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A Point Mutation in the Sendai Virus Accessory C Proteins Attenuates Virulence for Mice, but Not Virus Growth in Cell Culture

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A mutant Sendai virus (SeV^{MVC}), which grows much better than its progenitor virus (SeV^{M}) in cell culture, but, in strong contrast to SeV^{M}, is totally avirulent for mice, has been described. SeV^{MVC} contains two amino acid substitutions relative to SeV^{M}, namely, F170S in the C protein and E2050A in the L protein. We have examined which substitutions were responsible for the above phenotypes by exchanging the C gene of our reference strain Z with those of SeV^{H} (another reference strain), SeV^{M}, and SeV^{MVC}, in turn. We have found that the F170S mutation in the C^{MVC} protein is responsible both for enhanced replication in cell culture and for avirulence in mice. Avirulence appeared to be due to restricted viral replication primarily after day 1, implicating some aspect of innate immunity in this process. The SeV C proteins thus appear to be required for multiple cycles of replication in mice.

The paramyxovirus C proteins were first found in Sendai virus (SeV)-infected cells and, as they were apparently absent in virions, were termed nonstructural proteins (reviewed in Lamb and Paterson, 1991). Subsequent work with specific antisera showed that the C proteins could be detected in virions, associated with nucleocapsids. They are, however, greatly underrepresented in virions relative to their amounts intracellularly (Lamb and Paterson, 1991; Portner *et al.,* 1987; Yamada *et al.,* 1990). Further, unlike the other virion proteins which are translated from separate transcriptional units, the C proteins are translated from the P (phosphoprotein) gene mRNA, from an alternate ORF which overlaps the N-terminal half of the P protein ORF (Giorgi *et al.,* 1983) (Fig. 1).

The expression of the paramyxovirus C proteins can also be very complex. SeV is a member of the paramyxovirus genus of the *Paramyxovirinae.* This subfamily also includes the morbillivirus (e.g., measles virus) and the rubulavirus (e.g., mumps virus and SV5) genera. The more distantly related pneumoviruses, such as respiratory syncytial virus, are now classified in a separate subfamily (*Pneumovirinae*) of the *Paramyxoviridae* (Murphy *et al.,* 1994). The *Paramyxovirinae* have been recently reclassified based on their P gene organizations, including the presence of a C ORF. In SeV-infected cells, the C proteins are relatively abundant, and are the major products of the P gene on a molar basis. There are four independently initiated C proteins which are expressed (Fig. 1) via a non-AUG start site (C′) and both scanning dependent (C) and independent (Y1/Y2) ribosomal initiation (reviewed in Curran and Kolakofsky, 1990). However, morbilliviruses such as measles virus express only a single C protein, and in significantly lesser amounts than P (Alkhatib *et al.,* 1988; Bellini *et al.,* 1985). Rubulaviruses, in further contrast, do not contain a C ORF at all. The optional nature of this ORF is also apparent in other mononegaloviruses For the pneumoviruses, there is no overlapping ORF in the P gene of RSV, but the P gene of pneumonia virus of mice does contain a C-like ORF which expresses two small basic proteins (Barr *et al.,* 1994). Similarly, for the more distantly related *Rhabdoviridae,* vesiculoviruses like vesicular stomatitis virus express two C proteins, whereas lyssaviruses like rabies virus do not contain a C ORF of any size (Spiropoulou and Nichol, 1993). We note that the term "C protein" refers only to the origin of its coding sequence with reference to that of SeV; it is not as yet clear whether the different proteins carry out similar functions.

The SeV C proteins are relatively small (175–215 residues), basic proteins. There is little specificity in their localization intracellularly; they are evenly distributed in the cytoplasm by immunofluoresence and are present in all cytoplasmic fractions by cell fractionation (Portner *et al.,* 1987; Yamada *et al.,* 1990). Only a very small amount of the C protein is found bound to nucleocapsids intracellularly, or in reconstitution experiments with transfected cell extracts (unpublished), and this presumably accounts for their vast underrepresentation in virions. Consistent with the fact that some C protein is present on nucleocapsids, the C proteins play a role in viral RNA synthesis. Elimination of C protein coexpression from the

