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Involvement of the Leader Sequence in Sendai Virus Pathogenesis Revealed by Recovery of a Pathogenic Field Isolate from cDNA

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We previously demonstrated that a systematic passage of a pathogenic field isolate of Sendai virus (SeV), the Hamamatsu strain, in embryonated eggs caused attenuation of virulence to mice, and we isolated viral clones of distinct virulence (K. Kiyotani et al. Arch. Virol. 146:893-908, 2001). One of the clones, E15cl2, which was obtained from the virus at the 15th egg passage of E0, the parental Hamamatsu clone for egg passage, had 165-fold-attenuated virulence to mice and possessed only four mutations in the entire 15,384-base genome: in an antigenomic sense, U to A at position 20 ($U_{20}A$) and U to A at position 24 ($U_{24}A$) in the leader sequence, the promoter for transcription and replication, and A to G at position 9346 (silent) and A to U at position 12174 (Ser to Cys) in the L gene. To examine the possibility that leader mutations affect virus pathogenesis, we recovered live viruses from cDNA derived from the Hamamatsu strain. A mutant virus possessing either a mutation of $U_{20}A$ or $U_{24}A$ in the leader sequence showed a slightly lower pathogenicity than that of the parental virus, whereas a double mutant virus possessing both of the mutations showed 25-fold-attenuated virulence, accompanying a significantly lower virus replication in the mouse lung. Replications of the leader mutant viruses were also impaired in a primary culture of mouse pulmonary epithelial cells but not in chicken embryo fibroblasts. These findings suggest that leader mutations of SeV affect virus pathogenesis by altering virus replication in a host-dependent manner.

Sendai virus (SeV), which belongs to the family *Paramyxoviridae*, possesses a single-stranded negative-sense RNA genome. In the genome are six tandemly aligned genes that each encode the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L) from the 3′-to-5′ direction, individually flanked by both of the viral regulatory sequences: the transcription start and termination sequences. At the 3′ end of the genome is the 52-base leader sequence that acts as a promoter for transcription and replication of the viral genome (15).

SeV causes respiratory illness in rodents, and devastating infections in animal facilities have been sporadically reported. Experimental infection with SeV in mice—a natural host—has been used as a model of respiratory infection, and the mechanism of SeV pathogenesis has been extensively investigated (see, for example, references 13 and 25). The recent establishment of virus recovery systems from cloned cDNA for SeV has enabled us to manipulate the virus genome by site-directed mutagenesis and, together with infection experiments, to elucidate viral components and regulatory elements necessary for viral pathogenesis in mice (reviewed in reference 17). However, virus recovery systems have been available only for the Z strain (6, 11) and the Fushimi strain (16). Both are laboratory strains that have been extensively passaged in eggs and attenuated in virulence to mice. The Z strain's 50% mouse lethal dose (MLD_{50}) against 3-week-old male mice of the ICR/Crj (CD-1) strain is more than 10^5 cell infectious units (CIU)/mouse. On the other hand, fresh field isolates of SeV maintain high pathogenicity to mice with an MLD_{50} of ca. 10^2 CIU/mouse (8. 13). The Hamamatsu strain, isolated from an epidemic in an animal facility in Japan in 1976, has been passaged only two times in chicken eggs since its isolation and maintains high pathogenicity to mice (12, 21). This difference in pathogenicity suggests that experimental infection of mice with the laboratory strains does not reflect actual infection in mice. It is therefore essential to recover a virulent fresh isolate from cDNA.

We previously reported that a systematic egg passage of a highly virulent clone, E0, derived from the Hamamatsu strain caused attenuation of virulence to mice, and we suggested that the attenuation of virulence occurred due to the appearance and selection of virus variants showing poor propagation in mouse respiratory epithelial cells (12). In that study, we isolated virus clones by plaque purification from virus stocks after 15, 30, and 50 passages in eggs or after 30 passages in eggs and a further 15 passages in mouse lungs. E15 clone 2 (E15cl2), a virus clone obtained after 15 egg passages of E0, had ca. 165-fold attenuated virulence to mice with an MLD_{50} of 1.3×10^4 CIU/mouse, much higher than that of E0 (7.9×10^1 CIU/mouse). On the other hand, E15cl2 had a short passage history and was expected to have minimum mutations. To determine the molecular basis of the attenuation, we analyzed the entire nucleotide sequence of E15cl2 in the present study and found only four base substitutions in the entire 15,384-base genome. Two of the substitutions were in the leader region (U to A at position 20 and U to A at position 24), and the other two were

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in the coding region of the L protein [A to G at position 9346 (silent) and A to U at position 12174 (Ser to Cys)]. These findings raised the possibility that the mutations in the leader sequence as well as those in the L protein affect virus pathogenicity. We further sequenced leader regions of other viral clones obtained by the serial passages in eggs and mouse lungs and found no mutations other than those at positions 20 and 24. We focused our investigation on the leader mutations to determine their significance in viral pathogenesis by utilizing virus recovery of the Hamamatsu strain from cDNA, since the relationship between the leader sequence and viral pathogenesis has not been investigated extensively in paramyxoviruses.

