Original research

Comparison of the live attenuated mumps vaccine (Miyahara strain) with its preattenuated parental strain

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Abstract

Live attenuated mumps vaccines have been developed by passaging the field isolates in cells that differ from the natural host. To study mumps viral (MuV) attenuation at the genomic level, we compared a live attenuated mumps vaccine (Miyahara strain) to its preattenuated parental strain. These two strains exhibited several phenotypic differences. The parental strain grew faster, reached a higher titer, and formed larger plaques and syncytia in Vero cells compared to the vaccine strain. In addition, intracranial injection of parental strain in neonatal rats resulted in greater ventricular enlargement than that caused by the vaccine strain. Four nucleotide changes leading to amino acid substitutions were found between the two viral genomes. One change is present in the N and L genes, respectively, and two in the F gene. The fusogenic activity of the cloned parental F gene evaluated by a cell-cell fusion assay was weaker than that of the vaccine F gene, and did not correspond to the activity caused by the living parental and vaccine strains. The transcriptional activities of N and L proteins were monitored by a CAT minigenome assay. Cloned parental N gene produced almost the same CAT activity as vaccine N, and cloned parental L gene produced significantly higher CAT activity than vaccine L. Thus among four nucleotide changes, the three occurring in N and F were not found to relate to the viral outcome, but we confirmed that at least one change in the L protein was involved in the growth phenotype of parental MuV.

Keywords: attenuation; fusion; mumps virus; neurovirulence; replication; vaccine; Miyahara strain

Introduction

Mumps is an acute, contagious vaccine-preventable disease caused by the mumps virus (MuV). A mumps epidemic occurs every 3 to 5 years in unvaccinated populations, and humans are the only natural host for MuV infection. MuV is transmitted by the respiratory route and replicates primarily in the nasal epithelium, leading to viremia, and it triggers secondary viral replication [1]. Up to 30% of MuV infections are asymptomatic or show only nonspecific respiratory symptoms. In 70% to 90% of symptomatic infections, MuV invasion of glandular epithelium results in mumps parotitis. MuV enters the cerebrospinal fluid (CSF) during the viremia. CSF pleocytosis leads to a form of aseptic meningitis that occurs in about 10% of mumps cases. More serious complications, such as deafness and encephalitis, occur at a low frequency [1].

MuV belongs to the genus *Rubulavirus* of the family *Paramyxoviridae* and has a negative-strand nonsegmented RNA genome of 15,384 bases with seven genes encoding eight proteins: the nucleocapsid (N), V, phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins. The N, P, and L proteins participate in viral replication and, together with the genomic RNA, form the ribonucleocapsid (RNP). In the virion, RNP is surrounded by a host-derived membrane undercoated by the M protein. The HN and F

proteins projected onto the virion surface are responsible for cell binding and the entry of MuV. The functions of V and SH are less well understood [2–5]. The SH gene sequence is variable and is therefore used as the basis of MuV genotyping [6–8].

Live attenuated MuV vaccines have been developed by adapting the virus for different species of host or for different conditions of temperature, after repeated trial and error. The first vaccine, Jeryl Lynn strain, was introduced into the USA in 1967 and contributed to a dramatic reduction in mumps infection rates. To date, more than 10 vaccines have been produced. Some vaccines were found to retain a limited pathogenicity [9–11], and some tend to lose immunogenicity because of over-attenuation [12]. Five vaccines were developed in Japan (Urabe [13], Hoshino [14], Torii, Miyahara and NK-M46), and they were found to cause aseptic meningitis, although much less frequently than the natural infection rate [15, 16]. The virulence of MuV has recently been studied using the reverse genetic techniques established for Jeryl Lynn strain cDNA [17]. The genetic basis for attenuation has been vigorously studied [18–23], but a common genetic factor for MuV attenuation has not yet been identified.

In this study, we compared a Japanese mumps vaccine, Miyahara strain, with its parental strain in the preattenuated position to investigate the molecular basis of attenuation. The parental strain apparently has a different phenotype. The full genome sequences of the parental strain and the vaccine revealed four nucleotide substitutions. Those changes accompany the amino acid substitution, and one amino acid change occurring in the L protein was linked to replication capacity *in vitro*.

