early as the second passage. Site-directed mutagenesis from Ala to Cys at position 521 retained fusion inducibility but that from Ala to Lys reduced the extension of cell fusion. We suggest that HN 521 is an important site for modification of cell fusion without influencing hemagglutinin and neuraminidase activities.

Introduction

Acute infection by mumps virus is usually self-limiting with demonstrable parotid gland(s) swelling and several complications following parotitis, including aseptic meningitis, deafness, orchitis, and pancreatitis. The number of mumps patients has decreased since the introduction of an attenuated mumps vaccine combined with live measles and rubella vaccines (MMR) in the US and European countries, but in Japan, the use of MMR was discontinued in 1993 because of vaccine-associated aseptic meningitis[1),2)]. Mumps virus infects Vero cells, HeLa cells, and several cell lines in laboratory experiments and Vero cells are preferred for the primary isolation of mumps virus from clinical samples[3)]. Mumps virus is known to show characteristic cytopathic effects (CPE) in Vero cells. The current vaccine strains, including the Jeryl Lynn strain used in the US, were originally propagated in embryonic chicken cells[4)]. The Hoshino wild strain was isolated in the allantoic cavity of embryonic chickens in 1972 and a further attenuated vaccine seed strain (KO-3) was established through 22 passages in chicken embryonic cells at a lower temperature, 32°C[5)].

The mumps virus is a member of the family Paramyxoviridae and contains two surface integral membrane

Received August 20, 2001, Accepted December 18, 2001
Key words : Mumps virus, Fusion protein, Hemagglutinin-neuraminidase protein, Cell-fusion, HeLa cell
Reprint requests to : Rina Uzuka, Department of Pediatrics, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. Phone ; 03-3342-6111. FAX ; 03-3344-0643.
E-mail ; rinauzu@mti.biglobe.ne.jp

spike glycoproteins, the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein[3]. The HN protein is a type II integral membrane protein responsible for the attachment of the mumps virus to cellular sialic acid receptors. The F protein is a type I integral membrane protein and is synthesized as a biologically inactive precursor, F0, which is cleaved by cellular protease to form an active disulfide-linked heterodimer (F2-F1)[6]. The N-terminus of F1 has hydrophobic amino acid residues, which are highly conserved among paramyxoviruses and are believed to be the fusion domain involved in the fusion process[7),8]. As for the syncytium formation of mumps virus, extensive cell fusion was observed when the F and HN proteins were co-expressed. Replacement of Ser at position 195 of the F protein by Tyr reduced the fusion inducibility in COS7 cells[9),10] but the role of the region was not identified.

To investigate the genetic stability after passage in different tissue culture cell lines, we passaged the vaccine seed strain (KO-3) in HeLa cells which are different from the chicken embryonic cells used for vaccine production. No typical CPE was seen on passage in HeLa cells. We obtained culture fluid at each passage and constructed expression plasmids for the F and HN proteins. These were then co-transfected into HeLa cells and Vero cells using recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase[11),12] to identify the region responsible for the reduction in fusion inducibility.

Materials and Methods

Mumps virus strain

The Hoshino vaccine seed strain KO-3 was developed by attenuation through 22 passages in chick embryonic cells from wild type mumps virus isolated in 1972[5]. The seed virus was inoculated in HeLa cells and the culture fluid was obtained after seven days of culture. A small portion (0.1 ml) of culture fluid (5 ml) was serially passaged in fresh HeLa monolayers in 12.5 cm^2 tissue culture flasks and the strain 18HL was obtained after the 18th passage. The culture fluid was kept at −70°C. Infectivity was measured by plaque forming assay in Vero cells, using ABC staining. Briefly, cells were overlaid with 0.5% agar in MEM after virus inoculation. After 7 days culture in 5% CO_2, the plate was fixed with 0.25% glutaraldehyde. Hyperimmune rabbit polyclonal antibodies to mumps virus were added and after vigorous washing, biotinylated monoclonal antibodies against rabbit IgG were added. The plate was stained with ABC staining kit (Vectastain Elite ABC Kit, Vector Lab., Burlingame, CA, USA).

Construction of expression plasmids

Total RNA was extracted from the culture fluid of mumps virus and the F, and HN coding regions were amplified by reverse transcription polymerase chain reaction, as previously reported[13]. We synthesized a set of F primers (F-ATG ; 5′-TCCTGGTACCTTGATCA GTAATCATGAAGG-3, and F-TAA ; 5′-GCCG GAGCTC TTAGTACCTAATGAGAT-3), and HN primers (HN-ATG ; 5′-CTTTGGTACCTGCTCGA AAGATGGAGCC-3, and HN-TGA ; 5′-CCTA GAGCTCAAGTGATGGTCAATCT-3). The underlined sequences are linker restriction enzyme sites of *Kpn*I and *Sac*I and were attached to the sequence of mumps KO-3, as previously reported[13]. Expression plasmids were constructed by inserting the full coding region sequences of the F and HN regions into the pBluescript II SK- vector (Strategene Cloning Systems, La Jolla, CA, USA), using multicloning sites of *Kpn*I and *Sac*I located downstream of the T7 promoter.

Site-directed mutagenesis

Site-directed mutagenesis was used to construct mutant HN proteins in which Ala of HN 521 was changed to Lys or Cys. For the introduction of point mutations in the HN region, oligo-nucleotides were synthesized ; 521 Lys+(5′-TATGTCTCTAAACTT AATAAT-3), 521 Lys−(5′-ATTATTAAGTTTAG AGACATA-3), 521 Cys+ (5′-TATGTCTCTTGTC TTAATAAT-3), and 521 Cys−(5′-ATTATTAAGA CAAGAGACATA-3). The underlined letters represent the mutated amino acid, Lys or Cys, respectively. DNA fragments containing designed mutations were amplified by PCR and, using appropriate restriction enzymes, a mutated DNA fragment was inserted into pKO-3-HN. The two mutated clones were designated pHN-521 Lys and pHN-521 Cys. The constructed expression plasmids were sequenced using sequencing primers reported previously[13] by an ABI 377 sequencer (ABI PRISM™ 377, Perkin-Elmer Corp.).

