The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis

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The Sendai virus (SeV) V protein is characterized by the unique cysteine-rich domain in its carboxy-terminal half which is fused to the amino-terminal half of the P protein, but its function has remained enigmatic. The V protein-directing mRNA is generated by a remarkable process known as mRNA editing involving the pseudotemplated addition of a single G residue at a specific septinucleotide locus in the P gene, whereas the unedited exact copy encodes the P protein. Here, we introduced two nucleotide changes in the septinucleotide motif (UUUCCCC to UUCUCC) in a full-length SeV cDNA and were able to recover a virus from the cDNA, which was devoid of mRNA editing and hence unable to synthesize the V protein. Compared with the parental wild-type virus with regard to gene expression, replication and cytopathogenicity in various cell lines *in vitro*, the V(–) virus was found to be either potentiated or comparable but never attenuated. The V(–) virus, however, showed markedly attenuated *in vivo* replication capacity in and pathogenicity for mice. Thus, though categorized as a non-essential gene product, SeV V protein encodes a luxury function required for *in vivo* pathogenicity.

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Introduction

The non-segmented negative strand RNA genome characterizes the viruses in the superfamily Mononegavirales which comprises three families; Rhabdoviridae, Paramyxoviridae and Filoviridae. Their genomes are organized starting with a short 3'-leader region, followed by 5–10 genes, and ending with the short 5'-trailer region. They are tightly associated with nucleocapsid (N) protein subunits, forming helical ribonucleoprotein complexes (RNP). The RNP but not the naked RNA genome is the template for both transcription and replication, in contrast to positive strand RNA viruses whose naked RNA genome can serve as the functional genome and as mRNA. There is only a single promoter for the RNA polymerase at the 3'-end. By recognizing the stop (termination/polyadenylation) and restart signals, the polymerase gives rise to leader RNA and each mRNA. After translation of these mRNAs

and accumulation of the translation products, genome replication begins. Here, the same polymerase copies the same RNP template, but now ignores the successive stop signals for the leader RNA and mRNAs by an unknown mechanism to generate a full-length antigenomic RNP (for review, see Lamb and Kolakofsky, 1996).

Mononegavirus gene expression is generally monocistronic, each mRNA usually directing a single primary translation product. However, the P gene of the subfamily Paramyxovirinae in the Paramyxoviridae is a notable exception, because it gives rise to multiple protein species by means of overlapping frames and by a remarkable process known as RNA editing or pseudotemplated addition of nucleotides (for review, see Lamb and Kolakofsky, 1996). The P gene is the second proximal to the 3'-terminus in these viruses. RNA editing to insert pseudotemplated G residues is found for all the three paramyxovirus genera. It is a virus-specific event which takes place co-transcriptionally by reiterative copying of the short C stretch at a specific region on the genome template (Vidal *et al.*, 1990a). The septinucleotide consensus motif (3'-UUU/CUCCC-5') including the C stretch has been proposed to be a requirement for editing (Park and Krystal, 1992). A 'stuttering' model proposes that the polymerase pauses at this site and, when the pause is sufficiently long, slippage of the nascent mRNA occurs by one or two nucleotides, thereby reiteratively inserting one or two Gs (Thomas *et al.*, 1988; Vidal *et al.*, 1990b). For Sendai virus (SeV) (Figure 1), bovine parainfluenza virus type 3 (genus *Paramyxovirus*) and measles virus (*Morbillivirus*), the unedited mRNA that is the exact copy of the P gene encodes the P (phospho) protein, while the addition of one G residue produces an mRNA that encodes the V protein (Vidal *et al.*, 1990a; Galinski *et al.*, 1992). The edited version (with insertion of one or two G residues) encodes the P protein and the unedited version the V protein for the genus *Rubulavirus* including SV5 (Thomas *et al.*, 1988) and mumps virus (Paterson and Lamb, 1990). In either case, the P and V proteins are amino-co-terminal, while the –1 or –2 frame is used to generate the carboxy-terminal half from the edited mRNA. The P mRNA of SeV and other paramyxo- and morbilliviruses but not rubulaviruses further encodes the C protein in an overlapping frame at the 5'-end of the mRNA independently of editing status (Figure 1). SeV C protein seems to down-regulate viral genome amplification in a promoter-specific fashion (Cadd *et al.*, 1996), while that of measles virus did not affect the viral phenotype (Radecke and Billeter, 1996). The same frame of SeV additionally generates three other proteins termed C', Y1 and Y2 by using initiation codons at different positions, one of which is non-AUG (Curran and Kolakofsky, 1988). Furthermore, insertion of two Gs at the editing site also occurs with a much lower frequency (Vidal *et al.*, 1990a,b), which