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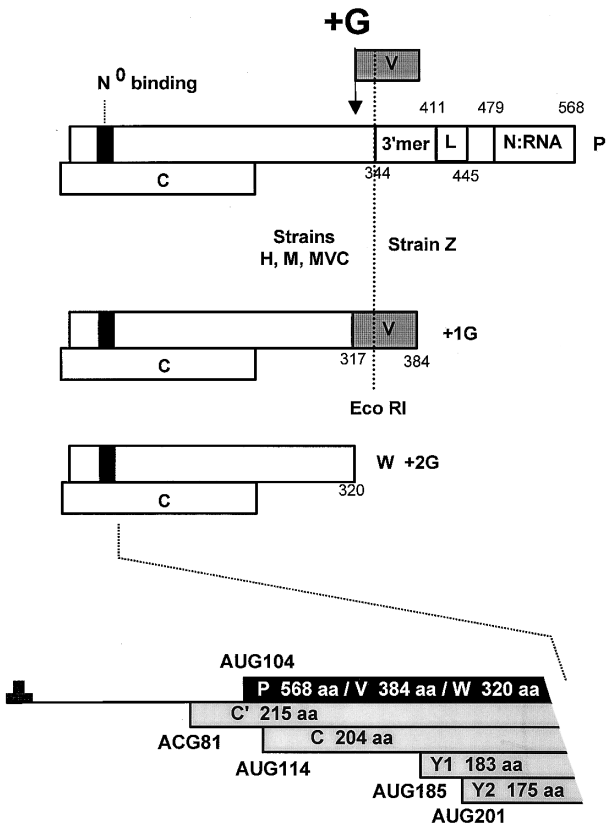

FIG. 1. The reading frame organization of the SeV P gene. The ORFs expressed as protein are shown as horizontal boxes, with their functional domains indicated (see text). The site (codon 317) at which G residues are added during mRNA transcription is indicated by the arrow above, and the consequences of the 1G and 2G insertions on the shuffling of the alternate C-terminal domains are shown below. Numbers refer to aa positions in the P,V, and W proteins. A blow-up of the 5′ end of the mRNA is shown below. Numbers associated with the five ribosomal start codons refer to nucleotide position in the P mRNA, and the seven primary translation products which result are indicated.

SeV P/C gene was found to strongly increase mRNA synthesis *in vitro,* and its reexpression from a separate plasmid eliminated this increase (Curran *et al.,* 1992). Further work showed that anti-genome synthesis from the genomic (leader+ region) promoter was also particularly sensitive to inhibition by C protein, in contrast to genome synthesis from the anti-genomic promoter (Cadd *et al.,* 1996). The promoter-specific inhibition of viral RNA synthesis can seriously compromise viral genome amplification when C protein accumulation is temporally disregulated in transfected cell systems, leading to abnormally high ratios of C proteins to nucleocapsid templates in the early stages of the reconstituted virus replicative cycle (Taparrel *et al.,* 1997). A presumed consequence of this is that in virus recoveries from DNA, C protein coexpression from the required P gene support plasmid (needed to initiate viral RNA synthesis) must be repressed for a successful outcome (Cadd *et al.,* 1996).

A mutant SeV with altered C protein function has recently been described. The Oh-M1 strain of Sendai virus (SeV^M) is a plaque-purified clone of the Ohita-M isolate, from a lethal epidemic of laboratory mice (Wang *et al.,* 1994). Oh-MVC11 (SeV^{MVC}) is a mutant derived from Oh-M1 (after five serial passages in LLC-MK2 cells) which formed clear plaques rather than the turbid plaques of the parent strain (SeV^M). SeV^{MVC} is highly adapted to LLC-MK2 cells; it produces 20-fold more virus than SeV^M in cell culture, and this phenotype was associated with significantly higher levels of viral mRNAs and proteins intracellularly (Itoh and Homma, 1993; Itoh *et al.,* 1997). Sequencing of the SeV^M and SeV^{MVC} genomes revealed a U to C change in codon 170 of the C ORF, where phe was changed to ser (this base change is silent in the P ORF) (Itoh *et al.,* 1997). Examination of these M strain C genes in transfected cell systems showed that C^M behaved similarly to C^Z or C^H (SeV^Z and SeV^H are our reference strains) in that they all inhibited viral RNA synthesis in a promoter-specific manner and prevented the recovery of rSeV from DNA, whereas C^{MVC} had mostly lost this inhibitory property (Cadd *et al.,* 1996). Remarkably, $rSeV^{MVC}$ was also found to be avirulent for mice, in marked contrast to SeV^M (Wang *et al.,* 1994).

This paper describes the preparation and properties of recombinant Z strain viruses (rSeV) in which the endogenous C gene has been replaced with those of strains M, MVC, or H and, in particular, their virulence for mice.