Fusion analysis of cells co-transfected with the F and HN proteins

The plasmid constructs were expressed in HeLa cells by the vaccinia virus T7 expression system, kindly supplied by Dr. B. Moss[11]. Monolayers of nearly confluent HeLa cells were infected with recombinant vaccinia virus v-TF7.3, which expresses bacteriophage T7 RNA polymerase, at a multiplicity of infection (m.o.i.) of 1 and incubated at 37°C for one hour in a 24-well plate. The cells were washed with Opti-MEM (GIBCO BRL, Life Technologies, Grand Island, NY, USA) and the F and HN expression plasmids were transfected with DMRIE-C (GIBCO BRL, Gaithersburg, MD, USA) at a dose of 0.2 μg/well for each. As for the expression in

Vero cells, recombinant vaccinia virus MVAT7 pol., kindly supplied by Dr. G. Sutter, was used at a m.o.i of $10^{12)}$ and plasmids were transfected with SuperFect Transfection Reagent (QIAGEN, Germany). After incubation overnight, the cells were fixed with 0.25% glutaraldehyde and were stained with Giemsa's solution (Merck Japan, Tokyo, Japan).

For the quantitative index for cell fusion, 5×10^5 Vero cells were cultured in 24-well plates as indicator cells transfected with pGEβgal, which was designed to express β-galactosidase under the control of T7 RNA polymerase. When cell fusion was demonstrated in the transfection experiments using mumps F and HN plasmids, indicator cells were trypsinized, and washed several times, then approximately 1×10^5 cells were added to the wells. After an additional 4-hr culture, cells were washed, and lysed with 200 μl of 0.05% Nonidet P-40 in PBS for the fluorescent assay[14]. The cell lysate of 20 μl was mixed with 80 μl of substrate solution, 0.2 mM 4-methylumbelliferyl-β-galactoside (Sigma Chemicals, St Louis, MO, USA) and after 30 min incubation at 37°C, 0.1 mM glycine buffer (pH 9.6) was added to stop the enzymatic reaction. The fluorescence was measured with a fluorometric microplate reader (Fluoroskan II, Labsystems, Japan) as previously reported[15].

Mock plasmid was used as a negative control.

Immunofluorescence assay

HeLa or Vero cells were cultured in the 8 isolated chambers of a Lab-Tek 8-well chamber slide (Nalge Nunc International, Naperville, IL, USA) and were transfected with F and or HN expression plasmids after infection with recombinant vaccinia virus expressing T7 RNA polymerase. The cells were then fixed with cooled acetone for 10 min. Indirect immunofluorescent assay was performed using monoclonal antibodies, kindly provided by Dr. K. Takeuchi, Department of Biological Products, National Institutes for Infectious Diseases of Japan[10]. After incubation with monoclonal antibodies, the cells were further processed for incubation with antiserum against mouse IgG labeled with FITC (Sigma, Steinheim, Germany).

Hemadsorption and neuraminidase activity assay

HN expression plasmid was transfected with or without F expression plasmid in Vero cells. After incubation overnight, guinea pig erythrocytes (0.5%) was added to the culture wells after being transfected with expression plasmids and incubated at 4°C for one hour. Then, they were washed and visualized. And transfected cells were lysed with 200 μl of 0.05% Nonidet P-40 in PBS. The cell lysate (50 μl) was added to a 96-well plate and an equal volume of 0.1 mM 4-methylumbelliferyl N-acetylneuraminic acid (Sigma Chemicals, St Louis, MO, USA) was added as substrate[16),17]. After 2 hr incubation at 37°C, the reaction was stopped by addition of 100 μl of 0.1 mM glycine buffer (pH 10.0). Neuraminidase activity was measured as fluorescence units, with a fluorometric microplate reader (Fluoroskan II, Labsystems, Japan).

Results

Difference in deduced amino acids among plasmid constructs

We constructed the F and HN expression plasmids, pKO-3-F and pKO-3-HN from the KO-3 seed vaccine strain and p18HL-F and p18HL-HN from the 18HL strain. Differences in the deduced amino acid sequences are shown in Fig. 1. In comparison with sequence results reported previously[13], pKO-3-F and pKO-3-HN showed no change. Among eight clones of 18HL F protein expression plasmids (p18HL-F), seven showed the same sequence as pKO-3-F but one had an amino acid substitution at position 90 of the F protein from Ile to Val (p18HL-F90Val). Direct sequencing of the PCR product of the F coding region of the 18HL strain revealed the amino acid at position 90 to be Ile. Thus, p18HL-F90Val appears a minor population of 18HL strain or mis-incorporation during the RT-PCR procedure. All four clones of p18HL-HN had an amino acid change from Ala to Asp at HN 521. Chimerical HN expression plasmids were constructed using the *Pst*I restriction enzyme site.

CPE and syncytium formation

The results of the culture of the KO-3 seed virus and 18HL are shown in Fig. 2. KO-3 induced extensive CPE in Vero cells but 18HL did not. During the passage of KO-3 in HeLa cells, no CPE was observed.

The expression plasmids for the F and HN were co-transfected into HeLa cells infected with recombinant vaccinia virus v-TF7.3 and into Vero cells infected with recombinant vaccinia virus MVAT7 pol. When HeLa cells were co-cultured after transfection with pKO-3-F and pKO-3-HN separately, no cell fusion was observed (data not shown). The results of co-transfection with different combinations of plasmids are shown in Fig. 2. When both pKO-3-F and pKO-3-HN were co-transfected into HeLa cells and Vero cells, extensive cell fusion was noted. No cell fusion was observed when HeLa cells were co-transfected with different combinations of pKO-3-F and p18HL-HN. However, in Vero cells, p18HL-HN induced less cell fusion than pKO-3-HN.