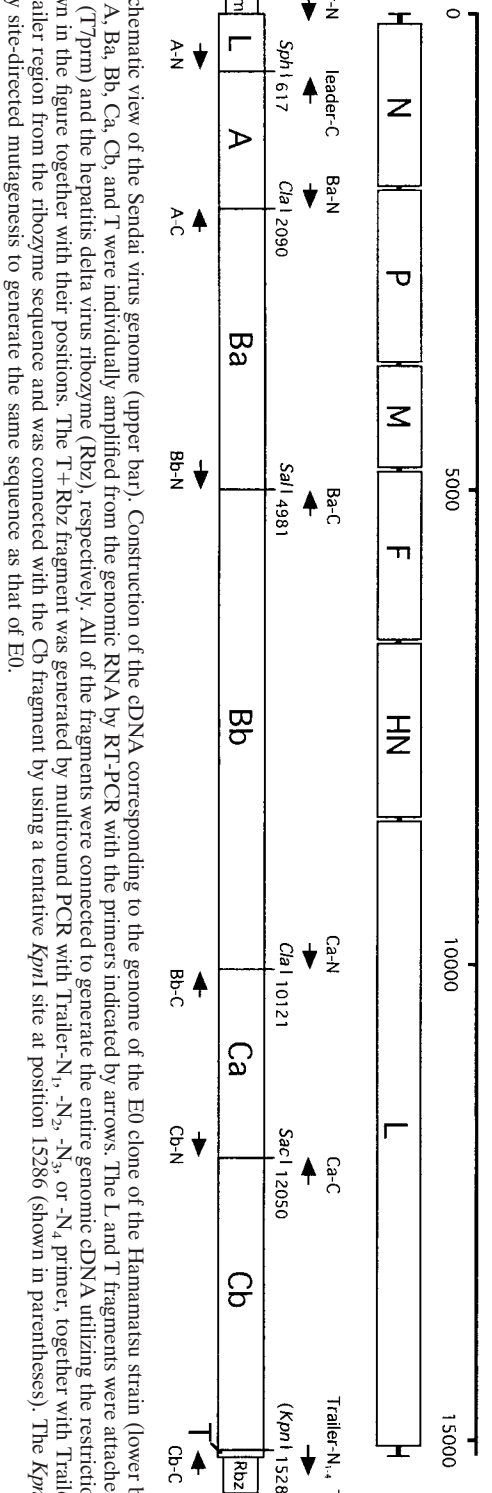


FIG. 1. Schematic view of the Sendai virus genome (upper bar). Construction of the cDNA corresponding to the genome of the E0 clone of the Hamamatsu strain (lower bar). The DNA fragments L, A, Ba, Bb, Ca, Cb, and T were individually amplified from the genomic RNA by RT-PCR with the primers indicated by arrows. The L and T fragments were attached to a modified T7 promoter (T7prm) and the hepatitis delta virus ribozyme (Rbz), respectively. All of the fragments were connected to generate the entire genomic cDNA utilizing the restriction enzyme sites that are shown in the figure together with their positions. The T+Rbz fragment was generated by multiround PCR with Trailer-N_1, -N_2, -N_3, or -N_4 primer, together with Trailer-C primer, to extend the trailer region from the ribozyme sequence and was connected with the Cb fragment by using a tentative *Kpn*I site at position 15286 (shown in parentheses). The *Kpn*I site was then eliminated by site-directed mutagenesis to generate the same sequence as that of E0.

MATERIALS AND METHODS

Cells and viruses. LLC-MK_2 cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. Primary mouse pulmonary epithelial (MPE) cells were prepared from 6-week-old ICR mice according to the method described previously (8). Primary chicken embryo (CE) cells were prepared from 10-day-old chick embryos by trypsin digestion as described previously (12).

The SeV clones used in the present study, E0 and E15cl2, were described previously (12). E0 is the parental SeV clone for serial egg passages that was isolated from the Hamamatsu strain and its MLD_{50} is 7.9×10^1 CIU/mouse. The Hamamatsu strain was originally isolated from mice by inoculating lung homogenates into the allantoic cavities of 10-day-old embryonated hen's eggs and by passaging once more in eggs. E0 was prepared by plaque cloning of the Hamamatsu strain on LLC-MK_2 cell monolayers and by amplifying once in eggs. Thus, E0 has been passaged three times in eggs in total. E15cl2 is an attenuated virus clone with MLD_{50} of 1.3×10^4 CIU/mouse that was isolated from the virus at the 15th egg passage of E0 by plaque cloning and amplified once in eggs. Infectivity of the SeV clones and that of recombinant viruses were determined by using an immunofluorescent infectious focus assay (9) and expressed as CIU/milliliter.

Nucleotide sequences of E0 and E15cl2 are available in the DDBJ/EMBL/GenBank database with accession numbers AB039658 and AB065187, respectively.

Nucleotide sequence determination. The entire genome of E15cl2 was sequenced as described previously (5). Briefly, the viral RNA extracted from the stock virus was reverse transcribed and amplified by PCR. The amplified DNA fragments were directly sequenced by an ABI 310 Genetic Analyzer (PE Biosystems, Foster City, Calif.) with virus-specific primers.

The 5′- and 3′-terminal regions of the genome were amplified by rapid amplification of the cDNA ends system (Roche Diagnostics, Indianapolis, Ind.). The genomic or antigenomic strand of viral RNA was reverse transcribed with a specific primer, and a poly(A) tract was attached to the 3′ end of the synthesized DNA by the terminal deoxynucleotidyltransferase. The tailed DNA was then amplified by PCR with a virus-specific primer and the oligo(dT)-anchor primer from the kit.