Materials and methods

Viruses

The MuV Miyahara isolate was originally obtained from a mumps patient in 1970 in Japan. A passage history of the Miyahara strain is indicated in Figure 1. The Miyahara field isolate had been lost, but the strain that locates on the preadapted position of chicken embryo fibroblasts (CE) passages (exact position was not known) was retrieved from the freezer of the National Institute of Infectious Diseases, Japan and used in this study as a parental strain with the approval of the Kaketsuken (the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). We purchased the live attenuated Miyahara MuV vaccine (lot 305) from the Chemo-Sero-Therapeutic Research Institute. Wild-type MuVodate-1 [24,25] and 02-49 were kindly provided by Dr. H. Saito (Akita Prefectural Institute of Public Health, Akita, Japan) and Dr. Y. Matsui (Niigata Prefectural Institute of Public Health and Environmental Science, Niigata, Japan), respectively. In all virus stocks used in this study, defective interfering particles that might influence the results were not found.
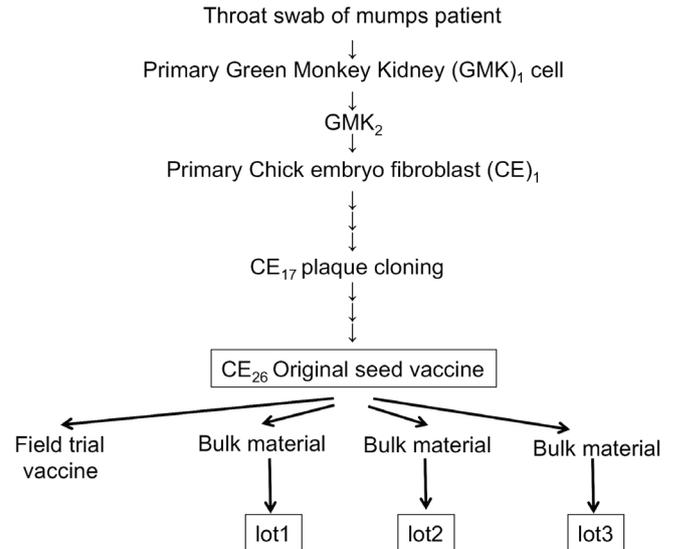
Figure 1 Passage history of the MuV Miyahara strain. MuV originally isolated from a throat swab of a 4-year old female mumps patient in primary green monkey kidney cells is shown as GMK_1. Another passage in the same GMK cells is shown as GMK_2. Passages in the primary cell culture of chick embryo fibroblasts are shown as CE. At the 17th passage of CE (CE_{17}), MuV was plaque purified. The test lot produced from CE_{26} was administered as the field trial vaccine. CE_{26} was licensed as the original seed for a live attenuated mumps vaccine, the Miyahara strain, in 1985 in Japan. The exact position of the Miyahara parental strain used in this study is not mapped due to the loss of records. Three bulk materials (lots 1, 2, and 3) have been produced, and the vaccine (lot 305) used in this study was produced from bulk lot 3.

Cell cultures and virus infection

Vero cells were grown in minimal essential medium (MEM) supplemented with 5% (v/v) bovine serum (BS) and inoculated with parental or vaccine strains at a multiplicity of infection (m.o.i.) of 0.01 cell infection unit (CIU)/cell unless otherwise noted. BHK-T7 cells [26] were kindly supplied by Dr. N. Ito (Gifu University, Gifu, Japan), and were maintained in Glasgow-MEM (Life Technologies Japan, Tokyo) containing 5% fetal BS.

For the growth kinetics study of parental and vaccine strains, 0.1 mL of culture supernatant was harvested daily until 5 days post-infection. The virus titer was then measured at least five times in each sample. MuV strains were titrated using Vero cells. Briefly, a diluted series of MuV was inoculated onto confluent cells in a 12-well plate after the removal of medium. After adsorption for 1 h, 1 mL of medium was added to each well. Thirty-six hours later, infected cells were treated with rabbit anti-MuV hyperimmune serum followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G. FITC-positive cells were measured, and the virus titer (CIU/ml) was calibrated.

For plaque formation, MuV was inoculated onto confluent Vero cells in a 6-well plate. After adsorption for 1 h, 1.0% (w/v) agarose in MEM containing 0.5% BS was overlaid on the cell culture and incubated at 37°C for 8 days. An agarose in MEM containing 0.01% (w/v) Neutral Red solution

(Sigma-Aldrich Japan, Tokyo) was again overlaid onto the first agarose layer and incubated for several hours until cells were stained. For the syncytium formation, MuV was inoculated onto cells at an input m.o.i. of 0.01 CIU/cell. Two days later, syncytia were observed by treating the cells with rabbit anti-MuV hyperimmune serum followed by FITC-conjugated anti-rabbit immunoglobulin G. FITC-positive fused cells were then counted.

Neurovirulence assay

Fertilized Lewis rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). One-day-old littermates were inoculated intracranially with 20 μL of each MuV strain containing 100 CIU or phosphate-buffered saline (PBS) and raised with their respective dams. This experiment was performed according to the Institutional Animal Care and Use Committee regulations (Permission number: 211072). Under isoflurane anesthetic inhalation, these rats were sacrificed 30 days after the inoculation. Their heads were removed and fixed in 10% buffered formalin solution. Hydrocephalus, characterized by the excessive accumulation of CSF in the cerebral ventricles, was scored as described [27]. The sizes of the brain and lateral ventricles in a digital picture were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The neurovirulence score for each strain is indicated by the mean score for all brains within an inoculated group. We analyzed differences in the scores using Student's t-test as described [19, 27].

PCR cloning and sequence analysis

The genome of MuV was extracted from the viral fluid using the High Pure Viral RNA Kit (Roche Diagnostics, Tokyo). Extracted RNAs were reverse-transcribed and amplified using the PrimeScript One Step RT-PCR Kit (Takara Bio, Otsu, Japan) with five sets of specific primer pairs that cover the entire sequence of the Miyahara vaccine strain as registered in the DNA database (accession number AB040874). These primer pairs amplified five regions that partially overlapped each other: 1(1-4592, the numericals in parentheses indicate the nucleotides from the 3' end), 2(3051-7413), 3(6049-10233), 4(8516-12935), and 5(11328-15384).