We constructed recombinant DNAs encoding chimeric proteins to ascertain the amino acid affecting fusion inducibility. The *Pst*I site was utilized for the chimeric

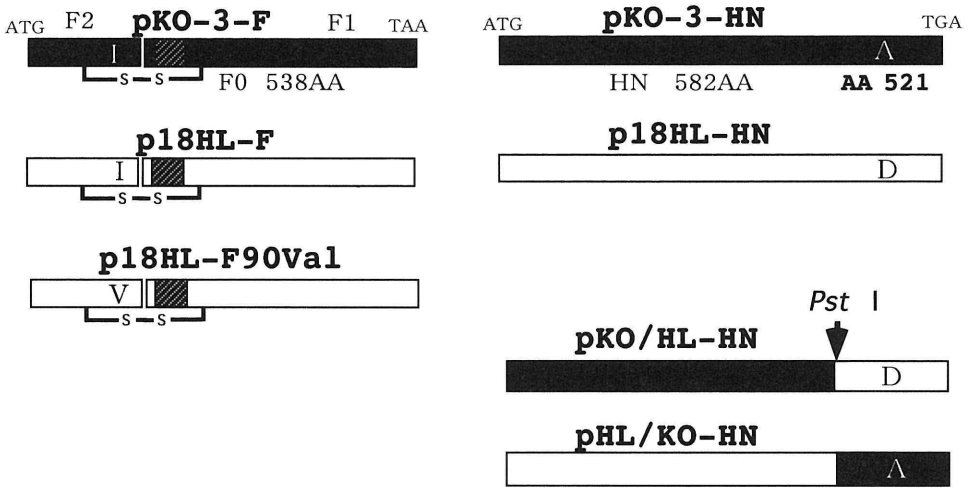

Fig. 1 Difference in deduced amino acid sequence of the plasmid constructs. For the construction of the chimeric plasmids, the Pst I site in the HN gene was used. Closed bars represent the plasmids from KO-3 strain. The shaded area represents the site of the fusion peptide domain.

construction in the HN region (Fig. 1). Asp at HN 521 of p18HL-HN was replaced with Ala in pHL/KO-HN and the fusion inducibility recovered. On the contrary, pKO/HL-HN chimeric plasmid reduced the fusion inducibility (data not shown).

Immunofluorescence assay

We examined the expression of the F and HN proteins using monoclonal antibodies, kindly supplied by Dr. Takeuchi. The results are shown in Fig. 3. The F protein was expressed when pKO-3-F was transfected in HeLa cells but not when p18HL-F90Val was used (Figs. 3-1, 2). In HeLa cells, the fluorescence intensity for HN protein was slightly weaker on transfection with p18HL-HN than pKO-3-HN (Figs. 3-3, 4). When pKO-3-HN was co-transfected with pKO-3-F as the expression partner for F protein, extensive fusion with intense fluorescence was observed in Vero and HeLa cells (Figs. 3-5, 6). When p18HL-HN was used as an expression partner for HN, the protein was expressed without any sign of cell fusion (Figs. 3-7, 8).

Hemadsorption, neuraminidase activity, and fusion index

The extent of hemadsorption in HeLa cells, the neuraminidase activity and fusion index for quantitative assay for cell fusion in Vero cells are shown in Fig. 4. Hemadsorption was observed on the syncytium cells when they were co-transfected with pKO-3-F and pKO-3-HN. HA activity was induced without cell fusion in HeLa cells co-transfected with pKO-3-F and p18HL-HN.

Fluorescence units (FU) were used as a measure of the enzymatic activity of neuraminidase. Culture fluid of the KO-3 virus (10^5 pfu/ml) had 889 FU of neuraminidase activity and that of 18HL, having the same infectivity, showed 823 FU. Cell lysate of Vero cells transfected solely with pKO-3-HN and with p18HL-HN had 294 ± 39 FU and 219 ± 61 FU of neuraminidase activity, respectively. When co-transfected with pKO-3-F, pKO-3-HN induced high levels of neuraminidase activity, 806 ± 126 FU. While co-transfection experiments with pKO-3-F and p18HL-HN showed a similar activity to pKO-3-HN or p18HL-HN alone. In mock-transfected cells, 95 ± 21 FU of base line activity was observed as background negativity. These values are the mean$\pm$1.0 S.D. of three independent experiments.

For quantitative assay for cell fusion, along with the co-transfection experiments, *lacZ*-containing cells were prepared as indicator cells. When indicator cells were mixed with cells having T7 RNA polymerase and expressing both F and HN proteins, a significant level of β-galactosidase activity was demonstrated. Only when pKO-3-F and pKO-3-HN were co-transfected, 335 ± 41 FU activity was observed. Whereas, when pKO-3-F and p18HL-HN were co-transfected, 153 ± 19 FU activity was demonstrated, similar to the background negativity for mock transfection or sole transfection experiments.

Change in amino acid at HN 521 during passage in HeLa cells

Direct sequencing revealed that HN 521 of the 18HL virus is Asp, suggesting that the majority of the virus had Asp at position 521 in HN protein. This change in amino acid was responsible for the reduction in cell fusion. We examined the distribution of the sequence diversity of the HN region on the first, second, third and fifth passage in the HeLa cell line (1HL, 2HL, 3HL, 5HL). We carried out another experiment with 17

