

# Isolation of an avirulent mutant of Sendai virus with two amino acid mutations from a highly virulent field strain through adaptation to LLC-MK<sub>2</sub> cells

Masae Itoh,<sup>1</sup> Yuji Isegawa,<sup>2</sup> Hak Hotta<sup>1</sup> and Morio Homma<sup>3</sup>

<sup>1</sup> Department of Microbiology, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan

<sup>2</sup> Department of Bacteriology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan

<sup>3</sup> Faculty of Home Economics, Kobe Women's University, 2-1 Aoyama, Higashisuma, Suma-ku, Kobe 654, Japan

A field strain of Sendai virus (SeV) Ohita-M1 (M1) was isolated from an epidemic in an animal laboratory by passaging in mice. A mutant strain, Ohita-MVC11 (MVC11), was then obtained by passaging M1 in rhesus monkey (LLC-MK<sub>2</sub>) cells. MVC11 was adapted to LLC-MK<sub>2</sub> cells and produced 20 times higher levels of infectious virus than M1. This increased production of infectious virus in LLC-MK<sub>2</sub> cells was associated with enhanced viral gene expression. However, MVC11 could not replicate efficiently in mouse lung and was not lethal to mice even when inoculated at a titre of  $8 \times 10^5$  cell-

infecting units (CIU) per mouse. On the other hand, with an inoculum of only  $4 \times 10^1$  CIU per mouse, corresponding to 1 LD<sub>50</sub>, M1 replicated well in mouse lung and was highly virulent to mice. Nucleotide and deduced amino acid sequence analyses of the entire genomes of M1 and MVC11 revealed that adaptation to LLC-MK<sub>2</sub> cells and the attenuation of mouse pathogenicity of MVC11 were associated with only two amino acid substitutions; one on the C protein (Phe substituted by Ser at position 170) and the other on the RNA polymerase, the L protein (Glu substituted by Ala at position 2050).

## Introduction

Sendai virus (SeV) is widespread among rodents, causing bronchopneumonia with high mortality (Bhatt & Jonas, 1974; Burek *et al.*, 1977; Ishida & Homma, 1978; Iwai *et al.*, 1980; Lucas *et al.*, 1987; Profeta *et al.*, 1969). In mice, the virus causes overt disease in some cases (Itoh *et al.*, 1978; Iwai *et al.*, 1977; Zurcher *et al.*, 1977) and subclinical infection in the others (Parker & Reynolds, 1968). Factors responsible for the difference may be related to the host, such as the age of mice at exposure (Zurcher *et al.*, 1977) and the genetically determined susceptibility of mouse strains to the virus (Brownstein, 1983; Parker *et al.*, 1978), as well as the virus. Although SeV strains exist that differ markedly in their pathogenicity to mice, the determinants of their virulence are largely unknown. In previous work, we demonstrated that

susceptibility of the fusion envelope glycoprotein (F) to trypsin and to the activating proteases in the mouse respiratory tract is one of the determinants of virulence, supporting multiple cycle replication through cleavage/activation of the F protein (Itoh *et al.*, 1990). The M protein is another determinant of virus pathogenicity. A mutant M protein was reported to cause budding of the virus from both the basolateral and apical sides of the cells, which resulted in a pantropic, systemic infection of mice through viraemia, whereas the parental virus, carrying the wild-type M protein, budded only from the apical surface causing local infection in the respiratory tract (Tashiro *et al.*, 1992). Recently, Kato *et al.* (1997) reported that an SeV mutant lacking the V protein had strongly attenuated pathogenicity in mice and concluded that the V protein markedly influences the pathogenicity of SeV.

In our previous work, we isolated the Ohita strain of SeV from an epidemic in an animal laboratory by passaging the virus in eggs and in rhesus monkey (LLC-MK<sub>2</sub>) cells; we found that the passage history of the virus had a noticeable influence on virulence. Plaque-purified clones obtained at different times during passage exhibited various degrees of pathogenicity to mice (Itoh *et al.*, 1992). This observation is compatible with the

**Author for correspondence:** Masae Itoh.

Fax +81 78 351 6347. e-mail masae@med.kobe-u.ac.jp

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empirically recognized phenomenon that SeV gradually loses virulence for mice upon continual passage in eggs. The mechanism(s) of the attenuation, however, has not been clarified. Sakaguchi *et al.* (1994) reported a highly virulent field isolate of SeV, the Hamamatsu strain, and sequenced its HN, F and M genes. However, because of the large genetic divergence from prototype strains, they could not determine which sequence difference(s) in the genome were responsible for high virulence.