The amplified fragments were cloned into the pCR2.1 vector (Life Technologies Japan), yielding pCR-1, pCR-2, pCR-3, pCR-4, and pCR-5, respectively. We sequenced plasmids obtained from three independent colonies of transformed *E. coli*. Both termini of the genomes were amplified using the 5' and 3' RACE System (Life Technologies Japan). Nucleotide sequences were read using a BigDye Terminator v3.1 cycle sequencing kit with a 3130xl Genetic Analyzer (Life Technologies Japan). The final sequence was determined if the three sequences obtained from independent clones were identical. If these were not concordant, sequences were determined by the

RT-PCR sequencing of bulk RNA. Obtained sequences were registered in the DNA database (accession number AB744048 and AB744049 for Miyahara vaccine lot 305 and parental strain, respectively).

The sequence of the Miyahara vaccine (AB744048) was not identical to the previously registered sequence (AB040874); it had a few nucleotide changes. We ignored these changes in our further studies as an error of the previous sequence, because these nucleotides were not found in the vaccine (AB744048) or in the parental strain (AB744049). The N, P, and L genes of each strain were cloned into the downstream site of the T7 promoter in a pGEM-3 vector (Promega, Tokyo), yielding pGEM-N, pGEM-P, and pGEM-L. The F and HN genes were cloned into the downstream site of the EF-BOS promoter in the pKS336 vector (AF403737, kindly provided by Dr. K. Sakai, National Institute of Infectious Diseases, NIID, Japan), yielding pKS-F and pKS-HN.

Fusion assay

One microgram each of pKS-F, pKS-HN, and pDsRed plasmids (Clontech, Takara Bio, Shiga, Japan) was cotransfected into confluent Vero cells using FuGENE 6 (Promega, Tokyo). Transfected cells were cultured for 24 h at 37°C or 39.5°C. Fused cells were observed by inverted fluorescent microscopy (AxioVert, Carl Zeiss Japan, Tokyo) and digitally captured using a CCD camera (AxioCam, Carl Zeiss Japan). The sizes of fused cells in the digital picture were scored using ImageJ software.

CAT minigenome assay

BHK-T7 cells, a baby hamster kidney (BHK) cell line stably expressing T7 polymerase [26], were seeded in 12-well plates at the semi-confluent level. Unless otherwise noted, 0.2 μg of pGEM-N, 0.2 μg of pGEM-P, 0.8 μg of pGEM-L, and 1.0 μg of pMuV-CAT were transfected using Lipofectamine 2000 (Life Technologies Japan). The plasmid pMuV-CAT is designed to produce a MuV-negative sense minigenome that codes the chloramphenicol acetyltransferase (CAT) reporter gene at an antisense manner [17]. CAT activity was assayed using the FAST CAT Chloramphenicol Acetyltransferase Assay Kit (Molecular Probes, Eugene, OR). Samples and the CAT reference standard were applied on a silica thin layer chromatography plate (Whatman, GE Healthcare Japan, Tokyo) and developed with chloroform:methanol (9:1) solvent.

Acetylation images were captured digitally using the luminescent image analyzer LAS-3000 (Fujifilm, GE Healthcare Japan). CAT activities were semiquantitatively indicated as a score by adding up the digital intensity of acetylated products at hydroxyl position 1 or 3 of chloramphenicol (CAP) and twice the intensity of those acetylated at both 1 and 3 hydroxyl positions, using ImageJ software. The experiments were performed in triplicate,

basically as described [17] except for our use of the non-radioisotope detection method.

Results

Characteristics of the Miyahara parental strain and vaccine in cells

Live attenuated MuV vaccines have been developed by adapting the virus for different species of host or for different conditions of temperature, after repeated trial and error. Five vaccines were developed in Japan through the adaptation to CE. During the vaccines' creation, candidates were designed to have the characteristic marker producing a smaller plaque than the parental field isolate in Vero cells. We compared the phenotypes of the preattenuated Miyahara parental strain to the live attenuated Miyahara commercial vaccine (Figure 1). The plaques caused by the parental strain were larger than those produced by the vaccine (Figure 2A). The parental plaque consisted of a clear edge and a colorless inside, but the vaccine plaque was unclear and turbid. This morphological character corresponded well with the developmental marker of the vaccine.

Differences in plaque sizes often reflect the viral capacity for fusogenicity, growth, or cytopathogenicity. We thus compared the syncytia of the two viruses at two days post-infection by staining the infected Vero cells with the anti-MuV antibody. The parent strain showed larger syncytia in Vero cells than the vaccine did (Figure 2B). Viral growth at the multistep condition (m.o.i. of 0.01) was then compared at three different temperatures (32°, 37°, and 39.5°C). The results from a representative of three independent experiments runs are shown in Figure 2C. At 32°C, the parental strain and the vaccine grew at almost the same growth rate.