MATERIALS AND METHODS

Generation of pFL3-Δ1

FL3 (Garcin *et al.,* 1995) was cut at the *Eag*I site (genome map position 2746, in the P gene) and the ends were filled in with the Klenow enzyme. This DNA was then cut with *Kpn*I (position 15,281, at the end of the L gene). The resulting fragment of 12,535 nt was purified from the full-length DNA and inserted into the blunted *Nco*I site (position 358, at the beginning of the N gene) and the *Kpn*I site of the vector pE307, which contains an internal deletion defective interfering genome of 1746 nt with the normal promoter sequences at both ends, the beginning of the N gene, and the end of the L gene (Engelhorn *et al.,* 1993). This generated a deleted version of the full-length clone, with a gap of 2388 nt between the *Nco*I site (position 358, at the beginning of the N gene) and the *Eag*I site (position 2746, in the middle of the P gene; see Fig.2)

Generation of the shuttle vector $pN/P^{Xho}/M$

The 2459-nt-long *Bal*I fragment (between position 1484 at the end of the N gene and position 3943 at the beginning of the M gene, including the entire P gene) was purified from the FL3 DNA and inserted into the *Bal*I site (position 1484, at the end of the N gene) of the support

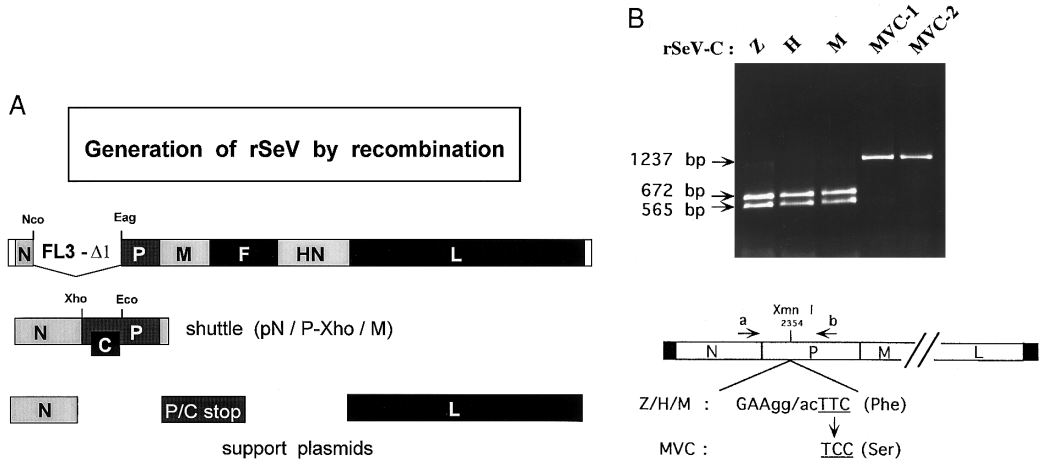

FIG. 2. Generation of rSeV by recombination. (A) The five plasmids cotransfected into vTF7-3-infected cells to generate the various rSeV-C by recombination are shown. These include three support plasmids which provide the N, P, and L proteins required to initiate the assembly and expression of the recombinant viral genome. For the P gene plasmid, C protein coexpression has been suppressed, in this case by a stop codon which interrupts the C proteins but not the P protein (P/C stop). The various C genes are first subcloned into the unique *Xho*I and *Eco*RI sites of the shuttle vector (pN/P^{Xho}/M). Neither SeV^{M} nor SeV^{MVC} contains the nearby *Eag*I site. Generation of an infectious genome requires the recombination of the deleted full length clone (pFL3-Δ1) and pN/P^{Xho}/M). (B) The relevant region of the various rSeV-C genomes was amplified by RT/PCR as shown below. The amplified DNA was then digested with *Xmn*I, and electrophoresed on an agarose gel. A photo of the ethidium bromide-stained gel is shown.

plasmid pGEM3-N (see Fig. 2). A unique *Xho*I site was then engineered in the 5′ untranslated region of the P gene by substituting C for G^{1809}. The sequences between the *Xho*I (position 1809) and the *Eco*RI (position 2870, in the P gene) sites of strains H, M, and MVC were amplified from their cloned genes with Expand high-fidelity *Tac* polymerase (Boerhinger) and inserted into the same sites of the plasmid pN/P^{Xho}/M, and the relevant sequences were verified by DNA sequencing.

Generation of rSeV expressing alternate C (and P) proteins

The recovery system was as described previously (Garcin *et al.,* 1995) except that one more plasmid, the shuttle vector pN/PXho/M, was included. Briefly, one 9-cm petri dish of A549 cells (ca. 80% confluent) was infected with 2 to 3 PFU/cell of vaccinia virus TF7-3 (Fuerst *et al.,* 1986) and transfected 1 h later with 1.5 μg of pGEM-L, 2.5 μg of pGEM-N, 4.5 μg of pGEM-P^{HA} (which does not express the C proteins), 15 μg of FL3-Δ1, and 5 μg of one of the pN/P^{Xho}/M shuttle vectors. Twenty-four hours later, ara-C (100 μg/ml) was added to inhibit vaccinia virus replication, and 24 h after that the cells were scraped into their medium and directly injected into the allantoic cavity of two to three 10-day-old embryonated chicken eggs. Three days later, the allantoic fluids were harvested and reinjected undiluted into eggs. For further passages the viruses were diluted 1/1000 before injection.