In order to study the mechanism of attenuation of SeV pathogenicity through adaptation to non-natural host cells, we isolated an exclusively mouse-passaged field strain of SeV, designated the Ohita-M strain, by inoculating the lung homogenate of an infected mouse into susceptible mice. We then obtained some mutant virus strains by passaging the Ohita-M1 (M1) strain, a plaque-purified strain of the Ohita-M, in LLC-MK<sub>2</sub> cells. One of the mutants, Ohita-MVC11 (MVC11), was highly adapted to LLC-MK<sub>2</sub> cells and showed increased virus production through elevated virus gene expression. On the other hand, MVC11 exhibited a limited replication in mouse lungs and had almost lost its virulence for mice. To specify the genetic mutations responsible for the phenotypic changes, we analysed the nucleotide sequence of the entire genome of MVC11 and compared it with that of the parental strain, M1. We report here that either one or both of the single point amino acid mutations found on the C and L proteins of SeV are responsible for adaptation to LLC-MK<sub>2</sub> cells and attenuation of mouse pathogenicity.

## Methods

### Isolation of SeV from an epidemic in an animal laboratory.

A 10% lung homogenate from a moribund mouse that had been suffering from pneumonia during an epidemic in an animal laboratory was inoculated intranasally into 3-week-old male BALB/c mice (Clea Japan). The lung was taken from a mouse that had shown loss of body weight; infection with SeV was confirmed by immunological staining of lung tissues using antiserum against the SeV Fushimi strain. The virus, designated the Ohita-M strain, could be passaged from mouse to mouse by intranasal inoculation of the infected lung homogenates. For the purpose of plaque purification, the Ohita-M strain was inoculated onto LLC-MK<sub>2</sub> monolayer cells and cultivated in Eagle's minimum essential medium (MEM) containing 2.5 µg/ml trypsin and 1% agarose. One of the representative plaques was cloned and designated the Ohita-M1 (M1) strain. Thereafter, M1 was maintained by passaging several times in mice and then propagated once in LLC-MK<sub>2</sub> cells to prepare stock virus. When M1 was passaged continually in LLC-MK<sub>2</sub> cells in the presence of 1 µg/ml trypsin, mutant plaques began to emerge (see Results), one of which was cloned and designated the Ohita-MVC11 (MVC11) strain. MVC11 was maintained only in LLC-MK<sub>2</sub> cell cultures.

Virus titres were determined as described previously and expressed as either cell-infecting units (CIU) per ml or p.f.u. per ml (Kashiwazaki *et al.*, 1965); CIU was essentially equivalent to p.f.u.

**Immunoprecipitation.** LLC-MK<sub>2</sub> cells infected with either M1 or MVC11 at an m.o.i. of 10 were labelled with [<sup>35</sup>S]methionine (20 µCi/ml) for 1 h at various times after infection. The labelled cells were disrupted in 25 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100, 0.5%

sodium deoxycholate and 5 mM NaCl. Nuclei were removed by centrifugation at 12000 g for 5 min and the resulting cell extract was subjected to immunoprecipitation analysis using antiserum against the SeV Fushimi strain or antiserum against the C protein (Omata-Yamada *et al.*, 1988) and Protein A-Sepharose 4B (Pharmacia). The precipitated proteins were electrophoresed in 8% or 14% (for the detection of the C protein) SDS-polyacrylamide gel.

**Northern hybridization.** Total RNAs were extracted from LLC-MK<sub>2</sub> cells infected with M1 or MVC11 (m.o.i. of 10) and mRNA was collected by passing through an oligo(dT)-cellulose column according to the conventional method. One µg of each mRNA preparation was resolved in a 1.5% agarose-formaldehyde gel, transferred to nylon membrane and probed with a <sup>32</sup>P-labelled cDNA of the entire genome of the SeV Z strain (a kind gift from the late H. Shibuta, Institute of Medical Science, University of Tokyo, Tokyo, Japan).

**Determination of pathogenicity in mice.** Specific-pathogen-free, 3-week-old male mice [ICR/CRJ(CD-1) strain; Charles River, Japan] were infected intranasally under ether anaesthesia with 25 µl serially diluted virus. The mice were observed for symptoms and weighed daily. The LD<sub>50</sub> was calculated by the method of Reed & Muench (1938). Mice inoculated with 1.25 × 10<sup>5</sup> CIU of the virus were sacrificed at intervals and infectivity in the supernatant of 10% lung homogenates was assayed (Tashiro & Homma, 1983). Immunohistological examination for viral antigens was performed on paraffin sections by an immunoperoxidase method using anti-SeV rabbit antiserum as the first antibody and peroxidase-conjugated goat anti-rabbit IgG as the second antibody.