Plasmid construction. The full-length cDNA of the clone E0 was constructed as follows. Viral RNA was extracted from allantoic fluid infected with E0 by using proteinase K digestion. The DNA fragments—L, A, Ba, Bb, Ca, Cb, and T—were individually amplified from the viral genomic RNA by reverse transcription-PCR (RT-PCR) by using ExTaq DNA polymerase (Takara Shuzo, Otsu Shiga, Japan) or KOD Dash DNA polymerase (Toyobo, Tokyo, Japan). The oligonucleotide primers for PCR and their corresponding positions are shown in Fig. 1. Sequences of the primers are available upon request. The L and T fragments were attached to a modified T7 promoter and the hepatitis delta virus ribozyme, respectively. All of the fragments were connected to generate the entire genomic cDNA utilizing the restriction enzyme sites that are shown in Fig. 1, together with their positions. The T+Rbz fragment was generated by multiround PCR with Trailer-N_1, -N_2, -N_3, or -N_4 primer, together with Trailer-C primer to extend the trailer region from the ribozyme sequence and was connected with the Cb fragment by using a tentative *Kpn*I site at nucleotide position 15286. The *Kpn*I site was then eliminated by site-directed mutagenesis to generate the same sequence as that of E0. After each cloning or mutagenesis during the assembly, the absence of an accidental mutation was confirmed by using a 310 Genetic Analyzer. The final sequence was completely identical to that of the E0 clone (5), and the plasmid containing the entire genomic cDNA for virus recovery was named pSeVH.

To introduce mutations into the leader region, a 634-bp *Apa*I and *Sph*I fragment from pSeVH containing the region was subcloned, and mutations were introduced into the plasmid by using "U.S.E. mutagenesis" (4). After sequence confirmation, the fragment was then returned to pSeVH.

Recovery of SeV from cDNA. Recombinant SeV (rSeV) was recovered from the pSeVH plasmid as described previously (11). Briefly, LLC-MK_2 cells were infected with the T7 RNA polymerase-expressing vaccinia virus vTF7.3 (a kind gift from Bernard Moss) and subsequently transfected with pSeVH and three plasmids, pGEM-N, pGEM-P, and pGEM-L (6, 11), thus supporting the initiation of the SeV life cycle from cDNA. After a 48-h incubation, the cell lysates were injected into embryonated chicken eggs and incubated at 33°C for 3 days. The recovered allantoic fluid, containing roughly 10^9 CIU/ml of SeV and 10^3 PFU/ml of vTF7.3, was diluted to 10^{-7} and inoculated again into eggs to eliminate contaminating vTF7.3. The harvested allantoic fluid was then used as a stock virus.

Protein analysis by SDS-PAGE. Virus-infected cells were labeled with [^{35}S]cysteine-[^{35}S]methionine ([^{35}S]Pro-Mix, 1.85 MBq/ml; Amersham Biosciences, Piscataway, N.J.) in cysteine- and methionine-free Dulbecco modified MEM and solubilized in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 150 mM NaCl). Polypeptides were then immunoprecipitated with anti-SeV serum and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with an 11% gel as described previously (22). An autoradiogram was analyzed by using a BAS2000 image analyzer (Fuji Film, Tokyo, Japan).

Infection of mice with SeV. Specific pathogen-free, 3-week-old male mice of the ICR/Crj (CD-1) strain weighing 8 to 11 g (purchased from Charles River Japan, Inc., Atsugi, Japan) were infected with SeV (25 μl) intranasally under mild ether anesthesia. At certain time intervals, the body weights and clinical signs of the animals were checked, and some of the mice were sacrificed.

Consolidation scores were marked by macroscopic appearance as described previously (10). Scores of 1, 2, 3, and 4 refer to consolidations of ≤25%, 26 to 50%, 51 to 75%, and 76 to 100% of the lung area, respectively. A score of 5 means death of the mouse. After scoring, lungs from each mouse were homogenized in 1 ml of MEM with a mortar and pestle and, after a low-speed centrifugation, infectivity in the supernatant of the homogenate was measured.

Five mice in a group were infected with each dilution of SeV and observed for 2 weeks, and MLD_{50} was then calculated, as described previously (13). All of the mice were kept under bioclean and regulated conditions in the facility for animal experiments of the Hiroshima University School of Medicine throughout the experiment.

Propagation of SeV in MPE or CE cells. Confluent monolayers of MPE or CE cells in a 35-mm dish were infected with SeV at an input multiplicity of infection (MOI) of 10 and then incubated in MEM without trypsin. The culture medium was replaced with a fresh medium every day, and the infectivity in the medium was measured after treatment with 10 μg of trypsin/ml at 37°C for 10 min.

Northern blot hybridization. Northern blot hybridization was performed as described previously (20) with some modifications. Briefly, the total RNA extracted from the SeV-infected cells was resolved by electrophoresis in an agarose gel containing formaldehyde and transferred onto a nylon membrane by capillary transfer. The membrane was used for hybridization with the pSeVH plasmid labeled with [α-^{32}P]dCTP (Amersham Biosciences). After a washing under stringent conditions, radioactive signals were visualized by using a BAS2000 image analyzer. DNA fragments corresponding to mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as an internal control.