Primer extension

RNA from either viral nucleocapsids or mRNA pellets purified on CsCl density gradients or total RNA extracted with Triazol (Gibco BRL) was analyzed by primer extension using MMLV reverse transcriptase (Gibco BRL) and 200,000 cpm of one of the following ^{32}P-5′- primers, which had been purified on sequencing gels: NP126 (5′-CGGCCATCGTGAACTTTGGC-3′), nucleotides 126–107, for estimation of anti-genomes and N mRNAs, or L15,270 (5′-GAAGCTCCGCGGTACC-3′), nucleotides 15,270–15,285, for genomes.

Determination of mouse pathogenicity

Specific-pathogen-free, 3-week-old male mice [ICR/CRJ(CD-1) strain; Charles River, Inc., Japan] were infected intranasally with 25 μl of serially diluted virus under ether anesthesia. The mice were observed for symptoms and weighed daily. LD_{50} was calculated by the method of Reed and Muench (1938). Mice inoculated with 1.25×10^{5} cell-infecting units (CIU) of the virus were sacrificed at certain intervals and the infectivity in the supernatant of 10 % lung homogenates was assayed (Tashiro and Homma, 1983). Virus titers were determined as described previously, and expressed as either cell-infecting units or plaque-forming units (Kashiwazaki *et al.,* 1965). The CIU was essentially equivalent to the PFU.

RESULTS

Generation of rSeV expressing alternate C (and P) proteins

Sendai viruses containing alternate C genes (rSeV-C) were recovered from DNA by taking advantage of the highly recombinogenic nature of the vaccinia virus-based recovery system, while taking into account that virus re-

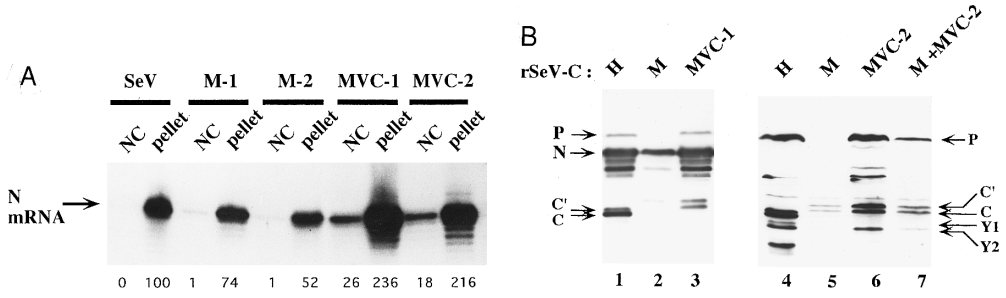

FIG. 3. Replication of the various Sendai viruses in BHK cells. (A) Parallel BHK cell cultures were infected with 10 PFU/cell of two clonal isolates of nonrecombinant SeV^M (M-1 and M-2) and SeV^{MVC} (MVC-1 and MVC-2) and of reference strain Z (SeV). Cytoplasmic extracts were prepared at 24 h p.i. and separated on CsCl density gradients into pelleted and banded (NC) fractions. The amounts of N mRNA present in 1/8 of each fraction from one 9-cm petri dish were determined by primer extension (Materials and Methods) and are given in arbitrary units relative to the SeV control. (B) Parallel BHK cell cultures were infected with 10 PFU/cell of the various rSeV-C as indicated or a mix of 10 PFU/cell of rSeV-C^M and rSeV-C^{MVC-2} (lane 7). Cytoplasmic extracts were prepared at 24 h p.i. and equivalent amounts (1/50 of a 9-cm dish) were separated on 15% protein gels, blotted onto membranes, and examined with a mixture of anti-P, anti-N, and anti-C (lanes 1–3) or anti-P and anti-C (lanes 4–7). The positions of the various proteins are indicated at the sides. Note that C′ and C of strain H migrate more quickly than those of strains M/MVC, whereas the mobilities of the Y2 proteins are the same.

covery here depends on the absence of (functional) C protein coexpression from the pGEM-P support plasmid (Garcin *et al.,* 1995; Cadd *et al.,* 1996). FL3 (full-length DNA clone) was modified to contain a deletion encompassing most of the N and P genes (FL3-Δ1; Fig. 2), such that a rSeV could not be generated simply by the recombination of FL3-Δ1 DNA with that of the individual pGEM-N and pGEM-P support plasmids. A shuttle vector was then constructed containing the deleted region plus flanking sequences (and a *Xho*I site in the 5′ untranslated region of the P gene as a marker and cloning site, pN/P^{Xho}/M), into which alternate C genes were cloned. The entire C protein ORF (and residues 1–325 of the P protein ORF) of SeV strains H, M, and MVC could then be placed in the Z strain background by recombination of pN/P^{Xho}/M with pFL3-Δ1. SeV^H is another of our reference strains, and its N and P gene sequences are virtually (99%) identical to those of SeV^Z. The growth of these two reference strains in cell culture and eggs is indistinguishable. N^H and N^Z, however, can be distinguished by mobility on SDS–PAGE. The sequences of strains M and MVC are only 85% identical to SeV^Z and their P, C′, and C proteins can be distinguished from those of strains Z and H by SDS–PAGE. The P genes of SeV^M and SeV^{MVC} can be distinguished, as the T to C transition (GAANNNNTTC, in bold), which results in the F170S mutation in the C ORF, fortuitously eliminates a *Xmn*I site (Fig. 2B). For all recovered viruses, the origin of their N and P genes was verified by the above criteria, to ensure that pGEM-P had not recombined with pN/P^{Xho}/M before the latter's recombination with pFL3-Δ1.