### Sequence analysis of the entire genome of M1 and MVC11.

The genomic RNAs were extracted from purified virus particles of M1 and MVC11. To obtain cDNAs corresponding to the end of the 3' leader region, poly(A) tails were added to the extracted RNAs by poly(A) polymerase (Takara Shuzo) and AMP. cDNAs were prepared from RNAs using random primers and an oligo(dT) primer with a cDNA synthesis kit (Pharmacia) according to the manufacturer's instruction. cDNA fragments corresponding to each gene of M1 and MVC11 were obtained by screening the cDNA libraries for M1 and MVC11, respectively, using <sup>32</sup>P-labelled cDNAs of the SeV Z strain. The positive cDNA fragments were subcloned into M13 mp18/mp19 and sequenced by the dideoxy termination method as described previously (Itoh & Homma, 1987). The nucleotide substitutions in the P and L genes were confirmed by sequencing at least two independent cDNA clones derived from each of M1 and MVC11.

**Primary culture of mouse pulmonary epithelial cells.** Tracheostomy was performed on 6-week-old male ICR/CRJ(CD-1) mice under ether anaesthesia and 1 ml protease type X (2 mg/ml; Sigma) was infused into the lung using a syringe. After incubation for 10 min at room temperature, the lung was taken and minced in phosphate-buffered saline. Blocks of the tissues were removed by filtration through four layers of sterilized gauze; single cells were collected by centrifugation, suspended in MEM supplemented with 10% foetal bovine serum and cultivated at 38 °C.

## Results

### Isolation of an LLC-MK<sub>2</sub>-adapted mutant of SeV (MVC11) and characterization of the growth properties of MVC11 and its parental strain (M1) in LLC-MK<sub>2</sub> cells

The M1 strain of SeV formed unclear, turbid plaques on LLC-MK<sub>2</sub> monolayer cells. When the M1 strain was suc-