RESULTS

Nucleotide substitutions of the E15cl2 clone. We sequenced the entire 15,384-base genome of E15cl2, a virus clone obtained after 15 passages of E0 in eggs, and found four nucleotide substitutions: U to A at position 20 ($U_{20}A$), U to A at position 24 ($U_{24}A$), A to G at position 9362, and A to U at position 12174. (Please note that the sequence is described in the RNA sequence of an antigenomic sense throughout this study.) The first two are in the leader sequence, and the last two are in the coding region of the L protein. Of the two L-frame mutations, the mutation at position 12174 directs an amino acid change from serine to cysteine at the 1,207th residue of the L protein, whereas the other mutation at position 9362 is silent. Accordingly, the two leader mutations, as well as the amino acid-changing mutation of the L protein, may contribute to the 165-fold attenuation of virulence of E15cl2. We further sequenced leader regions of other viral clones derived from serial passages in eggs and mouse lungs and found no mutations other than those at positions 20 and 24 (data not shown). We thus examined involvement of the leader mutations, $U_{20}A$ and $U_{24}A$, in viral pathogenesis by generating mutant viruses.

Recovery of a virulent field isolate of SeV from cDNA. To recover a field isolate of SeV from cDNA, we constructed the plasmid pSeVH containing a full-length genomic cDNA corresponding to the E0 clone derived from the Hamamatsu strain as described in Materials and Methods (Fig. 1).

SeV was recovered from pSeVH basically according to the previously reported method (11). LLC-MK_2 cells were infected with vTF7.3 and subsequently transfected with pSeVH and three plasmids: pGEM-N, pGEM-P, and pGEM-L. The cell lysates were injected into embryonated chicken eggs and further incubated at 33°C for 3 days. Virus was detected in the allantoic fluid by hemagglutination with chicken erythrocytes, and it was named rSeVH-WT. The recovery efficiency was equivalent to that of a simultaneous recovery of the control SeV Z strain.

To analyze proteins of rSeVH-WT, LLC-MK_2 cells were infected with SeV and metabolically labeled with [^{35}S]cysteine-[^{35}S]methionine for 20 min at 7 h postinfection (p.i.). Proteins were then solubilized with RIPA buffer, immunoprecipitated with anti-SeV antibody, and analyzed by SDS-PAGE (Fig. 2). The P, N, and M proteins of the E0 clone migrated differently from the respective counterparts of the Z strain, and this fact allowed us to discriminate the two viruses. rSeVH-WT evidently had a pattern identical to that of E0, demonstrating that E0 was actually recovered (Fig. 2). This result also suggests that genetic recombination between the genomic cDNA of E0 and the supporting plasmids of the Z strain due to the vaccinia virus was not the case at least for the P and N proteins.

We further confirmed the validity of rSeVH-WT by genome sequencing. DNA fragments corresponding to the leader region and a large part of the L coding frame were amplified by RT-PCR and sequenced by using virus-specific primers as described previously (5). All of the obtained sequence data were identical to those of pSeVH (data not shown), indicating that the E0 clone could be successfully recovered from cDNA. The high pathogenicity of the recovered virus rSeVH-WT will be described in a subsequent study.

Recovery of leader mutants of the SeV clone E0. We further recovered viruses with leader mutations. Mutations of either 20A, 24A, or 20A+24A were introduced into pSeVH (Table 1), and the viruses recovered from the cDNAs were designated rSeVH-20A, rSeVH-24A, and rSeVH-20A24A, respectively. The leader regions of the recovered viruses were amplified by RT-PCR, and the nucleotide sequences were confirmed (data not shown).

Virus replication of leader mutants in mouse lungs and pathogenicity to mice. Mice were infected with 10^3 CIU/mouse of the leader mutant viruses, and body weight gain, lung consolidation, and virus replication in the lung were monitored until 10 days p.i. (Fig. 3). Both E0 and rSeVH-WT replicated well in the mouse lungs, reaching a peak of more than 10^8

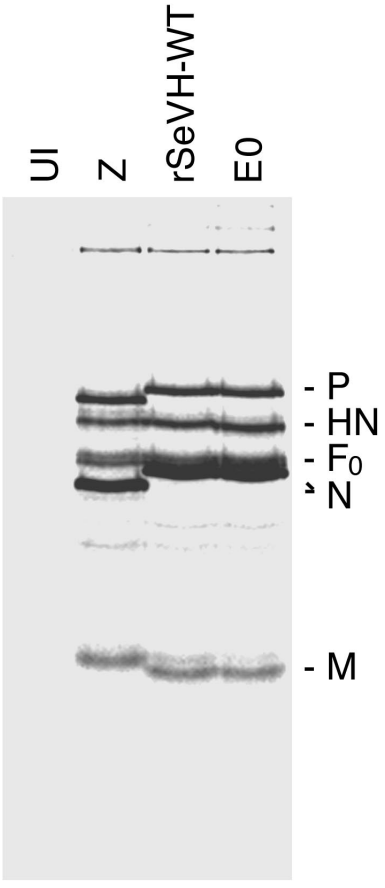


FIG. 2. Protein analysis of rSeVH-WT. LLC-MK_2 cells were infected with rSeVH-WT, as well as SeV Z strain, and the E0 clone of the Hamamatsu strain at an MOI of 20 and incubated for 7 h. The cells were then metabolically labeled with [^{35}S]cysteine-[^{35}S]methionine for 20 min. Proteins were analyzed by immunoprecipitation with anti-SeV Z antibody and SDS-PAGE on an 11% gel. Radioactive bands were visualized by using a Bioimaging Analyzer. The positions of SeV proteins are shown in the figure, and F_0 indicates the precursor of the F protein. UI, uninfected cells.