In the higher temperature conditions, however, the parental strain increased faster than the vaccine and reached a higher titer compared to the vaccine. The virus titers of the parental strain at 3 days post-infection were 8.0×10^5 (32°C), 7.9×10^6 (37°C), and 3.0×10^7 (39.5°C) CIU/mL (Figure 2C, filled black arrows). These results indicated the increased growth phenotype of the parental strain with increasing temperature. In contrast, the vaccine grew almost identically in the three temperature

(A) Plaques
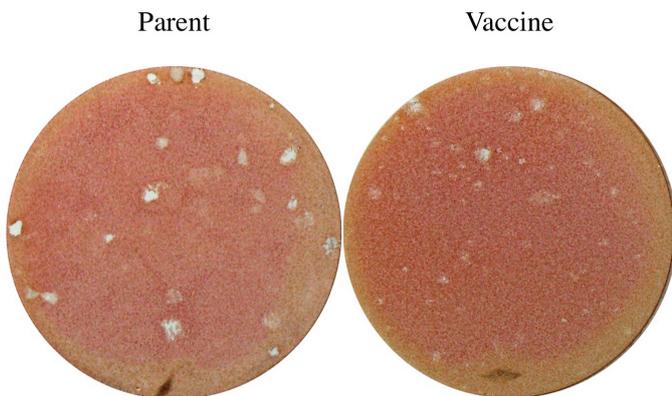
(B) Syncytia
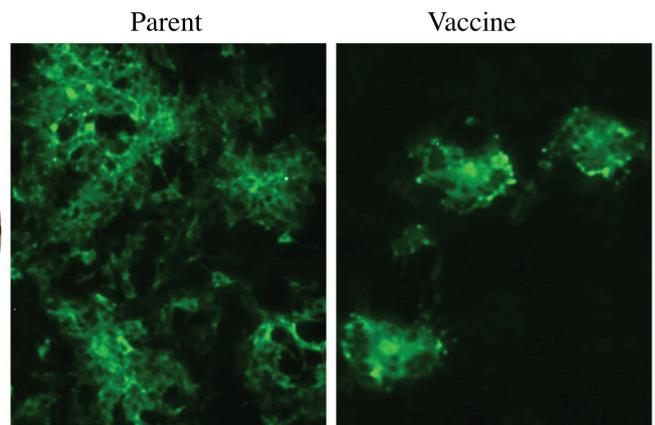
(C) Growth
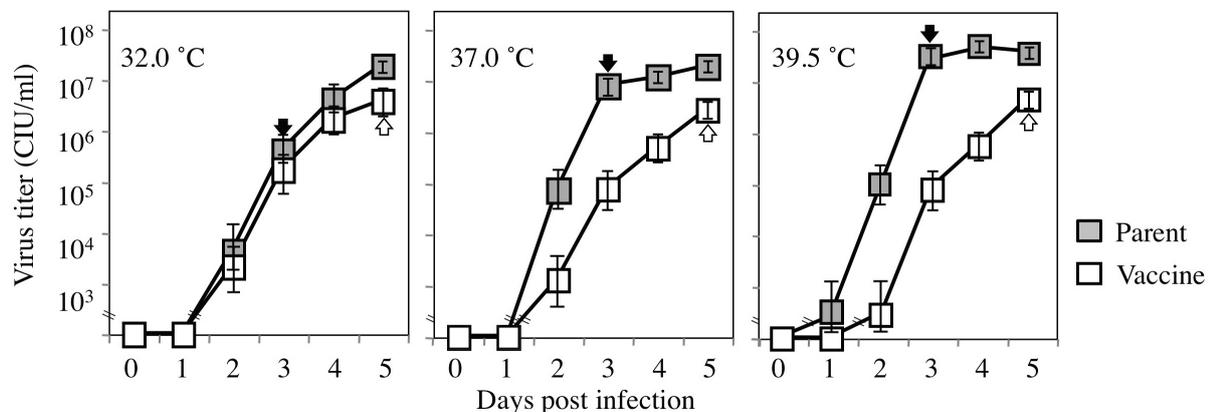
Figure 2 Phenotypic comparisons of parental strain and vaccine of MuV. Plaques produced on Vero cell culture (A). Syncytia found in Vero cell culture (B). Syncytia were reacted with rabbit anti-MuV hyperimmune serum as described in the materials and methods section. The growth of parental MuV and vaccine was compared under the temperature conditions indicated (C). A representative of three independent studies (32° and 37°C, 37° and 39.5°C, and 32° and 39.5°C) is shown. Closed boxes show the titer of parent MuV; open boxes show that of vaccine MuV. Each point indicates the mean CIU/mL titer of six calculations. Error bars: SEM.

conditions and reached identical titers (3.8×10^6 at 32°C, 2.7×10^6 at 37°C, and 4.3×10^6 CIU/mL at 39.5°C) at 5 days post-infection (Figure 2C, outlined white arrows). We tried to compare the viral growth at 42°C but failed to obtain growing viruses from the culture supernatant because of the high-temperature sensitivity of the Vero cells (data not shown).

Characteristics of neurovirulence in rats

Since humans are the only natural host for MuV, no routine method for assaying the pathogenicity of MuV has been established, although attempts have been made with the Cynomolgus monkey [28, 29], marmoset [30], and hamster [31]. We adopted a neurovirulence assay model using neonatal rats [27, 32]. In the present study, we injected two field isolates (Odate-I and 02-49) and PBS intracranially into littermates of rats as the positive and the negative controls along with the parental strain and vaccine (Figure 3). Odate-I was isolated from a meningitis patient during a mumps outbreak in 1993 with a high incidence of aseptic meningitis [24, 25], while 02-49 was isolated from a parotitis patient in 2002 in Japan.