Fig. 1. SeV P gene expression and introduction of mutations into the editing locus. The unedited version of the wild-type P gene transcript encodes the P protein. RNA editing with one non-templated G insertion at the editing site (+1G) produces an mRNA encoding the V protein, which is a fusion of the N-terminal portion of the P reading frame with the second (-1) reading frame (hatched rectangle). Insertion of the two Gs (+2G) generates the mRNA encoding the W protein. The W protein frame terminates immediately after the insertion. The carboxy-terminal portion of the V protein is cysteine rich (Curran *et al.*, 1991b; Pelet *et al.*, 1991), while that of P protein has the binding domains with L and N proteins as well as the trimerization site (Ryan *et al.*, 1991; Smallwood *et al.*, 1994). The P mRNA further encodes the C protein with the third (-2) reading frame at the 5'-end independently of editing status. The septinucleotide motif (UUUUCCC) is thought to be critical for these non-templated G insertions. Here, two mutations (U to C, C to U) were introduced (for details, see Materials and methods). The viruses recovered from the wild-type and mutated cDNAs were examined for actual frequencies of G insertions as described in Materials and methods. The two nucleotide exchanges were sufficient to reduce the frequency of one G insertion to an undetectable level (0/20), while the frequency was 25% (5/20) for the wild-type. No double insertions were detected (0/20) for not only the mutant but also the wild-type virus.

immediately creates a stop codon and gives rise to the W protein (Figure 1).

Of the distinct P gene products, the V protein is of particular interest because of its almost universal conservation in all three genera of Paramyxovirinae. To date, human parainfluenza virus type 1 seems to be the only exception; it has neither the consensus editing motif nor the V open reading frame (Matsuoka *et al.*, 1991; Power *et al.*, 1992; Rochat *et al.*, 1992; Tao and Ryan, 1996). The V protein is structurally characterized by a highly conserved cysteine-rich domain in its carboxy-terminal half which is fused to the amino-terminal half of the P protein (Figure 1). The cysteine-rich domain of the measles and Newcastle disease virus P protein can bind zinc (Liston and Briedis, 1994; Steward *et al.*, 1995), although its functional significance is unclear. The V proteins of SeV and measles virus are not present in virions and are not associated with RNP, probably because these V proteins are missing the trimerization site and L and NP binding sites, all of which are localized to the carboxy-terminal half of the P protein (Curran *et al.*, 1991a,b; Wardrop and Briedis, 1991; Smallwood *et al.*, 1994; Harty and Palese, 1995) (Figure 1). On the other hand, the V proteins of rubulaviruses appear to be present in virions (Paterson *et al.*, 1995). Moreover, unlike paramyxo- and morbilliviruses, their amino-terminal halves are predicted to be basic rather than acidic in character.

The function of V protein has been studied by using SeV DI (defective interfering) particles as a genome analog. The V protein was found to inhibit DI genome replication (Curran *et al.*, 1991a), possibly interfering with the RNA encapsidation step (Curran *et al.*, 1994), but did not appear to affect viral mRNA synthesis (Curran *et al.*, 1994). However, nothing is known about the role of V

protein in the context of replication of the full-length SeV genome in cells or the viral pathogenicity *in vivo*. It is not even known whether the V protein is essential or non-essential for virus replication. On the other hand, paramyxovirus P protein is essential and thought to be a modulator that plays a central role in both transcription and replication. Together with the L (large) protein, it forms the viral RNA polymerase (Hamaguchi *et al.*, 1983).

We recently established a system that allows efficient recovery of SeV from transfected cDNA. Here, using this system, we generated a V protein-negative [V(-)] virus by introducing mutations into the septinucleotide motif in the editing locus, proving the genuine role of the septinucleotide motif for SeV mRNA editing and establishing that the V is a non-essential protein for virus replication. We then examined what roles the V protein plays not only in viral gene expression and replication at a cellular level but also in *in vivo* replication and pathogenesis. The V(-) virus often displayed markedly increased gene expression and cytopathogenicity at the cellular level *in vitro*, but was strongly attenuated in pathogenicity for mice, indicating that V protein markedly influences both *in vitro* and *in vivo* phenotypes of SeV.

Results

Creation of SeV V(-) virus

The plasmid pSeV(+) generates full-length positive sense (+) RNA of SeV antigenome in transfected LLCMK2 cells by vaccinia virus (VV)-driven T7 polymerase (pol). From this cDNA, we were able to recover infectious SeV at high efficiency with the support of N, P and L proteins which were also expressed by T7 pol from the respective pGEM plasmids co-transfected with pSeV(+) (Kato *et al.*,

Table I. Infectious virus recovery from the wild-type and mutant pSeV(+)^a

Transfectant	Amplification		
	Inoculum size (No. of cells)	Virus yield	
		h.a.u.	p.f.u./ml
Wild-type pSeV(+)	10 ⁴	<2, <2	<10, <10
	10 ⁵	256, 512* (512)	3×10 ⁸ , 1×10 ⁹ * (2×10 ⁹)
Mutant pSeV(+)	10 ⁶	512, 512	1×10 ⁹ , 2×10 ⁹
	10 ⁴	<2, <2	<10, <10
	10 ⁵	256, 512* (512)	5×10 ⁸ , 1×10 ⁹ * (9×10 ⁸)
	10 ⁶	512, 512	1×10 ⁹ , 2×10 ⁹
	10 ⁷	512	1×10 ⁹