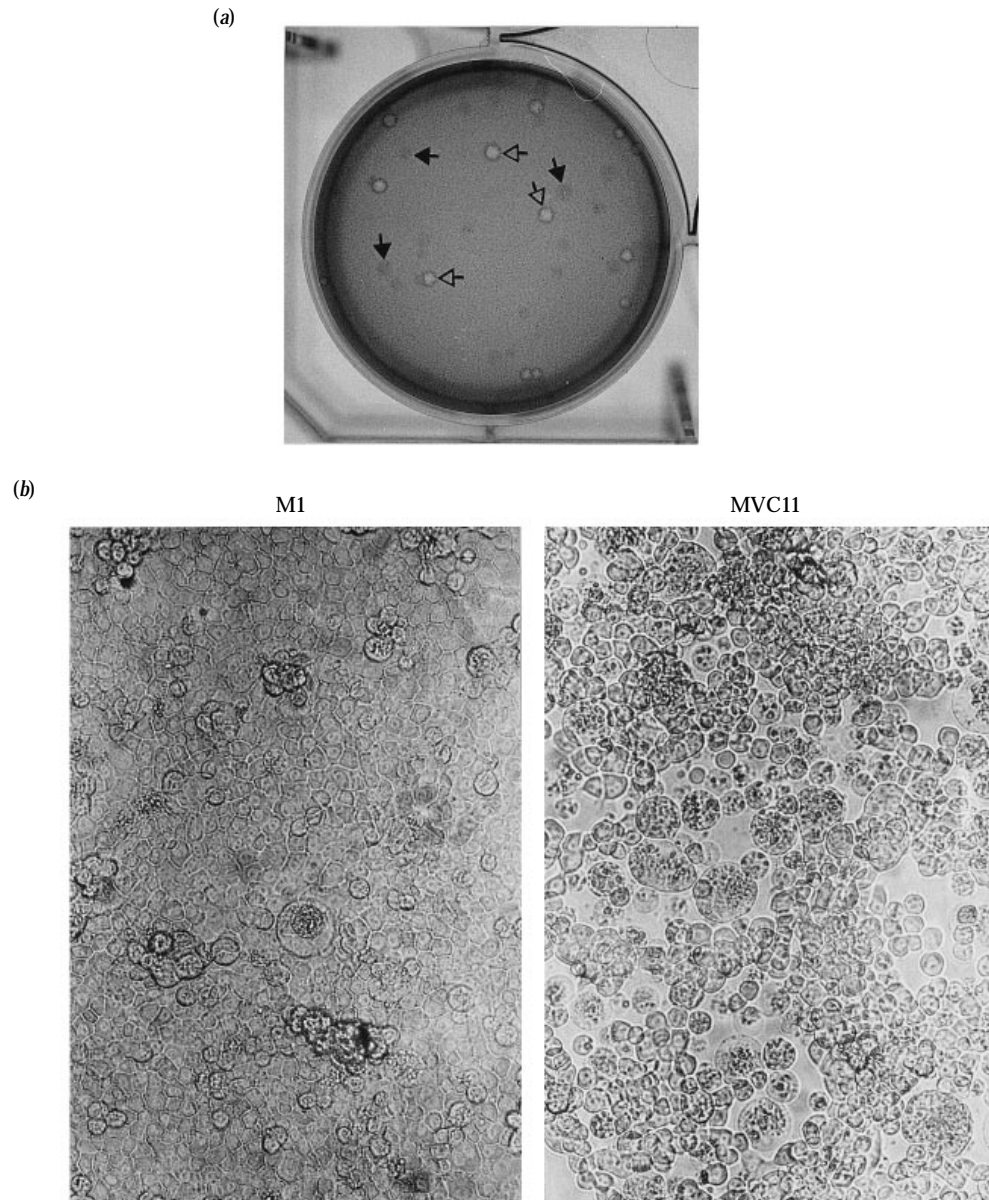


Fig. 1. Phenotypic properties of M1 and MVC11 in LLC-MK<sub>2</sub> cells. (a) Plaque morphology. The plaque-isolated M1 strain was inoculated onto LLC-MK<sub>2</sub> cells and passaged five times in the presence of 1  $\mu$ g/ml trypsin. The culture medium from the fifth passage was used for plaque formation. Solid arrows indicate the typical appearance of the plaques of M1 and hollow arrows indicate those of MVC11. (b) CPE 30 h after infection. LLC-MK<sub>2</sub> cells were inoculated with either M1 or MVC11 at an m.o.i. of 10 and incubated at 34 °C.

cessively passaged in LLC-MK<sub>2</sub> cells, mutants forming clearer plaques began to appear after several passages and increased in number as passage progressed (Fig. 1a). One of the mutants (MVC11) was stably maintained in LLC-MK<sub>2</sub> cells in the presence of 1  $\mu$ g/ml trypsin. As expected from the appearance of the plaques, MVC11 showed strong cytopathic effect (CPE), while CPE by M1 was very slight (Fig. 1b).

When one-step growth in LLC-MK<sub>2</sub> cells was compared, MVC11 produced about 20 times higher levels of infectious virus than M1 (Fig. 2a). We then examined the syntheses of

viral proteins and mRNAs in cells infected with M1 and MVC11. As shown in Fig. 2(b), synthesis of viral proteins, including C and V proteins, was enhanced throughout infection in MVC11-infected cells compared with that in M1-infected cells. No difference in the mobility of any proteins was found between M1 and MVC11; the M1 and MVC11 P, HN and NP proteins migrated more slowly and the M protein migrated faster than corresponding proteins of the Fushimi strain (data not shown; Cadd *et al.*, 1996). It should be mentioned that the Y2 protein was not detected in cells infected with either M1 or