CIU/mouse at 5 days p.i., and gradually decreased thereafter. Body weight gain began to decrease at 5 days p.i., and almost all of the mice had died by 9 days p.i. Lung consolidation scores increased progressively during the observation period. The results indicate that rSeVH-WT can replicate as efficiently as the parental E0, possessing similar pathogenicity.

In the rSeVH-20A and rSeVH-24A infections, virus growth was slightly impaired in mouse lungs with lower lung consolidation scores compared with E0 and rSeVH-WT (Fig. 3). Only one of the mice in a group had died by 9 days p.i. A single mutation in the leader sequence slightly suppressed virus growth and pathogenicity in the mouse lungs.

TABLE 1. The leader mutants of the Hamamatsu strain

Virus	Leader sequence[a]
E0	5′-ACCAAACAAG AGAAGAAAC**U** UGA**U**AGGAAU-
rSeVH-WT	5′-ACCAAACAAG AGAAGAAAC**U** UGA**U**AGGAAU-
rSeVH-20A	5′-ACCAAACAAG AGAAGAAAC**A** UGA**U**AGGAAU-
rSeVH-24A	5′-ACCAAACAAG AGAAGAAAC**U** UGA**A**AGGAAU-
rSeVH-20A24A	5′-ACCAAACAAG AGAAGAAAC**A** UGA**A**AGGAAU-
E15c12	5′-ACCAAACAAG AGAAGAAAC**A** UGA**A**AGGAAU-

[a] The leader sequence of the SeV mutants are shown in an antigenomic sense. Nucleotides at positions 20 and 24 are shown in boldface.

On the other hand, virus growth was suppressed severely in the rSeVH-20A24A infection (Fig. 3). The peak virus titer was less than 10^7 CIU/mouse, consolidation scores were low, and all of the mice survived without apparent body weight loss during the observation period. These parameters were close to those of the mice infected with E15cl2.

A series of dilutions of the viruses were intranasally inoculated in mice, and their MLD_{50} values were determined (Table 2). We tested two viral clones for rSeVH-WT: clone A1, which was used for protein analysis and nucleotide sequence confirmation as described above, and clone B3, which was recovered from another cDNA clone. The two viruses had the same MLD_{50} values, 2.0×10^2 CIU/mouse in two independent experiments, demonstrating that rSeVH-WT is extremely virulent to mice. The E0 clone showed an MLD_{50} value of 1.3×10^2 CIU/mouse in two parallel experiments. E0 has consistently shown MLD_{50} values of 7.9×10^1 or 1.3×10^2 CIU/mouse in experiments (12, 21). Thus, rSeVH-WT showed a slightly higher MLD_{50} value, 2.0×10^2 CIU/mouse, than that of the original strain. The difference depends on whether one mouse in a group of five died or not, but it appears fairly reproducible. We propose two hypotheses to explain the slight difference. One is that the E0 clone is heterogeneous and contains a minor population of more virulent virus that cannot be detected by nucleotide sequencing of the major population. The other is that a minor population of low virulence emerged in rSeVH-WT by the virus recovery procedures, including two passages in eggs. Considering the fact that a virulent phenotype is dominant when a virus is a mixture of different degrees of virulence (12), the former hypothesis seems more plausible.

MLD_{50} of rSeVH-20A24A was 3.2×10^3 CIU/mouse, indicating ca. 16-fold attenuation of virulence compared to that of rSeVH-WT. E15cl2, and was a further 4-fold attenuated in virulence compared to that of rSeVH-20A24A; this slight difference may be due to the additional mutation in the L protein of E15cl2. MLD_{50} values of rSeVH-20A and rSeVH-24A varied from experiment to experiment but were within the range of 2.0×10^2 to 8.0×10^2 CIU/mouse, demonstrating their intermediate pathogenicity between that of rSeVH-WT and that of rSeVH-20A24A. These data indicate that virus replication in the mouse lung and viral pathogenicity are affected by a mutation in the leader position 20 or 24 and particularly by a double mutation at both positions.

We analyzed the nucleotide sequence of rSeVH-20A24A again. It is known that a DNA homologous recombination, which is facilitated by vaccinia virus, could occur between the genomic cDNA and the supporting plasmids containing the cDNA of N, P, and L proteins during virus recovery (6). We used the supporting plasmids derived from an avirulent laboratory strain, Z, in this experiment. It is therefore possible that rSeVH-20A24A, the most attenuated clone, has a recombination in the N, P, and/or L proteins, leading to a lower pathogenicity of the virus. However, complete nucleotide sequence determination of the N, P, and L genes of rSeVH-20A24A demonstrated that there was no insertion of the Z strain se-