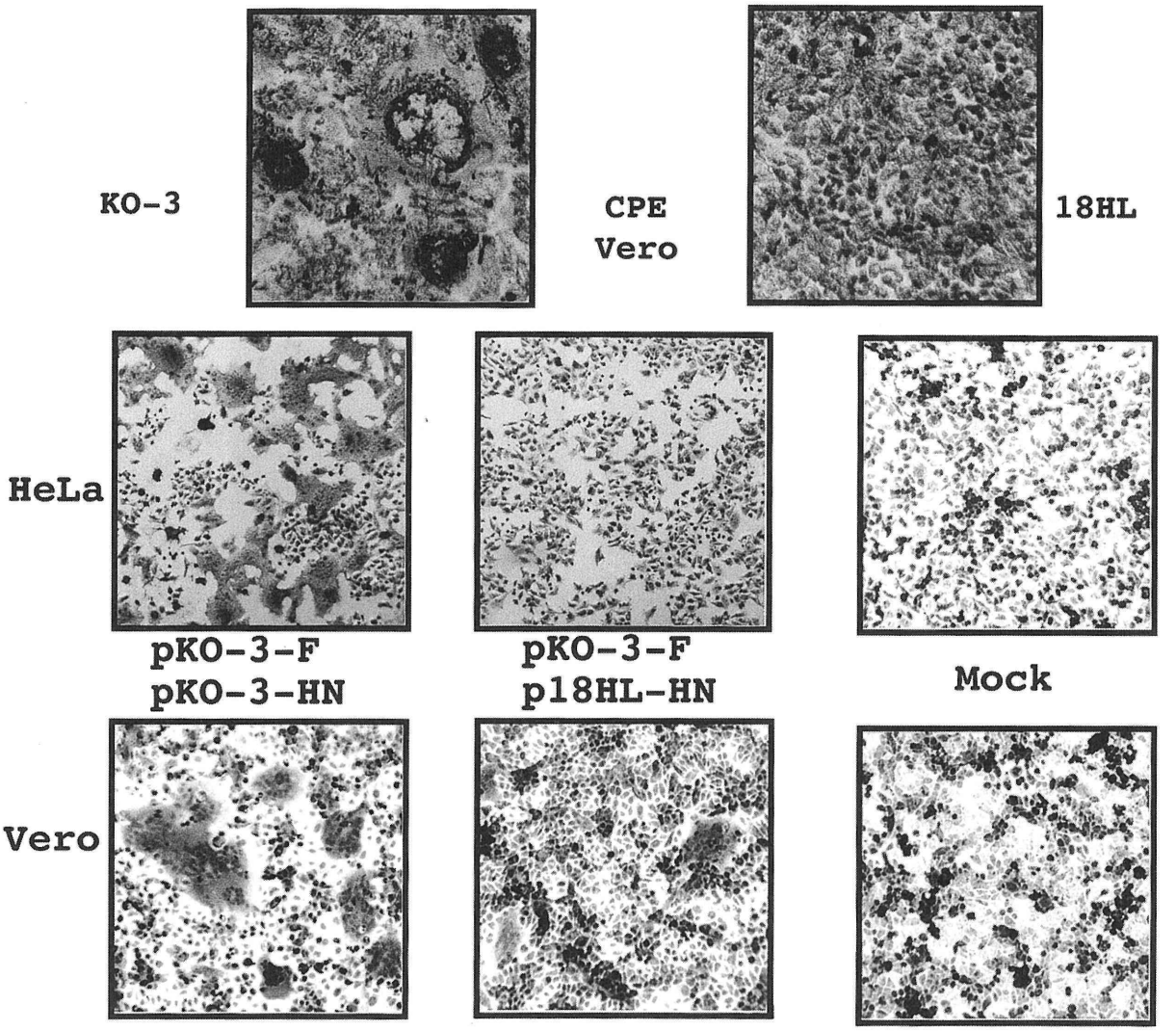

Fig. 2 CPE of the KO-3 and 18HL viruses and cell fusion inducibility of plasmids in HeLa cells and Vero cells. The plate was stained with Giemsa's solution and visualized with an Olympus microscope. All panels are at ×80. Upper panels show CPE in Vero cells infected with KO-3 and 18HL. Middle panels show the results of transfection experiments in HeLa cells and the lower panels, transfection experiments in Vero cells. As a negative control for mock transfection, pBluescript II SK- was transfected after the cells were infected with recombinant vaccinia virus.

passages of KO-3 in HeLa cells at 33°C (17HL 33°C). The distribution of plasmids having 521Ala or 521Asp is shown in Table 1. We obtained 14 clones after the first passage in HeLa cells (1HL) and all contained 521Ala. Among the 12 clones of 2HL, 7 had 521Ala and 5 had 521Asp. All clones from 3HL and 5HL contained 521Asp. And another experiment at the 17th passage in HeLa cells at 33°C showed that 9 of 11 clones had 521Asp. The change in amino acid at position 521 of the HN protein from Ala to Asp was a common feature in independent experiments for adaptation in HeLa cells.

Site-directed mutagenesis of HN 521

The pKO-3-HN induced cell fusion but p18HL-HN did not, despite having HA and neuraminidase activities. The p18HL-HN had an amino acid substitution at 521 from Ala (KO-3) to Asp (18HL). We constructed the mutant plasmids, pHN-521 Cys and pHN-521 Lys. The results of cell fusion and immuno-fluorescent assay using monoclonal antibodies against HN protein in Vero cells are shown in Fig. 5. When pKO-3-F was used as the expression partner for F protein, pHN-521 Cys induced extensive cell fusion, expressing the HN protein in Vero cells. However, pHN-521 Lys reduced the extension of cell fusion in Vero cells, although the HN protein was properly expressed. The results of neuraminidase and fusion index assays are shown in Fig. 5. When pHN-521 Cys or pHN-521 Lys was solely transfected, neuraminidase activity was induced at the same level, 232±61 FU and 205±36 FU, respectively. When co-transfected with pKO-3-F, pHN-521 Cys induced 431±66 FU, slightly higher level, but not significant, observed in co-transfection experiment with