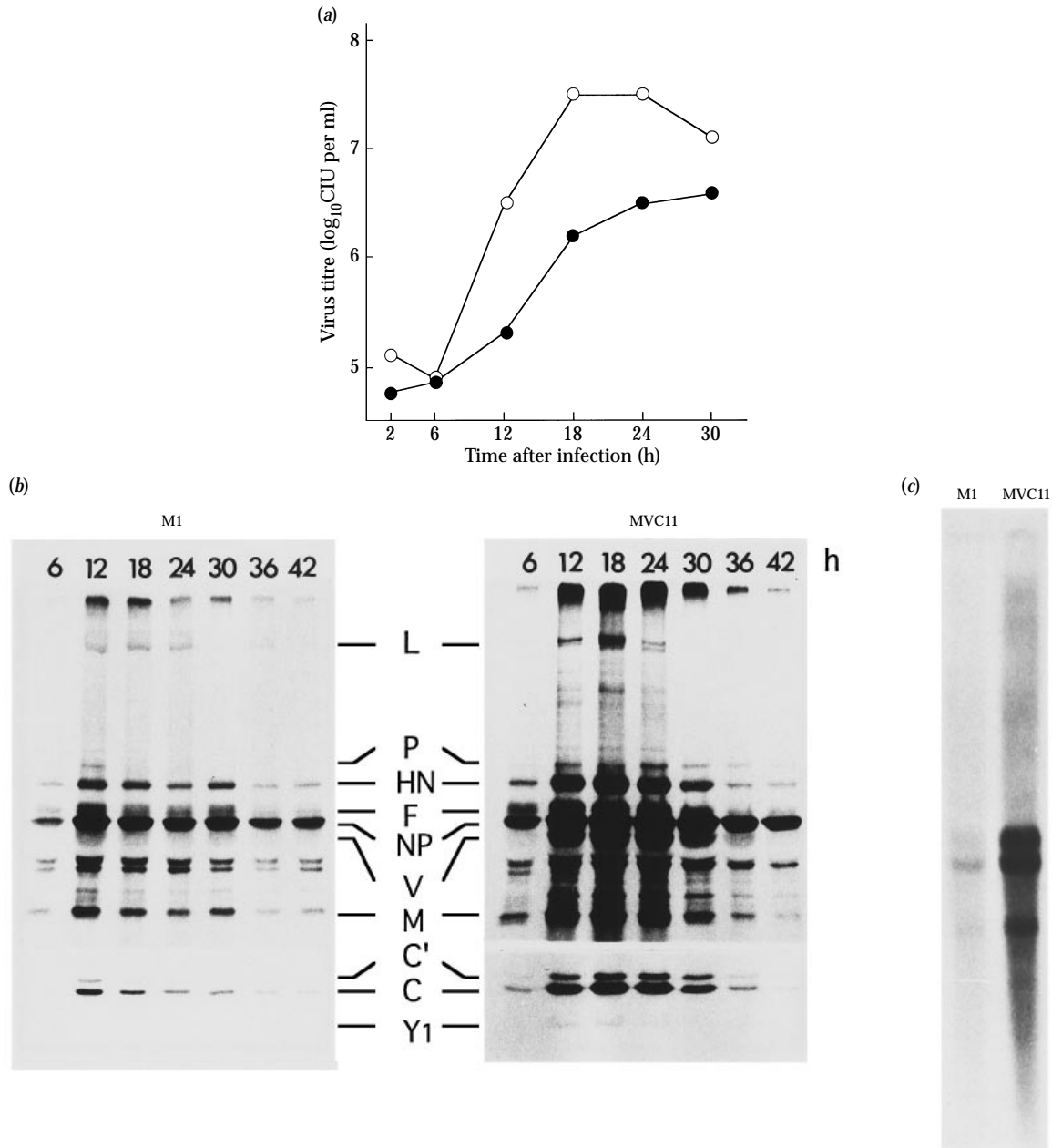


Fig. 2. Replication of M1 and MVC11 in LLC-MK<sub>2</sub> cells. (a) Production of progeny virus. LLC-MK<sub>2</sub> cells were infected with either M1 (●) or MVC11 (○) at an m.o.i. of 10 and incubated at 34 °C. (b) Time-course of virus protein synthesis. Virus proteins in LLC-MK<sub>2</sub> cells infected with either M1 or MVC11 were analysed by immunoprecipitation as described in the Methods. (c) Virus mRNA synthesis. Virus mRNA in LLC-MK<sub>2</sub> cells infected with M1 or MVC11 18 h after infection were detected by Northern hybridization as described in the Methods.

MVC11. The enhanced protein synthesis in MVC11-infected cells was associated with a high level of virus-specific mRNA synthesis (Fig. 2c). These results indicate that MVC11 is a mutant that is highly adapted to LLC-MK<sub>2</sub> cells in terms of virus replication.

#### Mouse pathogenicity of M1 and MVC11

To examine whether or not adaptation of MVC11 to LLC-MK<sub>2</sub> is accompanied by the attenuation of mouse patho-

genicity, MVC11 and M1 were each inoculated intranasally into mice. All the mice inoculated with M1 at a dose of  $1.25 \times 10^2$  CIU per mouse demonstrated symptoms of pneumonia and died within 10 days after infection. One LD<sub>50</sub> of M1 corresponded to  $4 \times 10^1$  CIU. On the other hand, even at a dose of  $8 \times 10^5$  CIU per mouse, the highest titre available for inoculation, MVC11 caused only a slight delay in body weight gain without causing apparent respiratory distress, and all mice survived the infection.

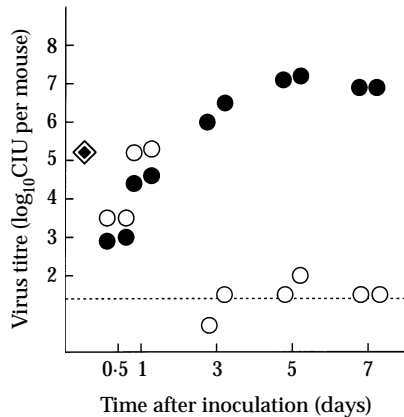


Fig. 3. Virus replication in the lung of mouse. Mice that had been inoculated with  $1.25 \times 10^5$  CIU (◆) of either M1 (●) or MVC11 (○) were sacrificed on the indicated days, and virus titres in 10% lung homogenates were determined in LLC-MK<sub>2</sub> cells. The lowest detectable titre in this assay system is depicted by a dotted line.

Replication of MVC11 in mouse lung was compared with that of M1 (Fig. 3). The virus titre of MVC11 on day 1 was a little higher than that of M1. While the virus titre of M1 increased in the ensuing days, that of MVC11 declined sharply to almost undetectable levels on day 3. It should be noted that progeny MVC11 virus was determined to be infectious by an

experiment using the primary culture of mouse lung epithelial cells, the target cells of MVC11 (data not shown). These results indicated that MVC11 was highly attenuated and had almost lost its mouse pathogenicity through extremely restricted replication in the lung.