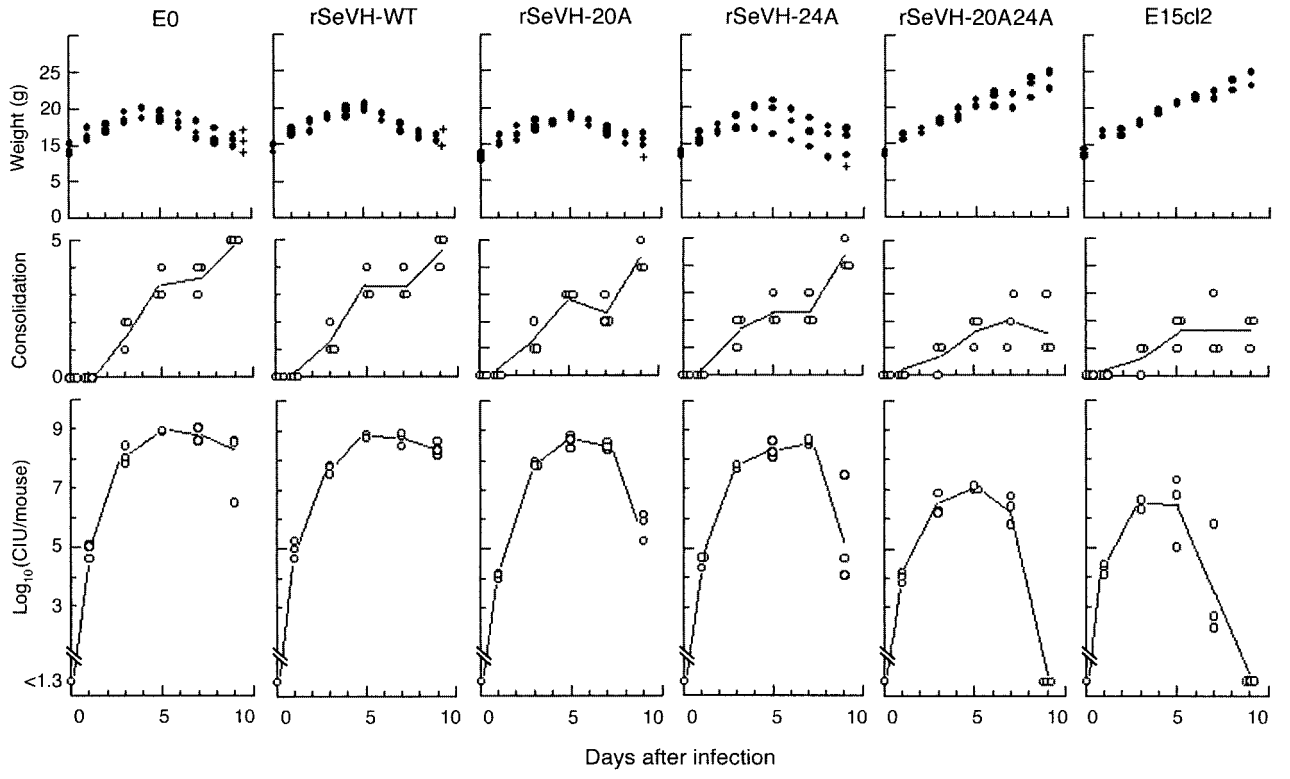


FIG. 3. Viral replication, lung consolidation, and body weight gain in the mice infected with the SeV leader mutants. Three-week-old ICR mice were infected intranasally with SeV at 10^3 CIU/mouse, and three mice were weighed daily. One dot shows a mouse, and the plus sign indicates the death of a mouse. Three mice in each group were sacrificed at the indicated days and examined for lung consolidation and viral infectivity. Each line is based on the mean of three mice.

quence in these genes, ruling out the possibility of genetic recombination (data not shown).

Virus replication in MPE cells. We previously showed that SeV replication in MPE cells correlated with that in mouse lungs and pathogenicity to mice (12). This correlation is not observed in other cell lines such as LLC-MK_2 cells, CV1 cells, or primary CE fibroblast cells. MPE cells are thus considered to be a suitable cell culture model for replication of SeV in mouse lungs.

MPE cells were infected with the leader mutants at an input MOI of 10, and the infectivity in the medium was examined

TABLE 2. MLD_{50} of the leader mutants of the Hamamatsu strain[a]

Virus (clone)	MLD_{50} (CIU/mouse)		
	Expt 1	Expt 2	Expt 3
E0	1.3×10^2	1.3×10^2	
rSeVH-WT (A1)	2.0×10^2	2.0×10^2	
rSeVH-WT (B3)	2.0×10^2	2.0×10^2	
rSeVH-20A	2.0×10^2	5.0×10^2	3.2×10^2
rSeVH-24A (7)	3.2×10^2	3.2×10^2	8.0×10^2
rSeVH-24A (9)		5.0×10^2	5.0×10^2
rSeVH-20A24A	3.2×10^3	3.2×10^3	
El5c12	1.3×10^4		

[a] The MLD_{50} endpoints (MLD_{50}) of the leader mutant viruses were determined in three experiments. Two viral clones of rSeVH-WT (A1 and B3) were recovered from two distinct cDNA clones, and those of rSeVH-24A (7 and 9) were also recovered from two distinct cDNA clones.