Because of the overlapping nature of the C gene ORF, one cannot exchange the C gene without exchanging at least part of the P gene. The P protein plays a central role in viral RNA synthesis, as an essential cofactor for the L protein polymerase and as a chaperone for unassembled N protein (N^0) during genome replication (Hamaguchi *et al.,* 1983; Horikami *et al.,* 1992). P is a highly modular protein of 568 residues, the C-terminal domains of which are shuffled by pseudotemplated G insertions during transcription, a form of mRNA editing (Vidal *et al.,* 1990; Curran *et al.,* 1991). Three P gene mRNAs result (Fig. 1); all express their C proteins identically but contain different P protein sequences beyond aa 317. Most of the P mRNAs are unedited (70%), and the reading frame downstream is open to aa 568. This C-terminal region contains a coiled-coil trimerization domain, a tight L protein binding site, and a nucleocapsid (N:RNA) binding site, and by itself is fully active with L protein in mRNA synthesis, i.e., this C-terminal 40% of the protein can act as the polymerase cofactor (Curran, 1996, and references therein). Around 25% of the mRNAs contain one additional G which shifts the reading frame to a highly conserved, Cys-rich domain (68 aa) which binds two Zn^{2+} atoms (Liston *et al.,* 1994; Paterson *et al.,* 1995) (V protein), while a small fraction (ca. 5%) contains a two-G insertion which adds but 2 aa to the polypeptide (W protein). Only one functional domain in the common N-terminal 317 residues of the P, V, and W proteins has been detected by deletion analysis (aa 33–41), that for chaperoning N^0 during the nascent chain assembly step of genome replication (Curran *et al.,* 1995). The three rSeV-C would then express C proteins (and the P protein N^0-chaperone domain) of the H, M, and MVC strains, but their P proteins would contain common Z strain L protein and nucleocapsid binding domains. All three rSeV-C contain the N gene of SeV^H as an electrophoretic marker.

rSeV with alternate C proteins

rSeV-C viruses could be recovered routinely from embryonated chicken eggs when all five plasmids (pGEMs-N, -P, -L, pFL3-Δ1, and one of the three pN/P^{Xho}/M) were cotransfected into vTF7-3-infected A549 cells. Two sepa-