The neurovirulence score for Odate-I was 17.54 ± 4.12 (n=18) and for 02-49 was 7.44 ± 3.22 (n=18). The decrease of neurovirulence observed in 02-49 was statistically significant ($P<0.01$). PBS-inoculated rats
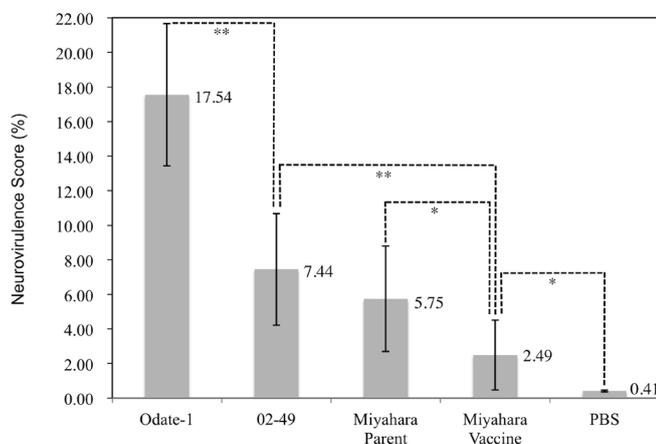
Figure 3 The severity of hydrocephalus in rats inoculated with the parental strain and vaccine of MuV. We tested the neurovirulence of parental MuV and vaccine as described in the materials and methods section. All bars and numerals represent the mean values of neurovirulence scores. Error bars: SEM. ∗∗, $P<0.01$; ∗, $P<0.05$. The Miyahara vaccine's score was 2.64 ± 2.41 (n=20), significantly lower than that of 02-49 ($P=0.006<0.01$) but higher than that of PBS ($P=0.026<0.05$).

did not show any apparent changes and the score was 0.41 ± 0.05 (n=24). The Miyahara vaccine's score was 2.49 ± 2.41 (n=20), significantly lower than that of 02-49 ($P<0.01$), indicating the avirulent phenotype of approved vaccine. The neurovirulence score of the parental strain was 5.75 ± 3.05 (n=18). This score was higher than that of the vaccine ($P=0.048<0.05$), but no significant

difference between the parental strain score and that of 02-49 was revealed ($P=0.436$). This result is in line with the finding that the Miyahara parental strain was moving toward adapting to CE cells and thus retaining the neurovirulence.

Differences in genome sequences

At this point, we had found some biologically significant differences between the Miyahara parent strain and the vaccine. A comparison of the genome sequence between the two strains was then necessary to better understand their biological functions at the molecular level. We cloned three independent fragments amplified from each genome and sequenced them carefully. Both termini were confirmed using the 3' and 5' RACE methods as described in the materials and methods section. Our analysis of the sequence data (AB744048 and AB744049) yielded four nucleotides that differed between the two strains: 1337 in the N gene, 5024 and 5129 in the F gene, and 14355 in the L gene (the numerals indicate the position from the 3' end). There was no deletion or insertion in the genome, and no nucleotide exchange had occurred in the noncoding region. All four exchanges were accompanied by the amino acid changes $1337N^{398}$(Val → Ile), $5024F^{160}$(Gln → Arg), $5129F^{195}$(Phe → Ser), and $14355L^{1973}$(Phe → Ser).

Fusogenicity of cells transfected with cloned F and HN genes

The F protein of MuV is responsible for the virus-cell and the cell-cell membrane fusion with the cooperation of the HN protein. To determine whether the amino acid exchanges in the F protein facilitated the fusion activity, we cotransfected plasmids coding each F and HN protein into Vero cells together with the pDsRed, and incubated them at 37°C or 39.5°C. The syncytia thus formed emitted red fluorescence (Figure 4). In marked contrast to the syncytia with live MuV-infected cells (Figure 2), the sizes of the syncytia associated with the parental F gene were smaller than those associated with the vaccine F gene.

The diameters of 50 randomly taken syncytia were measured and the histogram was drawn at 37°C and 39.5°C. The syncytia produced at 39.5°C were smaller than those obtained at 37°C. The temperature-independent syncytia formation by cloned F genes was the opposite of the higher growth rate of living parent MuV at 39.5°C compared to 37°C. These results showed that the vaccine phenotype was not explained by $5024F^{160}$(Gln → Arg) and $5129F^{195}$(Phe → Ser) mutations.

Minigenome activity of cloned genes

The P and L proteins form the viral RNA polymerase and the N protein surrounds the genomic RNA to form an