^aSeV multiplication was initiated from the wild-type pSeV(+) or the mutant pSeV(+) with two nucleotide exchanges in the mRNA editing site (see Figure 1), that was transfected into LLCMK2 cells as described in Materials and methods (for details, see Kato *et al.*, 1996). Transfected LLCMK2 cells were inoculated into chicken eggs after three cycles of freezing and thawing, and virus yields were determined after a 72 h incubation. Two eggs were used for each inoculum size, and titers in each egg are shown. The viruses amplified in one (*) of the eggs in each transfection–recovery experiment were once again propagated in several eggs at 10⁻⁷ dilution to eliminate helper VV, and the allantoic fluids were pooled and used as the stock virus. Their titers are shown in parentheses.

1996). This recovery system begins with encapsidation of the synthesized full-length (+) RNA with the N protein subunits to form the ribonucleoprotein complex (RNP) followed by initiation of the replication cycle using the RNP as the template and the *trans*-supplied polymerase (L-P). Once replication is initiated, the recovery process enters the normal virus life cycle in the transfected cells, ultimately generating mature infectious progeny.

Disruption of mRNA editing would be the only possible way to totally knock out V protein expression without affecting the P protein frame because the P and V proteins are amino-co-terminal. For this, we introduced two mutations into the consensus septinucleotide locus, where the polymerase is thought to stutter and co-transcriptionally insert G residues (Figure 1), as described in Materials and methods. We expected to recover a mutant, which would be unable to generate the edited V mRNA. To avoid reversion to the wild-type by recombination with the supporting plasmid, pGEM-P, the same mutations were introduced into pGEM-P. Another plausible approach might have been to introduce a stop codon in the V frame immediately after the editing site and thereby to delete the carboxy-terminal half of the V protein. This, however, was not done because it would result in overproduction of a molecule analogous to W protein whose copy number is quite low in the normal virus life cycle and whose function is unknown.

Remarkably, the rate of virus recovery from the mutated pSeV(+) was identical to that from the parental pSeV(+), yielding a full virus titer in embryonated chicken eggs following injection with ~10⁵ freeze-thawed transfected cells (Table I). These recovered viruses once again were propagated in eggs at a sufficiently high dilution (10⁻⁷) to eliminate the helper VV present at 10³–10⁴ plaque-forming unit (p.f.u.)/ml (Kato *et al.*, 1996), and used as stocks for

Fig. 2. Absence of V protein synthesis in CV1 cells infected with the mutant virus. The lysates of CV1 cells infected with the wild-type or mutant virus were analyzed by Western blotting with specific antisera against P, V and C proteins at various hours p.i. as indicated at the top of each lane. The V protein was undetectable with either anti-V or anti-P serum in the mutant-infected cells.

all subsequent experiments (Table I). That the introduced mutations were retained was confirmed by nucleotide sequencing of the mutant stock virus genome.

CV1 cells, a monkey kidney cell line, were infected with the mutant or the parental (wild-type) virus. The RNA was extracted from these cells and subjected to reverse transcription and PCR with a pair of primers which were designed to amplify the 1843–3556 nucleotide region of the P gene transcripts containing the inserted G residues (see Materials and methods). After cloning, the amplified sequences were determined. Twenty independent clones derived from the mutant- or wild-type-infected cells were used for sequence determination. A non-templated single G insertion at the expected position was found for 25% (5/20) of clones from the wild-type-infected cells (Figure 1). No double G insertions were detected, indicating that the W mRNA generation is indeed infrequent. On the other hand, all 20 clones from the mutant-infected cells were faithful copies of the P gene sequence without any G insertions, indicating that P mRNA editing did not occur or was suppressed to an undetectable level (Figure 1). CV1 cells infected with the mutant or the wild-type virus were then examined for P gene products by Western blotting with highly specific antibodies. The mutant was found to synthesize no detectable V protein, whereas P and C proteins were clearly detected (Figure 2). All these proteins were detectable in the wild-type-infected cells. However, the signals of P and C proteins were much weaker than those of the mutant (Figure 2). This suggested augmented gene expression by the mutant, and is discussed below in detail.

These findings indicated that the two nucleotide exchanges in the septinucleotide motif are sufficient to disrupt SeV P mRNA editing to generate V mRNA, and hence to abrogate V protein synthesis. Hereafter, we will refer to this mutant as V(-) virus. The high recovery rate of V(-) virus from cDNA and its high replication capability in eggs indicate that the V protein is not essential for SeV replication.