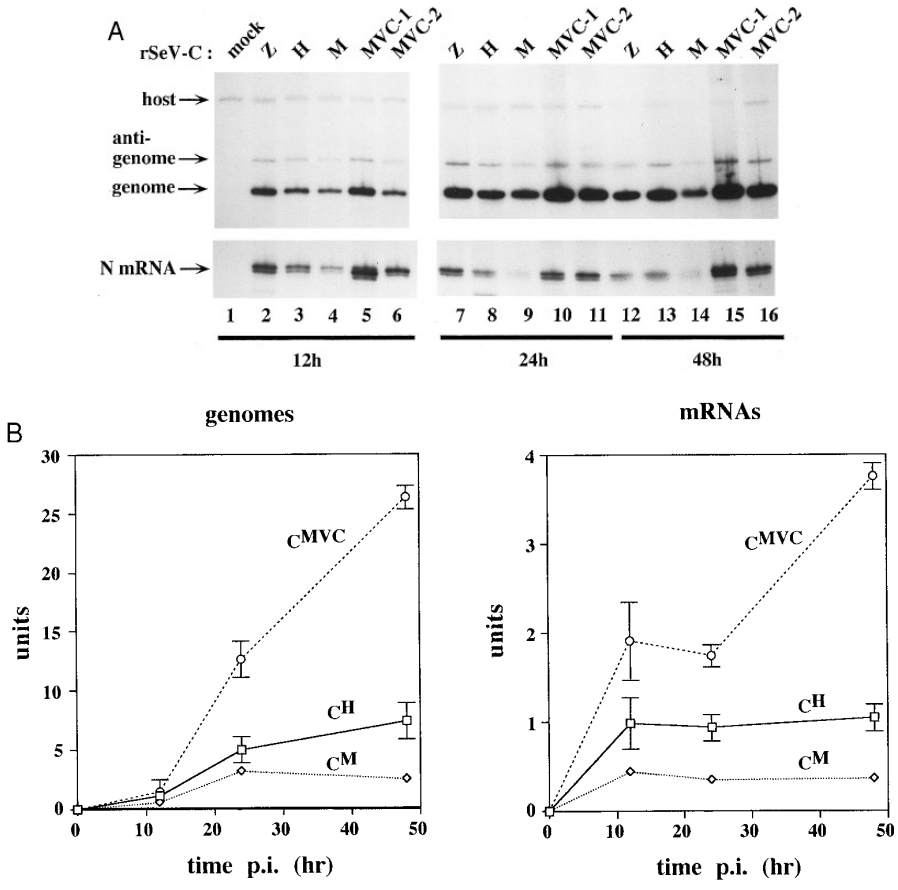

FIG. 4. The kinetics of accumulation of viral RNAs in rSeV-C-infected BHK cells. Parallel BHK cell cultures were infected with 10 PFU/cell of the various rSeV-C as indicated above. Total RNA was prepared at the times indicated below, and their levels of viral genomes, anti-genomes, and N mRNAs were determined by extension of a mixture of primers (Materials and Methods) (A). The intensities of the various bands in A were quantitated in a phosphorimager and these values are plotted in B. The viral RNA levels of the $rSeV^Z$ and rSeV-C^H infections, as well as those of the rSeV-C^{MVC-1} and rSeV-C^{MVC-2} infections, were similar and these are shown as the average (C^H and C^{MVC}, respectively). The bars indicate the range. Note that the scales of genomes and N mRNAs are different.

rate rSeV-C^{MVC} were generated from separate transfections with two independently constructed pN/P^{Xho}/M clones (MVC-1 and -2). In all cases, sufficient virus was present in 35 μl of the second passage allantoic fluid to be visible in Coomassie blue-stained protein gels, indicating that the viruses were generated at similar frequencies. However, there was clearly less rSeV-C^{MVC} present than the two others (not shown). The virus stocks were further passaged in eggs to remove residual vaccinia virus, the viruses were cloned by plaque isolation, and these viruses were found to be genetically stable with respect to the above criteria for at least seven passages. As before, less virus was present in the rSeV-C^{MVC} stocks (ca. 10^8 vs 10^9 PFU/ml for rSeV-C^M and rSeV-C^H), and this is similar to the original M and MVC strains (data not shown).

The relative replication of the original M and MVC viruses in BHK cells is shown for reference in Fig. 3A, in which the level of N mRNA in extracts of cells infected with 10 PFU/cell of two clonal isolates of either virus is estimated by primer extension. The SeV^M-infected cells had accumulated ca. half as much N mRNA as those infected with SeV^Z, whereas SeV^{MVC}-infected cells had accumulated more than twice as much mRNA as the SeV^Z control. These extraordinary levels of mRNA presumably account for their unusual presence in significant amounts in the banded nucleocapsid fractions of CsCl density gradients (lanes NC, Fig. 3A). rSeV-C^{MVC} also clearly grew better than $rSeV^M$ in cell culture, in contrast to their growth in eggs. When parallel cultures of BHK cells were infected with 10 PFU/cell of the respective rSeV-C, considerably more viral protein was present in the rSeV-C^{MVC}-than in the rSeV-C^M-infected cells (lane 2 vs lane 3, and lane 5 vs lane 6, Fig. 3B). Moreover, when cells were co-infected at a m.o.i. of 10 with both rSeV-C^{MVC} and rSeV-C^M, the intracellular level of the viral proteins most closely resembled that of rSeV-C^M-infected cells (lane 7, Fig. 3B), consistent with the dominant nature of the inhibitory effects of C. The amount of virus liberated extracellularly reflected the amounts of viral proteins inside the cells (not shown).

The time course of the accumulation of viral RNAs

TABLE 1

Mouse Pathogenicity of rSeV

Inoculum (CIU/mouse)	rSeV-C			
	H-wt	M	MVC1	MVC2
1.25×10^5	0/5[a]	0/5	5/5	5/5
1.25×10^4	1/5	0/5	5/5	ND[b]
1.25×10^3	5/5	5/5	5/5	ND
1.25×10^2	5/5	5/5	5/5	ND
1.25×10^1	5/5	5/5	ND	ND

[a] Surviving mice/total mice.
[b] ND, not done.