(A) F_{par}

37.0°C   39.5°C

(B) F_{vac}

37.0°C   39.5°C
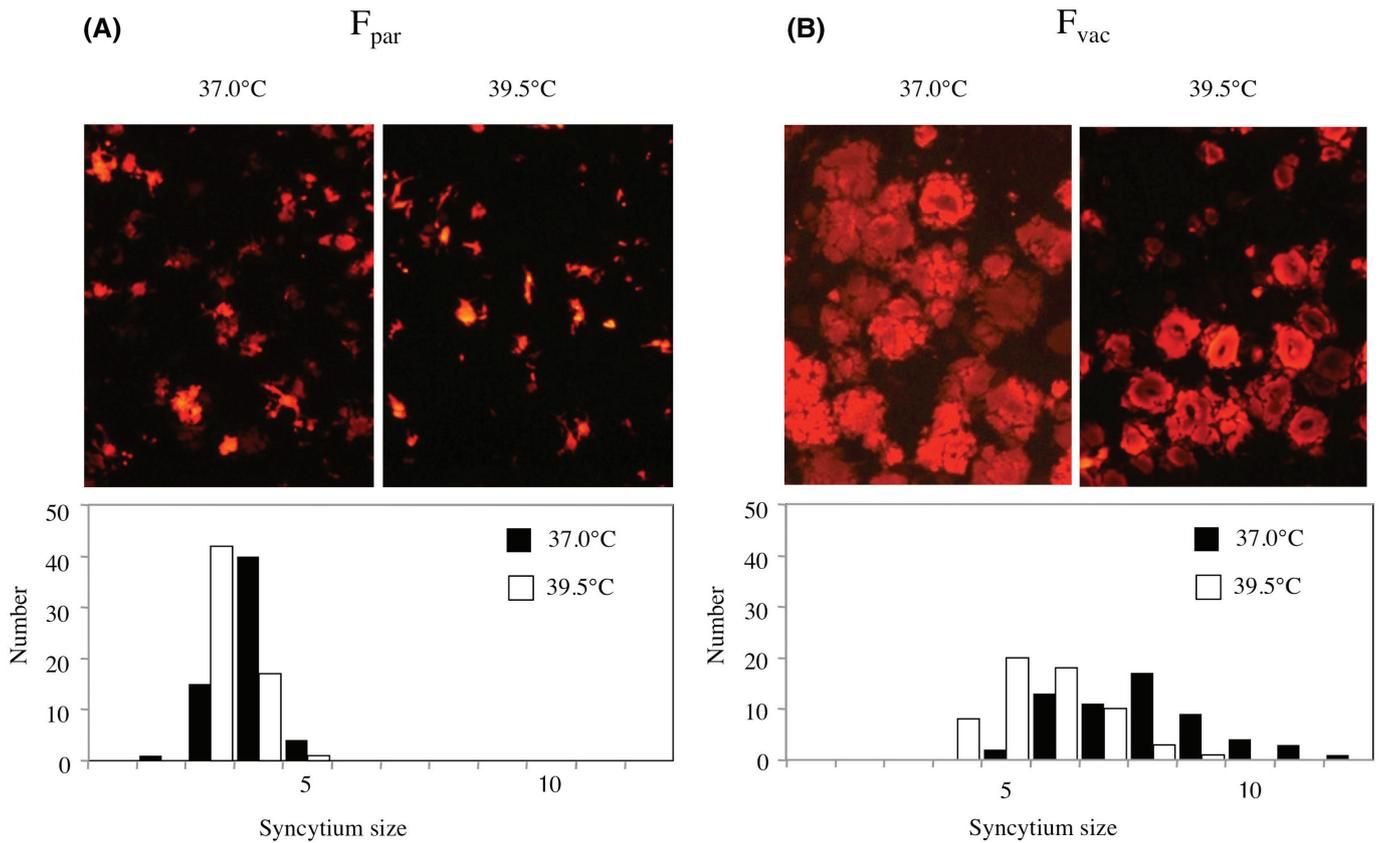
Figure 4 (A,B) Syncytium formation assay by cloned F and HN genes. Vero cell cultures were transfected with plasmids encoding cloned F gene from parent (F_{par}) or vaccine (F_{vac}) and HN gene together with pDsRed. Fused cells emitting fluorescence are shown. At 37°C (closed bar) or 39.5°C (open bar), the images of syncytia were digitally taken and the diameters were plotted as histograms.

active template. To evaluate the relevance of $1337N^{398}$ (Val → Ile) and $14355L^{1973}$(Phe → Ser) *in vitro*, we used a minigenome in which all MuV genes of the Miyahara strains were replaced with a CAT gene. CAT activity reflected the amount of CAT mRNA produced from the minigenome. We found no CAT activity in the experimental conditions when one of three proteins was depleted, but found that the use of 0.2 μg, 0.2 μg, and 0.4 μg of N, P, and L plasmids, respectively, resulted in the optimum CAT activity. Inputting the vaccine N (N_{vac}) or parental N (N_{par}) plasmid from 0 to 0.1 and 0.2 μg, the three clearer forms of acetylated products (1-, 3-, and 1,3-chloramphenicol (CAP)) were observed (Figure 5A). However, further input of the N plasmid (0.4 and 0.6 μg) did not result in an increase in acetylated products but rather their decrease. Maximum CAT activities were shown at 0.2 μg of N plasmid, and were almost the same between the two N plasmids. By the digital intensity of acetylated products, the amount of products was indicated as 34.1 (vaccine) and 35.1 (parent). These results showed that N_{vac} and N_{par} had no functional difference and that both were equivalent in the CAT assay. Any relevance of $1337N^{398}$(Val → Ile) was not identified under this condition.

We then added various amounts of vaccine L (L_{vac}) or parental L (L_{par}) plasmid together with N and P plasmids.