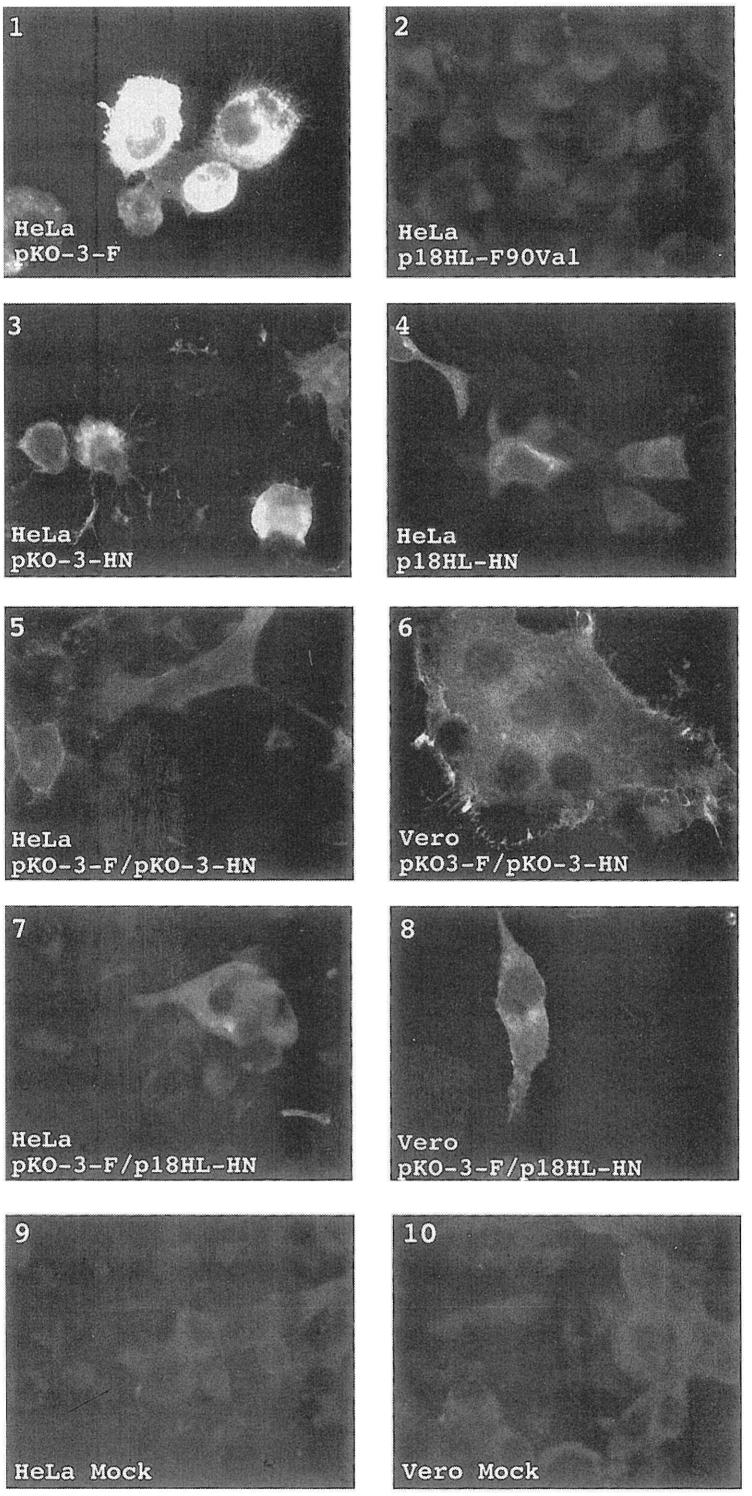

Fig. 3 Immunofluorescent assay of the expression of the F and HN proteins. HeLa cells were transfected with pKO-3-F, p18HL-F90Val, pKO-3-HN, or p18HL-HN (1, 2, 3, and 4). They were stained with anti F monoclonal antibodies (1 and 2) and with anti HN monoclonal antibodies (3 and 4). HeLa cells (5 and 7) or Vero cells (6 and 8) were co-transfected with pKO-3-HN and p18HL-HN, using pKO-3-F as an expression partner for F protein. They were stained with anti HN monoclonal antibodies (5, 6, 7, and 8). As a negative control for mock transfection, pBluescript II SK- was transfected after the cells were infected with recombinant vaccinia virus (9 and 10). Fluorescent-labeled cells were visualized with a Zweis Fluoro-Microscope at a magnification of ×63.

pHN-521 Lys (363±71 FU). Similar fusion index was obtained in co-transfection experiments, using pHN-521 Cys and pHN-521 Lys. Although pHN-521 Cys and pHN-521 Lys showed similar fusion index values, extension of individual syncytium cells was small in a co-transfection experiment using pHN-521 Lys. (Fig. 5