Fig. 3. Replication of the wild-type and V(-) viruses in CV1 cells under single (m.o.i. 10 p.f.u./cell) or multiple (m.o.i. 0.01 p.f.u./cell) cycle growth conditions (A), cytopathogenicity at 25 and 38 h p.i. under single cycle growth conditions (B) and plaque formation by these viruses in the same cells (C). In (A), virus titers are shown as p.f.u./ml (lines) and h.a.u. (bars) of the culture supernatants.

Gene expression, replication and cytopathogenicity of V(-) virus *in vitro*

We then compared the replication kinetics of V(-) virus in CV1 cells with that of the wild-type virus under the single cycle replication conditions with an input multiplicity of infection (m.o.i.) of 10 p.f.u./cell. The V(-) virus exhibited slightly faster kinetics than the wild-type. The V(-) virus titer was constantly higher by 10-fold or more than that of the wild-type at 6 and 14 h post-infection (p.i.). However, both showed comparable titers later at 26 and 38 h p.i. (Figure 3A). V(-) virus consistently showed much more severe cytopathogenicity than the wild-type (Figure 3B). Under multiple round replication conditions with an input m.o.i. of 0.01 p.f.u./cell, V(-) virus also exhibited slightly faster kinetics but its final titer was again comparable with that of the wild-type (Figure 3A). The V(-) and wild-type viruses showed almost the same plating efficiencies (Table I), their p.f.u. to hemagglutination units (h.a.u.) ratios being $\sim 4 \times 10^6$ and $\sim 2 \times 10^6$, respectively. However, they differed markedly in plaque morphology; the V(-) virus produced clearly edged plaques while those of the wild-type were turbid (Figure 3C).

The level of N and HN protein synthesis as detected by Western blotting was much higher in V(-) virus-infected cells throughout the observation period (Figure 4). This increased protein synthesis was already noted for P and C proteins (Figure 2) and further found for all other viral proteins (data not shown), except for the L protein whose generally low level of expression made comparison difficult. The augmented protein synthesis of V(-) virus was obviously due to increased gene expression as demonstrated by Northern blotting with an N mRNA-specific probe and a probe to detect all mRNA species (Figure 4). The 50S antigenomic and genomic RNAs were clearly detected by Northern blotting only late in infection and were comparable between the V(-) and wild-type viruses

Fig. 4. Gene expression and genome replication of the wild-type and V(-) viruses in CV1 cells. Infection was initiated at an m.o.i. of 10 p.f.u./cell. At various hours p.i. as indicated on the top of each lane, the gene expression was studied by Western blotting with polyclonal anti-SeV rabbit serum and Northern blotting with a negative sense viral RNA probe synthesized *in vitro*. Replication was studied by both Northern blotting and RT-PCR. To detect 50S antigenomic (+) RNA and 50S genome (-) RNA separately, antisense and sense probes were used, respectively. Two primer pairs to amplify the leader-N or the L-trailer region were used to detect the antigenomic or genomic RNA as described in Materials and methods.

Fig. 5. Moderation of cytopathogenicity of V(-) virus for CV1 cells by co-infection with the wild-type or by *trans*-supplied V protein from the pGEM plasmid. Infections were initiated with the V(-) virus at an m.o.i. of 10 p.f.u./cell. Co-infection with the wild-type was also performed at the same m.o.i. The pGEM plasmid encoding V or P protein was transfected and driven by T7 pol from the adenovirus vector. Photographs were taken 38 h p.i. For details, see Materials and methods.

(Figure 4). This is consistent with the observations that their maximum virus titers were similar to each other (Figure 3A). In earlier stages at 14 h p.i., however, both antigenomic and genomic RNA levels appeared to be higher in V(-) virus-infected cells than in wild-type-infected cells, as revealed by semi-quantitative PCR analysis (Figure 4). This is also consistent with the observation that V(-) virus replicated more efficiently in these stages than the wild-type virus (Figure 3A). These results suggest that SeV gene expression as well as the gene replication are augmented in CV1 cells in the absence of V protein synthesis. This appears to be correlated with the severe cytopathic effect and clear plaque morphology of the V(-) virus.

The disruption of mRNA editing not only resulted in the failure of V mRNA and V protein generation but could also lead to the elevation in P mRNA level. Assuming that V mRNA accounts for 25% of the total transcripts from the P gene in the normal life cycle of SeV in CV1 cells (Figure 1), the disruption of editing could increase the P mRNA level by ~1.3-fold. It has not been ruled out that this small increase in P mRNA and hence P protein rather than the absence of V protein could be the cause of the above-described phenotypes unique to V(-). However, no increased cytopathogenicity (Figure 5) nor elevated gene expression (data not shown) was seen in CV1 cells doubly infected with V(-) and wild-type viruses in which the increased P mRNA level was calculated to be 1.17-fold. This dominance of wild-type phenotype in doubly infected cells seems to be better explained by the complementation (supply of V protein) by the wild-type than the presumably small change in P mRNA level (from 1.3- to 1.17-fold). Cytopathogenicity of V(-) virus was also significantly moderated when V protein but not P protein was supplied *in trans* from the transfected plasmid (Figure 5). The V and P genes in transfected pGEM plasmids were driven by T7 pol expressed by a non-cytopathic adenovirus vector. Here, transfection was performed with another lipofection reagent (DOSPER), and its efficiency was satisfactorily high (no less than 30%) but not 100%, while 100% of cells were infected by V(-) virus. Therefore, abrogation of increased cytopathogenicity was not complete. From these observations, it appeared highly likely that the V(-) phenotypes were indeed caused by the absence of V protein.