Too many or too few L plasmids did not result in high CAT activity (Figure 5B). The maximum CAT activity was found under the conditions in which 0.4 μg or 0.8 μg of L_{par} or L_{vac} was added. The digital intensity of acetylated products was indicated as 51.8 (parent) and 32.8 (vaccine). Higher CAT activity was observed in L_{par}-transfected cells than in L_{vac}-transfected cells. These results indicate that L_{par} and L_{vac} did not have the same effect and that L_{par} had 1.6-fold (51.8/32.8) higher transcription and replication capacities. We tested and confirmed this higher capacity of L_{par} to L_{vac} by measuring acetylated products in three independent experiments. Thus, $14355L^{1973}$(Phe → Ser) was relevant under this condition.

Although an effect of the N mutation was not observed in the above experiment, it is possible that the mutation in the N gene might have an effect in combination with the mutation in the L gene. We therefore added various amounts of L_{vac} with a combination of fixed amounts of N_{vac}, and we also added L_{par} with a combination of fixed amounts of N_{par}. Higher CAT activity was observed in N_{par}/L_{par}-transfected cells than in N_{vac}/L_{vac}-transfected cells. Maximum CAT activities were obtained when 0.4 μg or 0.8 μg of L_{par} or L_{vac} was added. The scores shown by the digital intensity of acetylated products were 57.4 (parent) and 35.4 (vaccine) (data not shown). These results again

showed the importance of $14355L^{1973}$(Phe → Ser), but in a comparison of N_{vac}/L_{vac} and N_{par}/L_{par} (57.4/35.4 = 1.6 fold) to N_{vac}/L_{vac} and N_{vac}/L_{par} (51.8/32.8 = 1.6 fold, Figure 5B), the fold ratio of vaccine to parent gave the same value. Similar results were also obtained when various amounts

of N_{vac} were used in combination with fixed amounts of L_{vac} and when N_{par} was used in combination with fixed amounts of Lpar (data not shown). These findings show no contribution of $1337N^{398}$ (Val → Ile) mutation on CAT activity in combination with L protein.
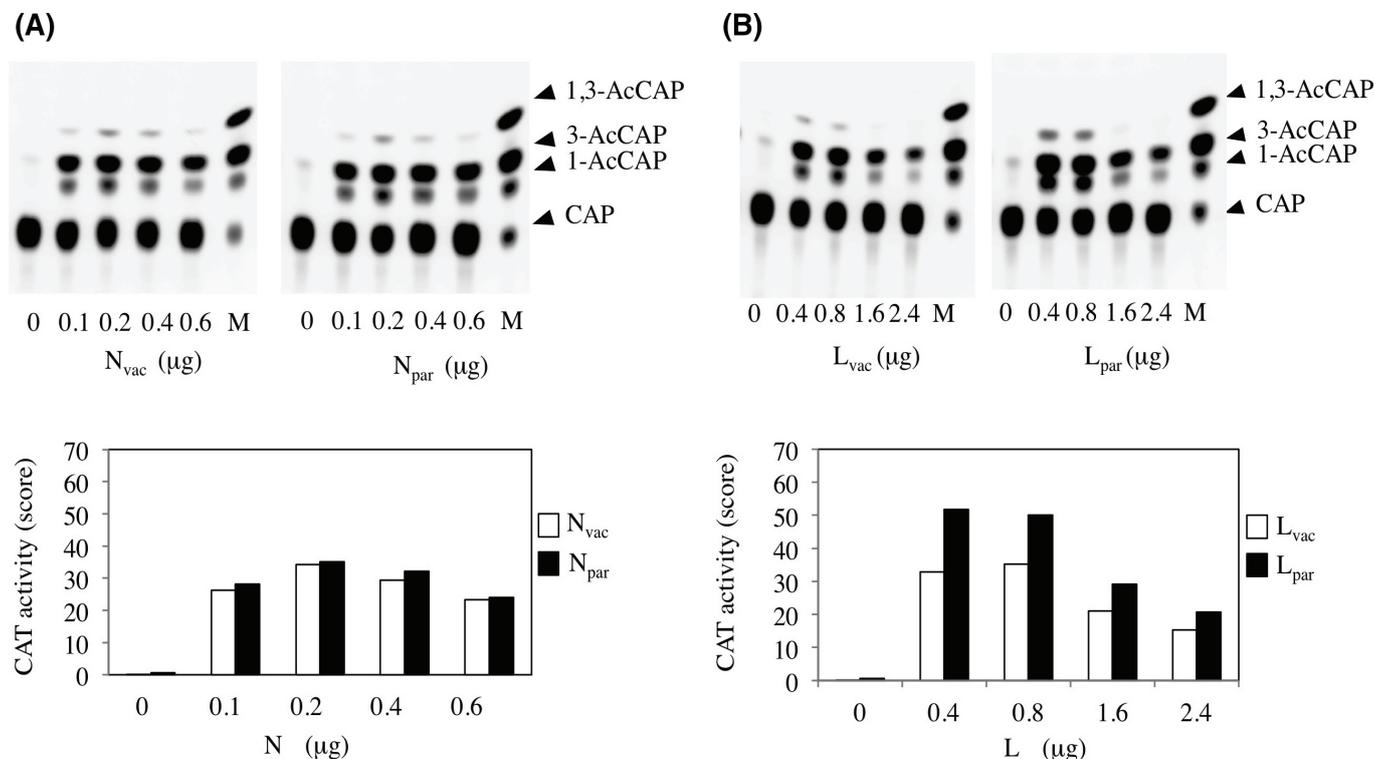
Figure 5 CAT minigenome assay for evaluating mutations in the N and L genes. Various amounts of the N gene from parent (N_{par}) or vaccine (N_{vac}) were transfected into the cells together with P and vaccine L (L_{vac}) genes and the CAT minigenome, as described in the materials and methods section (A). Various amounts of the L gene from parent (L_{par}) or vaccine (L_{vac}) were transfected together with P and N_{vac} genes and the CAT minigenome (B). The acetylated products developed in TLC plates are shown in the upper panel. The intensities of acetylated spots obtained from the digital photograph are indicated as the CAT activity bar graphs in the bottom panel. Open and closed bars show the results obtained by vaccine- and parent-derived gene transfection, respectively. Data are from a representative experiment performed in triplicate.