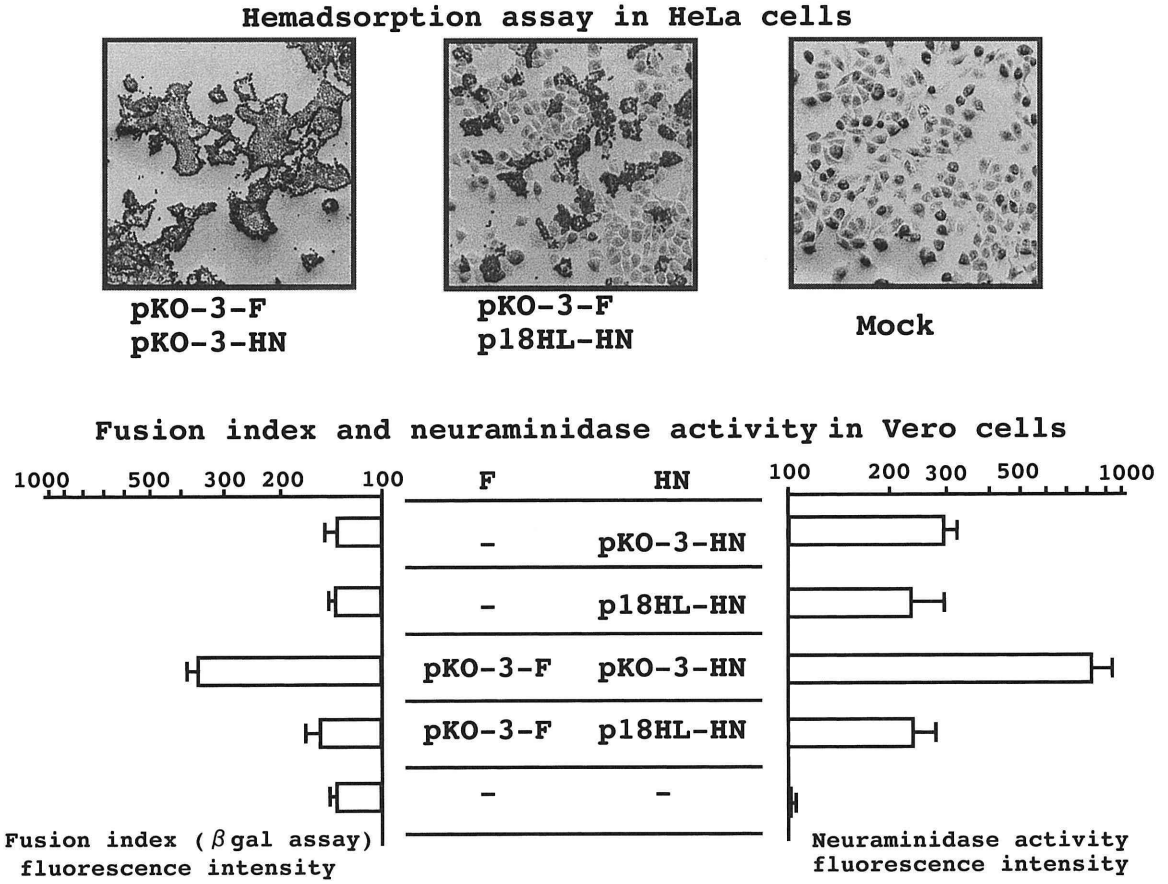

Fig. 4 Hemadsorption assay in HeLa cells and fusion index and neuraminidase activity in Vero cells. HeLa cells were transfected with pKO-3-HN and p18HL-HN using pKO-3-F as an expression partner for F protein. After incubation overnight, 0.5% of guinea pig erythrocytes were added. The plates were fixed with 0.25% glutaraldehyde and visualized with an Olympus microscope at ×80. For the fusion index and neuraminidase activity, Vero cells were transfected. Each bar represents the mean±1.0 SD in fluorescence units of three independent assays. For a negative control of mock transfection, pBluescript II SK- was transfected after infection with the recombinant vaccinia virus.

Table 1 Proportion of 521 Ala and 521 Asp of HN expression plasmids of different passage history in HeLa cells.

AA521	1HL	2HL	3HL	5HL	17HL33°C
GCC (Ala)	14	7	0	0	2
GAC (Asp)	0	5	14	12	9
Total	14	12	14	12	1

upper panel)

Mock plasmid was used as a negative control and neither HeLa cells nor Vero cells showed protein expression, HA activity or cell fusion (Figs. 2, 3, 4 and 5).

Discussion

Paramyxovirus cell fusion is critical to the infection and spread of a virus. Infectious particles bind to cell surface receptors and the fusion of cell membrane and virus allows the subsequent penetration of the virus genome into the cytoplasm. Infectious viruses proliferate by transmitting to adjacent cells through cell to cell fusion. The enveloped proteins are expressed on the membrane by intracellular transport with modification in the Golgi network apparatus after proper expression in the endo-reticular vesicles. The first step of viral infection is the binding of HN protein to the cellular receptor of sialic acid and it has been postulated that the interaction between HN and adjacent F protein molecules leads to a conformational change in the F protein, triggering the fusion cascade reaction. For the biological active form of the F and HN proteins, oligomerization of the proteins, possibly a trimer of the F protein and tetramer of the HN protein, was required[18]. Interaction between F and HN would cause the fusion peptide to extrude into the cellular membrane lipid layers[19].

In this study, we compared the Hoshino mumps vaccine seed strain and the fusion defective 18HL strain propagated through 18 passages in HeLa cells. Strain 18HL did not demonstrate a typical CPE during passage

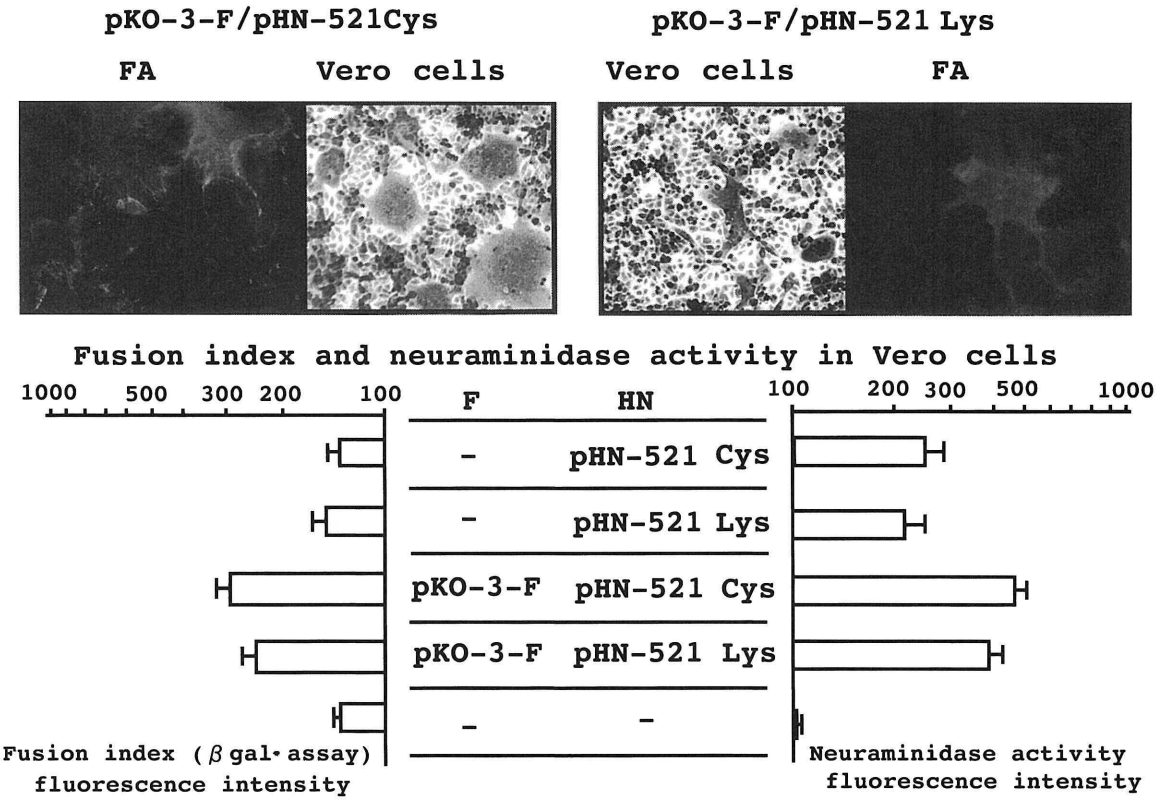

Fig. 5 Cell fusion inducibility and expression of HN protein of mutated pHN-521 Cys and pHN-521 Lys. The pHN-521 Cys and pHN-521 Lys were co-transfected with pKO-3-F into Vero cells (upper panel). After incubation overnight, cells were fixed for visualization of cell fusion and an immunofluorescent assay was done, using HN monoclonal antibodies. Vero cells were transfected for the fusion index and neuraminidase activity. Each bar represents the mean±1.0 SD in fluorescence units of three independent assay. As a negative control for mock transfection, pBluescript II SK- was transfected after infection with recombinant vaccinia virus.