intracellularly is shown in Fig. 4. At 12 h p.i., all the infected cultures contain roughly similar amounts of viral genomes, yet the rSeV-C^{MVC}-infected cultures (shown as the average of the rSeV-$C^{MVC1\&2}$) have accumulated twice as much N mRNA as the reference SeV^Z- or rSeV-C^H-infected cells (which were indistinguishable and are also shown as the average in Fig. 4B). rSeV-C^M-infected cells, on the other hand, had accumulated only half as much mRNA as the SeV^Z/rSeV-C^H-infected cells, such that the rSeV-C^{MVC}-infected cultures had 4 times as much N mRNA as the rSeV-C^M-infected cells at this time. This increased level of mRNA is presumably responsible for the increased levels of genomes and anti-genomes found at later times of infection, when there are 10 times as many rSeV-C^{MVC} vs rSeV-C^M genomes intracellularly. This experiment was repeated 4 times (with virus stocks which were independently generated) with identical results. Since the only known difference between rSeV-C^M and rSeV-C^{MVC} is the substitution of Phe^{170} with Ser (F170S) in the C ORF, we conclude that: (1) as for reconstituted viral DI-genome replication in transfected cells, the C proteins naturally limit viral RNA synthesis in infections of cultured cells and (2) Phe^{170} is critical for this inhibitory function. The reason why rSeV-C^M grows so poorly in cell culture remains unclear.

rSeV-C infections of mice

Sequencing of the M and MVC genomes showed that three nucleotide substitutions had occurred on adapting this strain to grow in cell culture: U^{2362} to C, leading to the F170S change of the C protein; $A^{14,704}$ to C (silent); and $G^{15,173}$ to A, resulting in the substitution of Glu^{2050} of the L protein by Ala (Itoh *et al.,* 1997). To determine whether one or both of these aa substitutions (and possibly also the silent mutation) were responsible for the attenuated virulence of SeV^M and SeV^{MVC} in mice, we examined the virulence of rSeV-C^{MVC} and rSeV-C^M for these animals. All the rSeV-C viruses were each inoculated intranasally into mice. As shown in Table 1, rSeV-C^H was pathogenic for mice as expected, with the LD_{50} corresponding to 8.3×10^3 CIU/mouse. rSeV-C^M was similarly virulent; all the mice inoculated with 1.25×10^4 CIU of rSeV-C^M died within 10 days of infection, and the LD_{50} corresponded to 6.9×10^3 CIU/mouse. On the other hand, all mice infected with 1.25×10^5 CIU/mouse of rSeV-C^{MVC} survived. These latter mice appeared to undergo only mild disease, with a transient loss of body weight.

Virus replication of these rSeV-C viruses in the mouse lungs was compared by determining the levels of infectious virus present in homogenates of these organs (Fig. 5). While the virus titers of rSeV-C^H and rSeV-C^M increased daily until ca. 10^8 CIU were present per lung, that of rSeV-C^{MVC} leveled off after day 1 and then decreased, becoming undetectable at ca. day 7 (Fig. 5). Again, since the only known difference between rSeV-C^{MVC} and rSeV-C^M is a single point mutation which substitutes Ser for Phe^{170} of the C protein (and C^H also contains Phe at this position), it appears that the F170S mutation is also responsible the attenuation of SeV^{MVC} for mice, via the restriction of multiple rounds of virus replication in the mouse respiratory epithelium.

DISCUSSION

Kato *et al.* (1997) have recently described the properties of a rSeV which cannot edit its P gene mRNA and therefore cannot express its V (or W) protein. This rSeV-$edit^{minus}$ induced cytopathic effects earlier and accumulated more mRNA intracellularly than normal SeV in some cell lines. On the other hand, rSeV-$edit^{minus}$ was highly attenuated in infections of mice. rSeV-$edit^{minus}$ appeared to replicate normally in mice for the first day, as virus titers in the lungs increased ca. 1 log relative to the input virus. However, whereas virus titers in mice infected with virulent SeV increased steadily until they reached 10^8 PFU/lung at ca. day 5, the virus titer in the lungs of rSeV-$edit^{minus}$-infected animals leveled off at ca. day 1 and then decreased steadily, until virus could no longer be detected at day 7 or 8. Remarkably, we have found almost the same properties for rSeV-C^{MVC}, a chimera of the virulent strain Z virus in which the C gene (and its overlapping P gene) of the avirulent SeV^{MVC} has been exchanged. Since an analogous exchange with the C gene of SeV^M (nor that of SeV^H) does not confer either the overtranscription phenotype in cell culture nor the avirulence for mice, we conclude that the C protein is responsible for both these traits and that Phe^{170} is critical for this effect.

rSeV-C^{MVC} is thus the second rSeV which is avirulent in mice, and in both cases it has been the disruption of one of the virus' accessory genes that has led to this phenotype. All *Paramyxovirinae* contain at least six genes (or mRNA transcription units), for N, P, M, two surface glycoproteins (F and HN), and L, representing the minimal essential genes of this virus subfamily. Many of the viruses also express accessory proteins (accessory in the sense that these proteins are not expressed by all the viruses of this subfamily), either from overlap-