To examine the differences between M1 and MVC11 in the spread of virus and their target cells, immunohistological investigation was carried out. In mice infected with M1, virus antigens were detected in bronchial epithelial cells on day 2 (Fig. 4*a*), which spread throughout the lung, even to the alveolar cells, on day 6 (Fig. 4*b*). M1-infected epithelial cells did not undergo cell lysis, but showed a tendency of cell-swelling and piling-up to narrow the lumen of bronchioles. In the lungs of mice infected with MVC11, virus antigens were detected in bronchial epithelial cells on day 2 to the same degree as that observed with M1. MVC11-infected cells exhibited strong CPE, undergoing cell lysis (Fig. 4*c*). In contrast to the case with M1, only trace amounts of virus antigens were detected on day 6 in MVC11-infected mouse lung (Fig. 4*d*).

#### Identification of mutations in the entire genome of MVC11

To specify the genetic changes responsible for the adaptation to LLC-MK<sub>2</sub> cells and the attenuation of the mouse

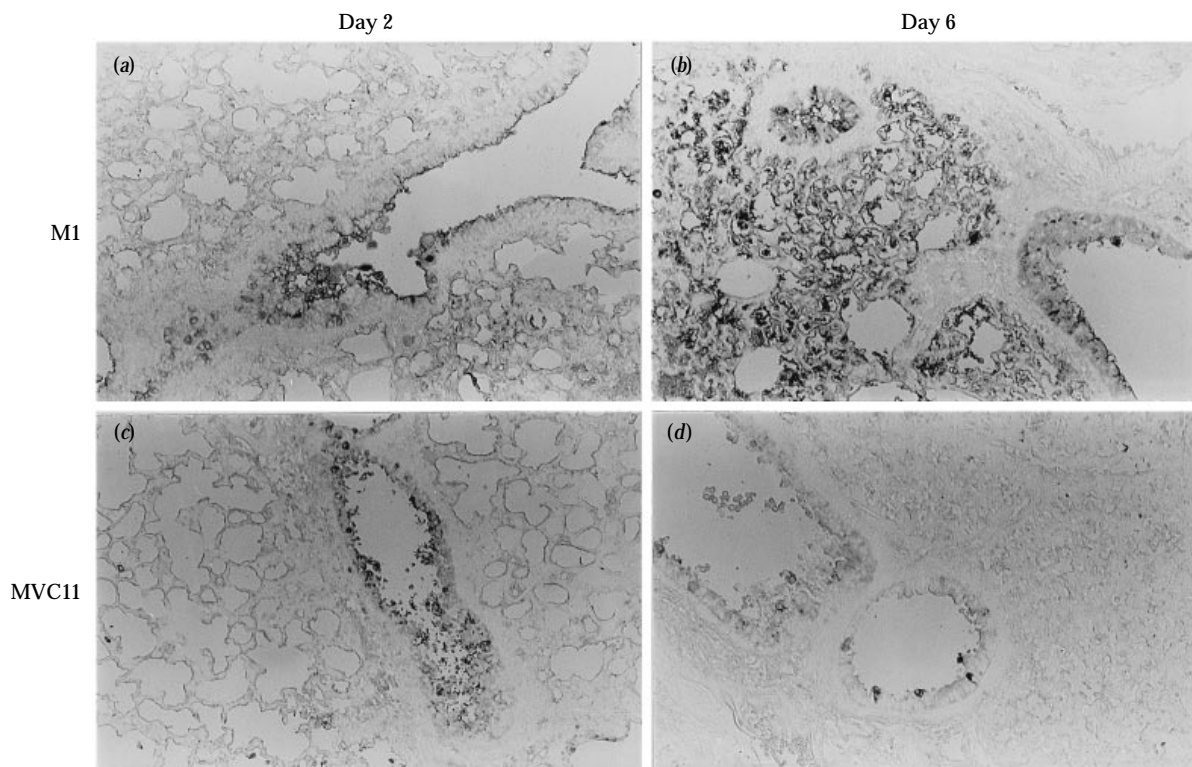


Fig. 4. Immunohistochemical analysis of virus antigens in the lung. The lungs of the mice infected with 25  $\mu$ l of the virus containing  $1.25 \times 10^5$  CIU were taken on days 2 (*a*, *c*) and 6 (*b*, *d*); immunohistochemical analysis was performed as described in the Methods.

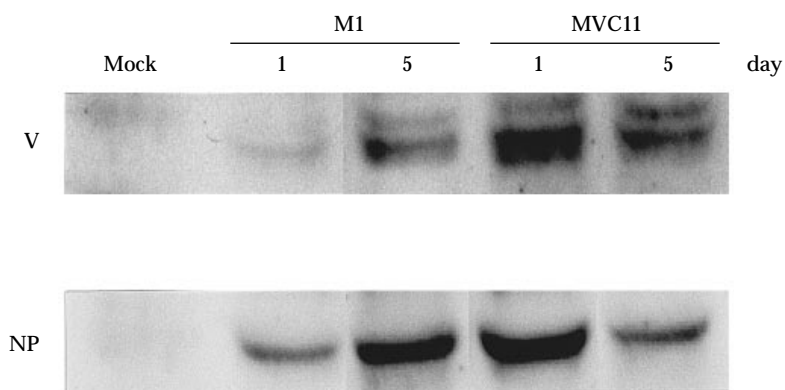
**Table 1.** Nucleotide and deduced amino acid mutations in MVC11 compared with the parental M1 strain

Nucleotide		Amino acid	
Gene (position*)	Mutation†	Protein (position‡)	Mutation
P/C (2362)	U → C	C (170)	Phe → Ser
L (14704)	A → C	L (2050)	Glu → Ala
L (15173)	G → A	L (2206)	Silent

\* Nucleotide position in the entire genome.