in HeLa cells. 18HL had a mutation at position 521 of the HN protein from Ala to Asp but no change in the F gene. We suppose that it was selected among quasi-species of the seed strain, rather than the accumulation of mutations, as early as the second passage in HeLa cells. KO-3 vaccine seed might not be homogenous, like the Jeryl Lynn mumps vaccine strain, which is a mixture of two different genotypes[20]. A receptor binding site of measles virus was reported at amino acid position 473–477[21] but that of mumps virus has not been identified. The HN protein has another important biological activity. The virus strains with low neuraminidase activity were highly fusogenic whereas the strains with high enzyme activity achieved limited or no fusion[22]. Culture fluid of the KO-3 and 18HL, having the same infectivity, showed similar neuraminidase activity in this study. The amino acid responsible for the neuraminidase activity was that at position 181[23]. To elucidate why the 18HL strain did not demonstrate typical CPE, we conducted expression experiments on the F and HN proteins under the control of T7 RNA polymerase. Although the HN protein was properly expressed by p18HL-HN, as ascertained by FA (Fig. 3) and HA experiments (Fig. 4), it did not induce cell fusion in HeLa or little in Vero cells. Neuraminidase activity was demonstrated at the similar levels in the cells transfected with pKO-3-HN or 18HL-HN. On co-transfection with pKO-3-F and pKO-3-HN, neuraminidase activity increased in proportion to the extent of cell fusion. Co-transfection with pKO-3-F and p18HL-HN induced similar levels of neuraminidase activity to those induced by pKO-3-HN or p18HL-HN alone. In the fusion index assay, β-galactosidase activity increased on co-transfection with pKO-3-F and pKO-3-HN. From the results of these experiments, mutation at 521 in the HN of p18HL-HN did not influence the hemagglutination and neuraminidase activities. We supposed that mutation at HN 521 might not influence the oligomerization of the HN protein because the HA activity was similar to that observed with pKO-3-HN. But p18HL-HN did not induce cell fusion, thus we suppose that the poor inducibility of cell fusion would be due to modification of the interaction between F and HN proteins.

Several critical sites for protein-protein interaction have been reported. Synthetic peptides of the heptad leucine zipper region blocked cell fusion in measles virus infection[24]. The cysteine-rich region, located between

the two heptad repeats of measles F protein, was reported to be the site for interaction with measles H protein in measles-cell fusion[25]. The N-terminal end of the HN protein contains the cytoplasmic domain, transmembrane, stem region of the HN protein stalk and follows terminal globular domain. Mutation of several conserved residues in the stalk of the Newcastle disease virus decreased the neuraminidase activity without having a significant effect on receptor binding[26]. Several reports demonstrated the stalk region of the ectodomain of paramyxovirus H or HN proteins may be essential for efficient fusion, probably mediating efficient dimerization[27]. The heptad leucine repeat region of the stalk domain of HN protein was important for fusion, promoting interaction between the heptad repeat regions of F and HN proteins[28),29)].

In this study, HN protein was expressed properly by both plasmids, pKO-3-HN, p18HL-HN, pHN-521 Cys and pHN-521 Lys, without influencing HA and neuraminidase activities (Figs. 2 and 4). At present, the functional properties of the COOH terminus of the HN protein around the 521 amino acid are not determined yet but the region might modify HN-F interaction and influence cell-to-cell fusion process.

Acknowledgements

We are obliged to Dr. B. Moss, for supplying recombinant vaccinia virus vTF7.3 and to Dr. G. Sutter, for MVAT7 pol. We thank Dr. K. Takeuchi for the monoclonal antibodies against mumps F and HN proteins.