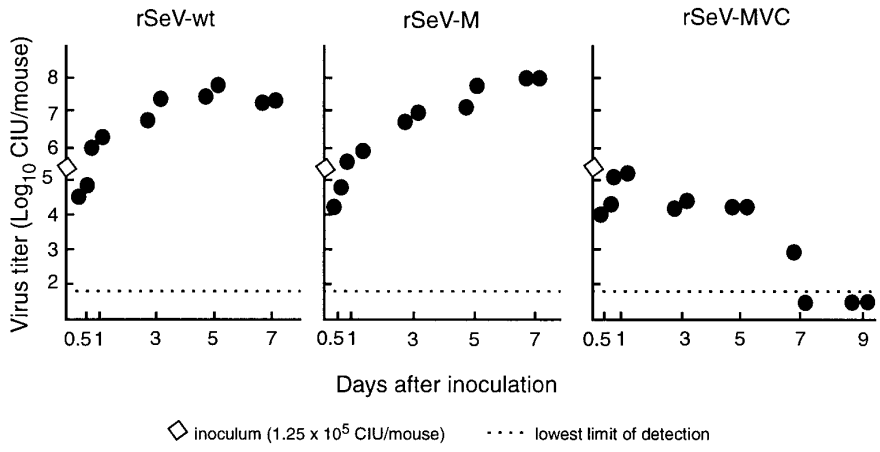

FIG. 5. rSeV-C replication in mice. Mice were inoculated with 1.25×10^5 CIU of rSeV-C^H (rSeV-wt), rSeV-C^M, or rSeV-C^{MVC}. Two mice each were sacrificed on the indicated days, and virus titers in 10% lung homogenates were determined. The dotted line indicates the lowest titer detectable in this assay system.

ping ORFs within the P mRNA or as separate transcription units. In addition to C, which is expressed by all but the rubulaviruses, the V proteins are expressed by all the *Paramyxovirinae* but hPIV1 and hPIV3 (both members of the paramyxovirus genus), the D proteins (extended W proteins, cf. Fig. 1) are expressed only by hPIV3 and bPIV3, and the SH protein is expressed only by some of the rubulaviruses (reviewed in Rochat *et al.,* 1992). Remarkably, the expression of these accessory genes can be quite different even among the most closely related viruses; e.g., SeV (also called murine PIV1) and hPIV1 and bPIV3 and hPIV3 are two pairs of very related viruses, yet SeV and bPIV3 express V proteins (Pelet *et al.,* 1991; Vidal *et al.,* 1990), whereas hPIV1 and hPIV3 do not (Galinski *et al.,* 1992; Matsuoka *et al.,* 1991). These accessory proteins presumably carry out specialized functions required for individual viruses.

It now appears that the accessory V and C proteins, both initially characterized as negative effectors of viral RNA synthesis in cell culture (Curran *et al.,* 1991, 1992), are both required for multiple cycles of infection in the mouse respiratory epithelium. It is unlikely that these essentially nonstructural P gene products play a role in virus entry or release, as rSeV with impaired V and C function grow very well in cell culture, and virus titers in the mouse lungs increase normally for the first day. Rather, Sendai viruses which cannot express either a V protein or a functional C protein appear to be particularly sensitive to the anti-viral defenses of the mouse. The containment of virus burden in these animals in the absence of V or C protein function is apparent as early as day 2 of the infection, and because of this timing, some aspect(s) of innate immunity is presumably playing a role in this containment and/or virus clearance, as previously suggested (Kato *et al.,* 1997, Nathan, 1995). Two aspects of innate immunity which could be important here are natural killer cells, which are thought to recognize general signatures of infectious pathogens, and interferons, which recognize general signatures specifically of viral infections. Protection of mice from lethal SeV pneumonia by low doses of anti-CD3 antibodies has been found to occur through activation of NK cells (Kast *et al.,* 1990), and induction of interferon in mouse lungs is a very early response to SeV infection (Robinson *et al.,* 1968). The accessory V and C proteins thus could well exert their effects on different arms of innate immunity, in keeping with their different primary sequences. On the other hand, rSeV missing either V or C function are associated with overtranscription in cell culture infections. If this is also so in infections of the mouse respiratory tract, it is not impossible that avirulence for both rSeV is due to unrestrained viral gene expression, which alerts the host defenses prematurely, leading to a more effective anti-viral response. It will be of interest to determine whether this link between overtranscription in cell culture infections and avirulence in mice is more than fortuitous.

Finally, we note that, in contrast to measles virus and vesicular stomatitis virus (Radecke and Billeter, 1996; Kretschmar *et al.,* 1996), we have been unable to prepare a rSeV without the ability to express any of its C proteins (T. Cadd, Latorre, and J. Curran, unpublished). Viruses which cannot express C and/or C′ are, however, viable, and their properties are under investigation.

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