Cell type dependency of V(-) virus phenotypes

Similar comparative studies of the phenotypes between the V(-) and wild-type viruses were carried out with several other cell lines of various origins. Remarkably, the phenotype displayed by V deletion was strongly cell type dependent. Augmented gene expression, which was readily demonstrable by Western blotting, and stronger cytopathogenicity of the V(-) virus were found with LLCMK2 and Vero cells, lines of non-human primate origin. On the other hand, V(-) and wild-type viruses manifested similar phenotypes in mouse fibroblastic L, rabbit kidney RK13 and canine kidney MDCK lines. Representative infection and Western blotting data obtained for LLCMK2 and L cells are shown in Figure 6. However, no cell type has been found in which the V(-) virus displays an attenuated phenotype with respect to gene expression, replication or cytopathogenicity. These results demonstrated that, although non-essential, SeV V protein strongly affects the viral phenotypes in a cell type-dependent manner, and thus probably interacts with a cellular factor(s).

In vivo replication and pathogenicity of V(-) virus

SeV causes pneumonia in the natural host, mice. To determine the role of V protein in SeV replication in and pathogenicity for mice, we examined the ability of the V(-) virus to replicate and produce lesions in the target organ, the lungs. The effects of infection on the body weight were also examined because this is a good indicator of SeV pathogenicity (Kiyotani *et al.*, 1990). Figure 7 summarizes the results obtained following intranasal inoculation with 10^7 p.f.u. of V(-) or wild-type virus. The wild-type virus significantly disturbed body weight gain and killed all mice by 9 days p.i. The infected lungs manifested severe pathological changes indicated by a rapid increase in and a high maximum value of consolidation scores. Macroscopic views corresponding to the scores from 0 (no consolidation) to 4 (maximum) are presented for reference (Figure 8A). When the mice died, one point was added (score 5) (see Materials and methods). In marked contrast, V(-) virus-infected mice all survived up to 9 days. Their body weight increased almost normally, and their consolidation scores were minimal or moderate throughout. The V(-) virus was thus significantly attenuated.

Fig. 6. Replication and gene expression of the wild-type and V(-) viruses in LLCMK2 and L cells. Cells were infected at an m.o.i. of 10 p.f.u./cell and harvested at various hours p.i. as indicated on the top of each lane. The viral proteins were analyzed by Western blotting with anti-SeV polyclonal antibody as in Figure 4 (top). Virus titers in the culture supernatants are shown as p.f.u./ml (lines) and h.a.u. (bars) (bottom).

Fig. 7. Replication of the wild-type and V(-) viruses in mice and their pathogenicity. Infections were initiated by intranasal inoculation of 10^7 p.f.u. At appropriate time points, five mice were weighed (left), and the consolidation scores (center) and virus titers in the lungs (right) were determined. When mice died, one point was added to the consolidation score (score 5). The dead mice are marked by x.

The virus titers in each lung in each experimental group were determined. The wild-type and V(-) viruses reached comparable titers on day 1 p.i. Thereafter, the V(-) virus titer sharply declined, while the wild-type continued to grow for an additional day and maintained a high titer throughout (Figure 7). We confirmed that the introduced mutations were retained correctly in the V(-) viruses recovered from the lungs. SeV targets the airway epithelium. Reflecting the above differences in replication kinetics, the spread of wild-type virus and accumulation of the viral antigens were extensive in the epithelium on day 2, while those of V(-) virus remained at the level of 1 day p.i. (Figure 8B). It was remarkable that the V(-) virus was largely cleared out of the body by day 7 p.i. and complete clearance was achieved by day 9 (Figure 7). There was thus a strong correlation between the attenuated phenotype of V(-) virus and its inability to maintain a high titer in the lungs. However, as the V(-) virus replicated as efficiently as the wild-type, at least on the initial day of infection, its replication potential did

not appear to have been attenuated. Rather, defense mechanisms were probably soon recruited to limit further replication of V(-) virus.