References

1) Plotkin SA, Wharton M : Mumps vaccine. Vaccines. 3rd edn. (Eds) Plotkin SA and Orenstein WA, W.B. SAUNDERS, Philadelphia, 267～292, 1999
2) Sugiura A, Yamada A : Aseptic meningitis as a complication of mumps vaccination. Pediatr Infect Dis J **10** : 209～213, 1991
3) Wolinsky JS : Mumps virus. Virology, 3rd edn. (Eds) Fields BN, Knipe DM and Howley PM, Lippincott-Raven, New York, 1243～1266, 1995
4) Buynak EB, Hilleman MR : Live attenuated mumps virus vaccine. 1. vaccine development. Proc Sci Exp Biol Med **123** : 768～775, 1966
5) Sasaki K, Higashihara M, Inoue K, Igarashi Y, Makino S : Studies on the development of a live attenuated mumps virus vaccine. The Kitasato Arch Exp Med **49** : 43～52, 1976
6) Lamb RA : Minireview : paramyxovirus fusion : a hypothesis for changes. Virology **197** : 1～11, 1993
7) Horvath CM, Lamb RA : Studies on the fusion peptide of a paramyxovirus fusion glycoprotein : roles of conserved residues in cell fusion. J Virol **66** : 2443～2455, 1992
8) Horvath CM, Peterson RG, Shaughnessy MA, Wood R, Lamb RA : Biological activity of paramyxovirus fusion proteins : factors influencing formation of syncytia. J Virol **66** : 4564～4569, 1992
9) Tanabayashi K, Takeuchi K, Okazaki K, Hishiyama M, Yamada A : Expression of mumps virus glycoproteins in mammalian cells from cloned cDNAs : both F and HN proteins are required for cell fusion. Virology **187** : 801～807, 1992
10) Tanabayashi K, Takeuchi K, Okazaki K, Hishiyama M, Yamada A : Identification of an amino acid that defines the fusogenicity of mumps virus. J Virol **67** : 2928～2931, 1993
11) Fuerst TR, Moss B : Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc Natl Sci USA **83** : 8112～8126, 1986
12) Sutter G, Ohlmann M, Erfle V : Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. Fed Eur Biochem Societies **371** : 9～12, 1995
13) Kashiwagi Y, Takami T, Mori T, Nakayama T (1999) Sequence analysis of F, SH, and HN genes among mumps virus strains in Japan. Arch Virol **144** : 593～599, 1999
14) Nussbaum O, Broder CC, Berger EA : Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. J Virol **68** : 5411～5422, 1994
15) Nakayama T, Aizawa C, Kuno-Sakai H : A Clinical analysis of gelatin allergy and determination of its causal relationship to the previous administration of gelatin-containing acellular pertussis vaccine combined with diphtheria and tetanus toxoids. J Allergy Clin Immunol **103** : 321～325, 1999
16) Myers RW, Lee RT, Lee YC, Thomas GH, Reynolds LW, Uchida Y : The synthesis of 4-methylumbelliferyl α-ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase. Anal Biochem **101** : 166～174, 1980
17) Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB : Fluorometric assay of neuraminidase with a sodium (4-Methyl-umbelliferyl-α-D-N-acetylneuraminate) substrate. Analytical Biochem **94** : 287～296, 1979
18) Sergel T, McGinnes LW, Peeples ME, Morrison TG : The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. Virology **193** : 717～726, 1993
19) Dutch RE, Leser GP, Lamb RA : Paramyxovirus fusion protein : characterization of the core trimer, a rod-shaped complex with helices in anti-parallel orientation. Virology **254** : 147～159, 1999
20) Afzal MA, Pickford AR, Forsey T, Heath AB, Minor PD : The Jeryl Lynn vaccine strain of mumps virus is a mixture of two distinct isolates. J Gen Virol **74** : 917～920, 1993

21) Patterson JB, Scheiflinger F, Manchester M, Yilma T, Oldstone MBA : Structural and functional studies of the measles virus hemagglutinin : identification of a novel site required for CD46 interaction. Virology **256** : 142～151, 1999
22) Merz DC, Wolinsky JS : Biochemical features of mumps virus neuraminidase and their relationship with pathogenicity. Virology **114** : 218～227, 1981
23) Waxham MN, Aronowski J : Identification of amino acids involved in the sialidase activity of the mumps virus hemagglutinin-neuraminidase protein. Virology **183** : 226～232, 1988
24) Wild TF, Buckland R : Inhibition of measles virus infection and fusion with peptides corresponding to the leucine zipper region of the fusion protein. J Gen Virol **78** : 107～111, 1997
25) Wild TF, Fayolle J, Beauverger P, Buckland R : Measles virus fusion : role of the cysteine-rich region of the fusion glycoprotein. J Virol **68** : 7546～7548, 1994
26) Wang Z, Iorio RM : Amino acid substitutions in a conserved region in the stalk of the Newcastle disease virus HN glycoprotein spike impair its neuraminidase activity in the globular domain. J Gen Virol **80** : 749～753, 1999
27) Plemper RK, Hammond AL, Cattaneo R : Characterization of a region of the measles virus hemagglutinin sufficient for its dimerization. J Virol **74** : 6485～6493, 2000
28) Deng R, Wang Z, Mahon PJ, Marinello M, Mirza A, Iorio RM (1999) Mutations in the Newcastle disease virus hemagglutinin-neuraminidase protein that interfere with its ability to interact with the homologous F protein in the promotion of fusion. Virology **253** : 43～54, 1999
29) Stone-Hulslander J, Morrison TG : Mutational analysis of heptad repeats in the membrane-proximal region of Newcastle Disease virus HN protein. J Virol **73** : 3630～3637, 1999

HeLa 細胞に適合したムンプスワクチン株の HN 領域 521 位は細胞融合を抑制する

宇塚里奈[1)] 駒瀬勝啓[2)] 中川正治[3)]
中山哲夫[3)]

[1)]東京医科大学小児科学教室
[2)]北里研究所生物製剤研究所開発研究部
[3)]北里生命科学研究所ウイルス感染制御 I

【要旨】 ムンプスウイルス星野ワクチンシード株 (KO-3) を HeLa 細胞で継代すると，典型的な細胞壊死変性効果 (CPE) は観察されなかった．細胞融合が減弱した原因を明らかにするため，KO-3 株と KO-3 株を HeLa 細胞で 18 代継代した 18HL 株の細胞融合 (F) 蛋白，赤血球凝集・ノイラミニダーゼ (HN) 蛋白翻訳領域発現プラスミドを構築した．pKO-3-F, pKO-3-HN は KO-3 株から構築し，p18HL-F, p18HL-HN は 18HL 株から構築した．p18HL-F ではアミノ酸変異は認めなかったが，p18HL-HN では 521 位にアラニン (Ala) からアスパラギン酸 (Asp) への変異を認めた．T7 RNA Polymerase 発現下で HeLa 細胞に pKO-3-F と pKO-3-HN (521Ala) を Transfection させた場合，広範な細胞融合が観察された．一方，p18HL-HN (521Asp) を用いた場合，赤血球凝集能，ノイラミニダーゼ活性は認めているにもかかわらず，細胞融合誘導能は減弱していた．HeLa 細胞継代によるアミノ酸の変化を解析した結果，HN 領域 521 位の変異は 2 代継代後の早期から認められた．521 位をアラニンからシステイン (Cys) に変異を導入すると，細胞融合誘導能は保持されるが，アラニンからリジン (Lys) に変異させた場合，細胞融合誘導能は減弱した．これらの結果より，HN 領域 521 位は赤血球凝集能，ノイラミニダーゼ活性には影響を与えず，細胞融合を修飾する重要な部位と考えられた．

〈**Key words**〉 ムンプスウイルス，細胞融合蛋白，赤血球凝集—ノイラミニダーゼ蛋白，細胞融合，HeLa 細胞