† Mutation in the mRNA sense.

‡ Amino acid position in each protein.



**Fig. 5.** Synthesis of V protein in the primary culture of mouse pulmonary epithelial cells. Cells infected with either M1 or MVC11 (m.o.i. of 10) were collected on days 1 and 5, disrupted and subjected to immunoblot analysis using polyclonal antiserum specific to the V protein (Curran *et al.*, 1991) or with polyclonal anti-SeV antiserum.

pathogenicity, we compared the sequence of the entire genome of MVC11 with that of M1.

Both of the genomes of M1 and MVC11 consisted of 15 384 nucleotides. When the nucleotide sequences of M1 and MVC11 were aligned and compared with that of the Z strain, neither nucleotide deletions nor insertions were found. Although the gene-end, intergenic and gene-start sequences were highly conserved between our present isolates and the Z strain (Hidaka *et al.*, 1984; Middleton *et al.*, 1990; Shioda *et al.*, 1983, 1986), relatively low sequence homology was observed in each gene between the M1 and the Z strains (86.7–90.2% homology), which was in contrast to the high degree of sequence conservation observed between the Fushimi and Z strains (98.8–99.4% homology). The putative translation start and termination codons were all conserved in M1 and MVC11, except for the start codon for the Y2 protein in the P/C gene, which had been eliminated. This explains the absence of the Y2 protein in Fig. 2(b).

When the nucleotide sequence of MVC11 was compared with that of M1, only three nucleotide substitutions were found; one was on the P/C gene and the other two were on the L gene (Table 1). The single nucleotide substitution on the P/C gene at nt 2362 from U to C (in the mRNA sense) was silent in the P protein, but it did result in an amino acid change in the C protein from Phe to Ser at residue 170 (C170F → S

mutation). Since the mutation was located upstream of the RNA editing site (at nt 2793), it would not affect the sequences of the V or W proteins. Only one of the two nucleotide changes in the L gene caused an amino acid mutation from Glu to Ala at residue 2050 (L2050E → A mutation).

#### Synthesis of the V protein in the primary culture of pulmonary epithelial cells infected with MVC11

Kato *et al.* (1997) reported that depletion of the V protein conferred attenuation of SeV pathogenicity in mice. The mutation in the P gene of MVC11 is located upstream of the RNA editing site for expression of the V protein and, in fact, the V protein was detected in MVC11-infected LLC-MK<sub>2</sub> cells (Fig. 2b). A possibility still remained, however, that the C170F → S mutation and/or the L2050E → A mutation could abrogate V protein synthesis in mouse lung. We therefore examined V protein synthesis in mouse lungs infected with M1 and MVC11, but failed to detect the V protein in MVC11-infected mouse lung, probably due to the limited degree of infection (data not shown). We then analysed V protein synthesis in the primary culture of pulmonary epithelial cells infected with M1 and MVC11. Immunoblot analysis using anti-V antiserum revealed that, on day 1, more V was detected in MVC11-infected cells than in M1-infected cells (Fig. 5). On day 5, the amount of V protein increased significantly in M1-

infected cell cultures, whereas it decreased in MVC11-infected cell cultures, probably due to the strong CPE caused by the virus. Similar kinetics were observed with the level of NP protein, as determined by immunoblot analysis using anti-SeV antiserum.

## Discussion

Adaptation of a virus to non-natural host cells is often accompanied by the attenuation of the virulence against natural host animals, as has been reported with many viruses such as influenza virus (Brown, 1990), Japanese encephalitis virus (Cao *et al.*, 1995), hepatitis A virus (Graff *et al.*, 1994), Borna disease virus (Rubin *et al.*, 1993) and human immunodeficiency virus (Fujita *et al.*, 1992; Sawyer *et al.*, 1994). The mechanisms of attenuation/adaptation have been intensively studied to make use of them for designing new vaccines. We reported the possible use of MVCE51, a protease activation mutant derived from MVC11, as a safe and potent live vaccine (Wang *et al.*, 1994). However, the mechanism of the attenuation/adaptation of MVC11 was as yet unclarified.