Discussion

Our system to recover infectious SeV from transfected cDNA allowed us to create a V(-) virus by introducing mutations into the genomic locus which has been suggested to be critical for the reiterative insertion of a non-templated G residue to generate the edited mRNA encoding the V protein. Previous studies with a synthetic SeV minigenome expressing chloramphenicol acetyltransferase demonstrated that a 24 nucleotide sequence spanning the G insertion site was sufficient for editing (Park and Krystal, 1992). Our present study demonstrated that only two nucleotide exchanges, one at the insertion site (C stretch) and the other in the U stretch three nucleotides upstream of the insertion site, were sufficient to disrupt editing completely (Figure 1). This provides strong support for the

Fig. 8. Lesions and viral antigens in the lung. Actual macroscopic views of the lung corresponding to the consolidation scores 0 (no lesion) to 4 (maximum) are presented for reference (A). The infected lungs were also analyzed immunohistochemically with polyclonal anti-SeV serum, and the staining patterns on days 1 and 2 p.i. are presented (B).

importance of the septinucleotide motif (UUU/CUCCC) conserved at the editing locus of paramyxoviruses. Previous studies showed that, during normal SeV infection, 20–31% of P gene transcripts had a single G insertion, while 5–7% had multiple G insertions (Vidal *et al.*, 1990a; Pelet *et al.*, 1991). In good agreement with this, in our study five (25%) had a single G insertion and no multiple G insertions were detected in the 20 independent PCR clones with wild-type virus infection.

V(-) virus was recovered from cDNA as efficiently as the wild-type, and the recovered V(-) virus replicated as efficiently as or even better than the wild-type in cells in culture and in eggs (Table I), indicating that V protein is non-essential. One remarkable finding was that V(-) virus displayed greatly increased gene expression combined with much more severe cytopathogenicity in some cell lines (Figures 3, 4 and 6). The virus replication monitored by infectious virus production as well as by genome replication was also augmented in V(-) virus-infected cells at least early in infection. Later, virus titers and replicated genome amounts became comparable between the wild-type and V(-) viruses, while higher levels of gene expression of the latter were maintained. This selectivity at the late stage is difficult to explain, but the V protein may somehow differentially recognize and modulate the transcription and replication processes. However, as no selectivity was observed in earlier stages, it is also likely that the replication process was more strongly affected than the transcription process by damaged cellular metabolism due to severe cytopathogenicity late in V(-) virus infection. The dependency of transcription augmentation

by V deletion on cell type (Figure 6) suggests that V protein works along with cellular factor(s). This is interesting in view of previous observations suggesting a requirement for cellular factors for transcription and replication of mononegaviruses including SeV (Moyer *et al.*, 1986). It was shown previously that *trans*-supplied V protein was inhibitory to SeV DI genome replication but did not affect transcription (Curran *et al.*, 1991a, 1994). This conclusion, which contradicts ours, was reached mainly using an *in vitro* assay system with extracts from cells infected with SeV DI and transfected with the V protein expression plasmid, differing greatly from our present system.

The V(-) virus transcription, replication and cytopathogenicity were either potentiated or unaltered, but never appeared to be attenuated at a cellular level. Thus, its greatly reduced pathogenicity in mice was unexpected. This indicates that although V protein is a non-essential gene product, its auxiliary effect *in vivo* is marked. This is reminiscent of accessory genes such as *nef* of simian immunodeficiency virus type 1 which is sometimes unnecessary for the virus replication *in vitro* but is essential for *in vivo* pathogenicity (Kestler *et al.*, 1991). The same is true for some dispensable genes in cell culture of herpes simplex virus (Roizman and Sears, 1996). At present, we have no answer to the question of why V deletion ultimately leads to attenuation of *in vivo* pathogenicity. Virus titration in the lung revealed that the V(-) virus replicated as efficiently as the wild-type at least for 1 day at the beginning of infection (Figure 7). Thereafter, the V(-) virus titer rapidly declined. In contrast, the wild-type virus continued to grow for an additional day to reach the

peak titer and then maintained a high titer until it killed the mice. These results suggested that infected mice were able to clear the V(-) virus but not the wild-type. Interferons and natural killer cells are known to be generally important for host defense early in virus infection, while cytotoxic T lymphocytes play a major role late in infection. The V(-) virus could induce these early host defense mechanisms more strongly than the wild-type. If gene expression is also potentiated by V deletion in mice early in infection, the increased gene products could have contributed to stimulating neighboring cells to recruit early defense mechanisms (Ito *et al.*, 1978; Ito and Hosaka, 1983). Unfortunately, however, histochemical and other procedures were not sensitive enough to study this possibility. Even if gene expression is not augmented, intracellular viral RNAs may somehow be rendered more capable of induction of interferons by the absence of V protein. In this context, it may be noteworthy that interferon activity in V(-) virus-infected lungs was elevated by several-fold on day 1 p.i. compared with the wild-type infection (data not shown).