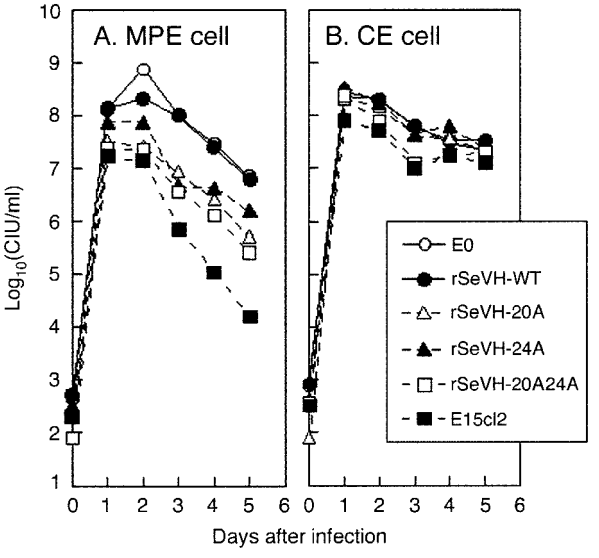
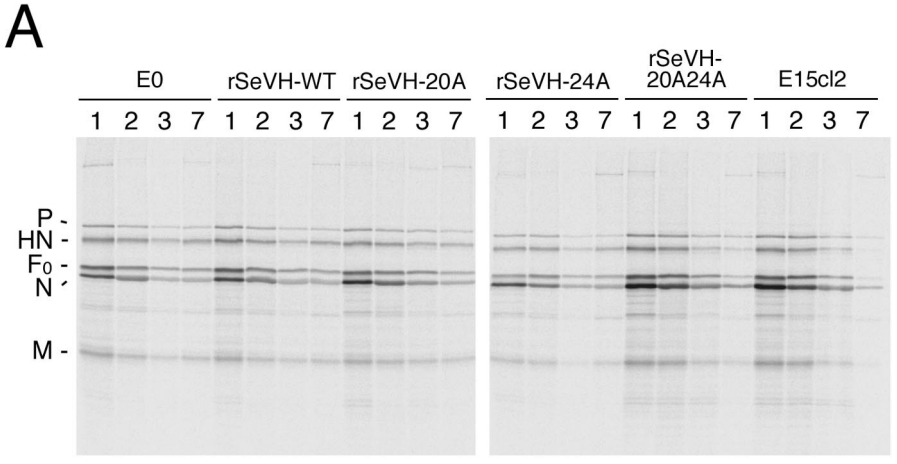


FIG. 4. Propagation of the SeV leader mutants in primary cultures of MPE cells (A) and CE cells (B). Monolayers of MPE or CE cells on 35-mm dishes were infected with each of the SeV clones at an MOI of 10 and then incubated in 1 ml of serum-free MEM at 36°C. The culture medium was harvested, and the cells were refed with 1 ml of fresh medium every day. Viral infectivities in the medium were assayed after activation with 10 μg of trypsin/ml.



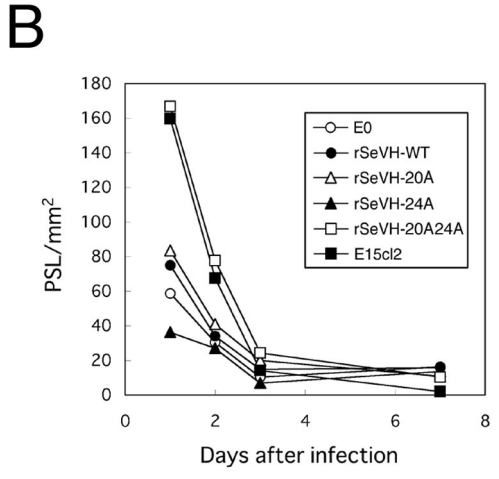


FIG. 5. Viral protein synthesis in MPE cells infected with the SeV leader mutants. Monolayers of MPE cells on 3.5-cm dishes were infected with the SeV leader mutants at an MOI of 10. At various days after infection (indicated at the top of panel), the culture medium was replaced with 1 ml of methionine- and cysteine-free MEM, and the cells were metabolically labeled with 1.85 MBq of [^{35}S]methionine-[^{35}S]cysteine/ml for 15 min. (A) Viral proteins were immunoprecipitated with anti-SeV rabbit serum and processed for SDS-PAGE and autoradiography by using a BAS2000 image analyzer. (B) The radioactivity of the viral proteins P, HN, F_0, N, and M in each lane was quantified by using an image analyzer and plotted in the graph. PSL is a unit of radioactivity defined by the MacBAS software used for the image analyzer.

daily (Fig. 4A). E0 and rSeVH-WT propagated well and were released efficiently into the medium. In contrast, E15cl2 did not propagate well in MPE cells, and the infectivity in the medium was >10-fold lower than those of E0 and rSeVH-WT at 1 day p.i. and later. Replications of rSeVH-20A, rSeVH-24A, and rSeVH-10U24A were at an intermediate level between those of E0 and E15cl2. Although no notable difference was found between the replication of rSeVH-20A24A and those of rSeVH-20A and rSeVH-24A, the leader mutations affected virus replication in MPE cells in a fashion almost compatible with their growth in mouse lungs. On the other hand, all of the viruses had a similar growth pattern in CE cells (Fig. 4B) as was predicted from the result of a previous study (12).

Protein and RNA syntheses in MPE cells infected with the leader mutants. MPE cells were infected with the respective SeV mutants at an MOI of 10, and proteins were pulse-labeled with [^{35}S]cysteine-[^{35}S]methionine for 15 min at 1, 2, 3, or 7 days p.i. Migrations of the viral proteins of the mutant viruses in SDS-PAGE were similar to those of E0. Viral protein synthesis was almost equivalent among the viruses except that protein syntheses of rSeVH-20A24A and E15cl2 were enhanced compared to those of the other mutants at 1 and 2 days p.i. (Fig. 5). However, these findings do not explain the low virus growth of E15cl2, rSeVH-20A, rSeVH-24A, or rSeVH-20A24A in MPE cells.

We next analyzed intracellular viral RNA by Northern blot hybridization. MPE cells were infected with SeV at an MOI of 10, and total RNA was extracted from the cells at 12 and 24 h p.i. There were no significant differences in amounts of mRNA and intracellular full-length genomic RNA (data not shown).