In the present study, we showed that enhanced viral mRNA synthesis in MVC11-infected LLC-MK<sub>2</sub> cells was mediated by two mutations at the most, i.e. in the C and L proteins (C170F → S and L2050E → A mutations, respectively). The L protein is considered to execute all of the catalytic steps of synthesis, capping and methylation of RNA (Lamb & Kolakofsky, 1996). On the other hand, the C protein has been reported to play a regulatory role, as an accessory protein, in inhibiting viral mRNA synthesis, although it is not essential for the viral RNA polymerase activity (Curran *et al.*, 1992). Recently, we reported that the chimeric C protein with Phe at position 170 inhibited genome replication 4-fold more strongly than that with Ser in the same position (Cadd *et al.*, 1996). In addition, the C protein of M1 (C170F) expressed by recombinant vaccinia virus inhibited transcription of SeV RNA and production of progeny virus more strongly than that of MVC11 (C170S) (data not shown). Taken together, these results suggest the possibility that the C170F → S mutation is responsible for alleviation of C protein activity that is supposed to inhibit RNA polymerase activity, and thereby, play an important role in the adaptation of MVC11 to LLC-MK<sub>2</sub> cells through augmentation of viral mRNA synthesis. The significance of the L2050E → A mutation on the RNA polymerase activity has not yet been elucidated. It is also possible that both of the mutations are indispensable for the adaptation of MVC11 to LLC-MK<sub>2</sub> cells.

MVC11 was highly attenuated in terms of mouse pathogenicity, through decreased production of infectious progeny virus, especially in the late stage of infection (Fig. 3). It has been suggested that a host cell factor(s) such as tubulin is required for the full expression of viral mRNA synthesis (Mizumoto *et al.*, 1995; Moyer *et al.*, 1986). It is not unreasonable to assume that such a cellular factor(s) varies with cell types or species and

that the RNA polymerase complex of a virus interacts with the factor(s) of the inappropriate host cells less effectively than with that of the appropriate host cells. This may be one of the reasons why MVC11 does not replicate efficiently in mouse lung. Also, it is possible that, because of the function of the C protein of M1, i.e. suppression of the otherwise unregulated production of viral proteins that are toxic to the host cells, M1-infected cells are protected from cell death for certain period of time, during which more progeny virus can be continuously produced to spread throughout the lung. From this point of view, it is notable that MVC11 caused strong injury to bronchial epithelial cells in mouse lung on day 2, while M1 did not (Fig. 4). This hypothesis is in line with the observation that a measles virus mutant lacking the C protein induced lytic injury of infected cells more strongly than the wild-type measles virus possessing the C protein (Radecke & Billeter, 1996). At present, we are unable to draw a conclusion as to whether the C170F → S mutation, the L2050E → A mutation or both is responsible for attenuation of virulence in mice. To make this point clear, generation of recombinant SeV carrying either the C170F → S mutation or the L2050E → A mutation by reverse genetic technology would be necessary.

MVC11 bore some resemblance to SeV lacking the V protein [V(-)] (Kato *et al.*, 1997) which displayed markedly increased gene expression and cytopathogenicity in cultured cells, but was strongly attenuated for pathogenicity in mice compared to the wild-type Z strain. Figs 2(b) and 5 clearly demonstrated that MVC11 expressed the V protein in LLC-MK<sub>2</sub> cells as well as in mouse pulmonary epithelial cells. It is possible that involvement of the V protein in the expression of those phenotypes varies with different SeV strains. We used virus freshly isolated from an epidemic, which was genetically distinguishable from the laboratory strains such as Fushimi and Z strains. The M1 strain exhibited extremely high virulence (1 LD<sub>50</sub> corresponded to  $4 \times 10^1$  CIU) and MVC11 was attenuated by more than 10<sup>4</sup>-fold compared to M1. On the other hand, V(-) was derived from cDNA of the Z strain that had been already considerably attenuated with 1 LD<sub>50</sub> corresponding to more than 10<sup>5</sup> CIU (Sakaguchi *et al.*, 1994). Thus, MVC11 was much more highly attenuated than V(-) when compared with their respective parental strains. Therefore, the mechanism(s) of the attenuation might differ for MVC11 and V(-).

The sequences of M1 and MVC11 presented in this report are the first data of the entire genomes of a mouse-derived fresh isolate of SeV and its LLC-MK<sub>2</sub>-adapted mutant. Phylogenetic analysis of all the genomic regions has confirmed that SeV can be classified into at least two lineages, fresh isolates such as the M1 strain and laboratory strains such as the Fushimi, Z, Harris and Enders strains (data not shown; Wang *et al.*, 1994). At present, we do not know whether multiple lineages of SeV prevail among rodents or if the genetic divergence is due to alterations that have accumulated during long-term passages in non-natural host cells such as chicken

eggs. In any case, because all the recent field isolates belong to the same lineage as M1 and because the laboratory strains have been considerably attenuated through passages in non-natural hosts, field isolates like M1 would be preferable for the analysis of the mouse pathogenicity of SeV.

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