In summary, our present study illustrated the usefulness of SeV engineering in evaluating the role of an auxiliary gene product in determining the viral phenotype *in vivo* as well as *in vitro*. Of extreme importance is that it allows analysis of the significance of mutations of interest in the context of not only virus replication in cells in culture but also *in vivo* replication and pathogenicity. It should also be noted that even *in vitro* the function of a non-essential gene has to be studied with many different cell types, because the results obtained with a single cell type could lead to incorrect conclusions. This is also reminiscent of investigations on the auxiliary genes of human and other primate lentiviruses.

Materials and methods

Cell cultures and virus infection

Monkey kidney-derived cell lines LLCMK2, CV1 and Vero cells, the rabbit kidney-derived line RK13, mouse connective tissue-derived line L, and canine kidney-derived line MDCK cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Monolayer cultures of these cells were infected with the cDNA-derived parental Z strain or the V(-) virus at an input m.o.i. of 10 p.f.u./cell unless otherwise noted, and maintained in serum-free MEM.

Mutagenesis of full-length SeV cDNA

The plasmid pSeV(+) contained the cDNA copy of the full-length SeV antigenome (Kato *et al.*, 1996). Two nucleotide changes were introduced into the editing locus (UUUUUCCC to UUcUUuCC) in the P gene of pSeV(+) (see also Figure 1). For this, a pair of primers, PF (5'-²⁷⁵⁸CCCGGGTCTAGAGACCGACTCAACAAAgAAAGGCATAGG-AGA²⁸⁰¹-3'), where the lower case letters represent the mutations, and PR (5'-³⁵⁵⁶CCCGGGATCTAGTTGGTTCAGT³⁵³⁶-3'), were synthesized. The nucleotide numbering is according to Shioda *et al.* (1983). The mutations were expected to be silent with respect to the P open reading frame. Using these primers, PCR was performed with the template, pSeV(+), for 20 cycles of 92°C, 1 min, 55°C, 2 min and 74°C, 3 min. The amplified fragments were digested with *Sma*I, and inserted into *Sma*I-digested pSeV(+). The same mutations were introduced into pGEM-P plasmid by the same procedure. Before use, these plasmids were purified by CsCl centrifugation.

Recovery of viruses from cDNAs

Viruses were recovered from cDNAs essentially according to the previously described procedures (Kato *et al.*, 1996). Briefly, 1.2×10^7 of LLCMK2 cells were infected with vaccinia virus (VV) vTF7-3, a gift from Dr B. Moss (Fuerst *et al.*, 1986), at 2 p.f.u./cell. Then, 60 µg of

pSeV(+) or the mutated pSeV(+) and the plasmids encoding *trans*-acting proteins, pGEM-N (24 µg) and pGEM-P, or the mutated pGEM-P (see above) (12 µg) and pGEM-L (24 µg) were transfected simultaneously with the aid of the lipofection reagent DOTAP (Boehringer-Mannheim). The cells were maintained in serum-free MEM in the presence of 40 µg/ml araC and 100 µg/ml rifampicin to minimize VV cytopathogenicity and thereby maximize the recovery rate (Kato *et al.*, 1996). At 40 h after transfection, cells were harvested, disrupted by three cycles of freezing and thawing and inoculated into 10-day-old embryonated chicken eggs. After 3 days of incubation, the allantoic fluid was harvested. The titers of recovered viruses were expressed in h.a.u. and p.f.u./ml as described previously (Kato *et al.*, 1996). The allantoic fluid of the eggs contained 10^8 – 10^9 p.f.u./ml of the recovered viruses along with the helper VV, vTF7-3, at 10^3 – 10^4 p.f.u./ml. The latter was eliminated by the second propagation in eggs at a dilution of 10^{-7} . Complete elimination was ascertained by the absolute lack of VV antigen-expressing cells following infection of LLCMK2 by the undiluted egg fluid of the second passage. This second passage was thus used as the stock virus for all the experiments.

Identification of the mutations and determination of the frequency of non-templated G insertions

Total RNAs extracted from infected CV1 cells at 26 h p.i. were reverse-transcribed using oligo(dT) primer and Superscript (Gibco-BRL, Gaithersburg) at 42°C for 90 min according to the manufacturer's recommendations. The cDNAs were amplified by PCR using P1 (5'-¹⁸⁴³ATGGATCAAGATGCCTTC¹⁸⁶⁴-3') and PR (see above) primers. Amplified cDNA fragments were digested with *Sma*I and *Eco*RI and ligated into pGEM-4Z (Promega, Madison) digested with the same enzymes. The cloned plasmids from 20 independent colonies were sequenced with the reverse primer using an AFLII automated DNA sequencer (Pharmacia, Uppsala) and examined for the presence or absence of G insertions.