DISCUSSION

Virus recovery of the Hamamatsu strain. We could recover a virulent field isolate of SeV, the Hamamatsu strain, from cDNA by using supporting plasmids containing the N, P, and L genes of the SeV Z strain. SeV strains so far sequenced can be divided into two evolutionary lineages by nucleotide sequence comparison (21, 26). Conventional laboratory strains such as Z and Fushimi are in one lineage, and those isolated in the 1970s in Japan such as Hamamatsu and Oh-ita are in the other lineage. The Hamamatsu and Z strains in distinct lineages are 11% different in nucleotides over the entire genome (5, 8). This difference seems to be a maximal level for a virus of a common serotype of the paramyxovirus (23). Despite the genetic divergence, the N, P, and L proteins of the Z strain could initiate transcription and genome replication of the Hamamatsu strain. This is reasonable considering the fact that regulatory elements such as the leader sequence are relatively conserved between the two strains (5).

Conzelmann et al. reported rescue of rabies virus from cDNA in 1994, the first reported recovery of a mononegavirus from cDNA (2). Since then virus recovery systems have be-

come available for almost all of the *Mononegavirales* (reviewed in references 1, 17, 18, and 19). The initial recovery of laboratory strains has extended to the recovery of field pathogenic strains, as seen in the recovery of a pathogenic measles virus from cDNA (24). Our recovery system of a field isolate of SeV, the Hamamatsu strain, from cDNA will be useful for investigation of the pathogenesis of virulent SeV in the context of the natural course of infection.

On the other hand, we must be careful about unexpected mutations in this virus recovery system. It is known that DNA homologous recombination by a coexisting helper vaccinia virus can occur between the genomic cDNA and the supporting plasmids containing the cDNA of N, P, and L proteins during virus recovery (6). We used the supporting plasmids derived from the Z strain, an avirulent laboratory strain. It is therefore possible that the recovered viruses have been recombined to be of attenuated virulence. However, it was not the case for rSeVH-20A24A, the most attenuated clone among the mutant viruses, because of the results of nucleotide sequence confirmation in the N, P, and L genes of rSeVH-20A24A. Protein migrations of the N and P proteins of the viruses in SDS-PAGE and the fact that rSeVH-WT maintained high pathogenicity to mice also suggests a lack of recombination. Homologous recombination may be rare because of the heterogeneity between the Hamamatsu and Z strains (11% in nucleotides). However, since there is a possibility of recombination, this virus recovery system must be further improved by employing the supporting plasmids derived from the Hamamatsu strain and not the Z strain.

Host-dependent replication and leader mutations. In generating a live attenuated vaccine, the virus is usually passaged in an unnatural host. We conducted a model experiment in which an SeV field isolate was passaged in embryonated eggs (12) and found leader mutations by egg passage. Actually, recombinant SeV with leader mutations at positions 20 and 24 had attenuated virulence to mice. The double mutant rSeVH-20A24A showed particularly low virus growth and low pathogenicity compared to those of rSeVH-WT, while single mutants, rSeVH-20A and rSeVH-24A, were attenuated intermediately between the levels of rSeVH-WT and rSeVH-20A24A. According to the results of scanning mutagenesis of the leader sequence of human parainfluenza virus type 3, which is classified into the same genus as that of SeV, the first 12 nucleotides of the leader sequence are essential for efficient genome transcription and replication, whereas the nucleotide at position 13 and its downstream nucleotides are of moderate or no importance (7). Thus, positions 20 and 24 may not be essential for the leader function, but they may be important for modification of virus replication in mice and pathogenicity.

Leader mutations were originally obtained by selection in eggs, and they were expected to be advantageous for efficient replication in eggs. We tried to find a growth advantage of rSeVH-20A24A and E15cl2 in the time course of growth in eggs, but no such advantage was found (data not shown). This is probably because the E0 clone of the Hamamatsu strain has already been passaged in eggs three times, and thus the strain may have adapted to eggs to some extent. We are not able to confirm this because we do not have an original Hamamatsu strain that has not been passaged in eggs. It appears that since embryonated chicken eggs allow SeV to grow so efficiently, it is difficult to detect the growth difference in this system. In contrast, we detected an impediment to multiplication of rSeVH-20A24A in mouse lungs. Inhibition of the virus replication by the 20A24A mutation was also seen in MPE cells but not in CE cells (Fig. 4), and it was thought to be host dependent. So far, we have not detected a significant difference in protein and RNA syntheses in MPE cells between the leader mutant viruses. Further study is needed to determine the reason for the difference in virus replication.

It is possible that leader-binding proteins may be involved in host dependence. De et al. reported that GAPDH and a host autoantigen, LA protein, can bind leader RNA and the ribonucleocapsid from human parainfluenza virus type 3 (3). Other host factors that associate with viral RNA have also been described (reviewed in reference 14). These studies suggest that the leader region coated with the N protein can interact with a host factor in infected cells. We postulate that association of the host factor with the leader region in the viral nucleocapsid enables efficient virus replication in MPE cells. Mutations at positions 20 and 24 in the leader region may impair this association.

In summary, a field isolate of SeV, highly pathogenic to mice, has been recovered from cDNA, and reverse genetics to elucidate virus pathogenicity has become available. By utilizing this system, base substitutions at positions 20 and 24 of the leader sequence found in an egg-passaged attenuated virus were shown to be sufficient to inhibit virus growth in the mouse lung and to impair viral pathogenicity. This was also observed in cultured cells in a host-dependent manner. We hypothesize the presence of a host factor that associates with this leader region and accelerates virus replication in mice. The molecular mechanism remains to be clarified.

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