Discussion

Wild-type MuV exhibits neurovirulence, and even attenuated vaccines carry some risk of neurovirulence approved attenuated vaccines carry some risk of neurovirulence and can cause aseptic meningitis, although the frequency of its occurrence with the mumps vaccine is low compared to the natural infection. Approximate frequencies are specifically determined by data from administered vaccine strains [1, 9], which indicate some genetic factor dictating MuV's neurovirulence. The identification of such a gene or genes is thus important for developing a safe vaccine.

It was reported that the Urabe mumps vaccine is a mixture of viruses differing at amino acid 335 of the HN gene, and one form was associated with neurovirulence [33]. Some virological studies supported this finding [21, 22]. However, the complete nucleotide sequences of several stocks of the Urabe strain did not support this finding [34, 35], and reverse genetic work finally showed that HN^{335}

was not a crucial determinant of Urabe neurovirulence [36].

The Jeryl Lynn vaccine strain adapted to human neuronal cells had three amino acid substitutions in N^{468}(Phe → Pro), M^{85}(Val → Ala) and L^{1165}(Glu → Asp), and the wild-type 88-1961 strain adapted to CE fibroblasts had three amino acid substitutions in F^{91}(Ala/Thr → Thr), HN^{466}(Ser → Asn) and L^{736}(Ile → Val) [37]. Swapping of the respective gene from the Jeryl Lynn strain to the 88-1961 strain resulted in a gradual attenuation with the increase in the number of genes and yielded no critical gene for viral virulence, and swapping those from the wild-type to the Jeryl Lynn strain resulted in a gradual virulence to the contrary. [23]. These results suggest that there is no specific gene determining viral attenuation and neurovirulence, and that viral attenuation and neurovirulence are determined by a sum or a certain combination of noncritical genes in the context of viral life cycle.

We started to study MuV attenuation using one of

the Japanese vaccines, the Miyahara strain, and its preattanuated parental strain. We found that the plaque size, viral growth, temperature sensitivity, fusogenicity and neurovirulence of the two strains were different (Figurs 2 and 3). The genome sequences of the parental strain and vaccine were carefully determined by two-step experiments. In the first step, three independent cDNA clones of both strands were sequenced and the sequence was determined only when the results were identical. In a few non-concordant cases, sequences were determined by the RT-PCR sequencing of bulk RNA as a second step. The results indicated that each virus source was highly homogeneous.

Considering that the Jeryl Lynn vaccine contains JL2 and JL5 component stains which differ by over 400 nucleotides [38], it was surprising for us that these phenotypes were probably caused by four nucleotide exchanges between the genomes. Among the four exchanges, $5024F^{160}$(Gln → Arg) and $5129F^{195}$(Phe → Ser) seemed not to be related to the Miyahara phenotype but were epiphenomena (Figure 4). Moreover, $5129F^{195}$(Phe) is found among known mumps strains independent of vaccines or field isolates. For example, the Odate-1 strain used for the rat neurovirulence test as a virulent strain in the present study has $5129F^{195}$(Ser), while the Urabe strain approved for the mumps vaccine in Japan has $5129F^{195}$(Phe). The relevance of $5129F^{195}$(Phe) to low fusogenic activity has been reported in other strains [39,40]. The discrepancy in fusogenic activity between the living virus and the cloned F gene was not resolved in this study.

We found that $14355L_{par}^{1973}$(Phe) resulted in higher CAT activity than $14355L_{vac}^{1973}$(Ser), but that $1337N_{par}^{398}$ (Val → Ile) mutation did not result in a significant difference (Figure 5). We concluded that an amino acid substitution occurring in $14355L_{par}^{1973}$(Ser) is associated with attenuation by reducing replication and transcription activity. Six conserved L domains (I-VI) are known between two different families of *Mononegavirales, rhabdoviridae* and *paramyxoviridae*, and are postulated to constitute the specific enzymatic activities [41]. However, the amino acid change observed herein in L^{1973} does not belong to any of the conserved regions. How the amino acid change in L^{1973} might alter transcriptional activity is unknown. Another possibility also remains that the mutations contribute to viral attenuation in a way that the fusogenic or the minigenome assay *in vitro* does not capture. The importance of amino acid mutations during the attenuation process will be revisited as a next step using the reverse genetics technique in the context of viral multiplication in cells and of viral neuropathogenecity for neonatal rats.

Conflict of interest

All the authors declare that they have no conflict of interest.

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