Antibody preparation and Western blotting

For the production of P- and C-specific antigens, the viral-specific sequences derived from the regions of *Nar*I¹⁸⁹⁴-*Sma*I³⁵⁵³ (P) and *Nar*I¹⁸⁹⁴-*Sma*I²⁷⁶¹ (C) were inserted into the pQE vector (Quagen, Chatsworth) to be fused to the His×6 tag. The recombinant proteins were produced in *Escherichia coli* and purified according to the manufacturer's protocol. After dialysis against phosphate-buffered saline (PBS), the respective recombinant proteins were injected into rabbits three times at intervals of 1 or 2 weeks, and anti-P and anti-C sera were obtained. The anti-P serum reacted with both P and V proteins. Anti-V serum was a gift from Dr D. Kolakofsky, Geneva (Curran *et al.*, 1991a). Anti-SeV rabbit polyclonal serum was described previously (Kato *et al.*, 1996). For Western blotting with these sera, infected cell lysates were electrophoresed in 12.5% SDS-polyacrylamide gels (Laemmli, 1970). The proteins in the gels were electrotransferred onto PVDF membranes (Millipore, Bedford) and probed with the above specific antibodies as reported previously (Kato *et al.*, 1995).

Northern hybridization and semi-quantitative RT-PCR

RNAs were extracted by the guanidine isothiocyanate method from ~ 10^6 CV1 cells infected with wild-type or V(-) virus at various time points p.i. The RNAs were ethanol precipitated, dissolved in formamide/formaldehyde solution, then electrophoresed in 1.5% agarose-formamide/MOPS gels, and capillary transferred onto Hibond-N filters (Amersham, Amersham). They were probed with ³²P-labeled riboprobes made from pGEM-N (linearized with *Nae*I or *Bam*HI), pSeV(+) or pSeV(-) (both linearized with *Mlu*I) using T7 or SP6 RNA polymerase. To amplify genomic and antigenomic RNA fragments, two pairs of primers, pHv1 (5'-¹ACCAACAAGAGAAAAACA²⁰-3') and pHvNPr1 (5'-³⁵⁸CCATGGCAAACAGCAAGACG³³⁹-3') for the leader-N region, and pHvL1 (5'-¹⁴⁹⁰⁷TCTAGAAGACTTGTGCTATC¹⁴⁹²⁶-3') and pHvT (5'-¹⁵³⁸⁴ACCAGACAAGAGTTTAAGAG¹⁵³⁶⁵-3') for the L-trailer region were synthesized. The same RNAs used above for Northern blotting were reverse-transcribed either with pHvL1 primer for the genomic RNA detection or pHvNPr1 for the antigenomic RNA detection. The reverse transcripts were then amplified for 15 cycles with primers specific for each genomic or antigenomic strand. The cDNAs reverse transcribed from RNAs extracted at 38 h p.i. were serially 10-fold diluted and amplified under the same conditions. By this procedure, PCR cycles which gave a linear correlation between the amounts of RNA template and PCR product were defined.

Trans-supply of V and P proteins to cells infected with V(-) virus

Monolayer cultures of CV1 cells were infected with a non-replicating and non-cytopathic adenovirus expressing T7 pol (AdT7), which was a gift from Dr Y.Matsuura (NIH, Japan), at a m.o.i. of 20 infectious units per cell. The T7 pol with CAG promoter was inserted into the E1A gene locus. The recombinant adenovirus is non-replicating and non-cytopathic. The virus stock was produced in 293 cells with the helper function. After virus adsorption for 1 h at 37°C, 2 µg of pGEM-P or pGEM-V plasmid was transfected with the aid of a new lipofection reagent, DOSPER (Boehringer-Mannheim). The culture media were removed 24 h p.i. and then the wild-type or V(-) virus was inoculated at 10 p.f.u./cell. Cytopathogenicity was examined at 38 h p.i. The V protein-expressing plasmid, pGEM-V, was made from pGEM-P by inserting a C residue into the position corresponding to the editing site in the viral genome. The expression of P and V proteins from the respective plasmids in CV1 cells was verified by Western blotting with the respective specific antibodies described above.

Infection of mice

Three-week-old, male ICR/Crj (CD-1) mice were purchased from Charles-River, Japan and used for the experimental virus infection. For mock infection, five mice were used. Thirty five mice were used for infection with 10⁷ p.f.u. of wild-type or V(-) virus. At 0, 1, 2, 3, 5, 7 and 9 days p.i., five mice from each experimental group were sacrificed, and their body weights, consolidation scores and virus titers in the lungs were measured individually. The consolidation scores are expressed as follows: 0 = no visible lesions or atrophy, 1 = <25% of follicles affected, 2 = 25–50% follicles affected, 3 = 50–75% follicles affected, 4 = >75% follicles affected. When the mice died, one point was added (score 5). Infectivity in lung homogenates was determined by cell infectious units (c.i.u.) assay. The c.i.u. is essentially equivalent to p.f.u. (Kiyotani *et al.*, 1990). Histochemical staining with anti-SeV rabbit serum was performed using a staining kit (Boehringer Mannheim, Mannheim) according to the manufacturer's recommendations.

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References

- Cadd,T., Garcin,D., Tapparel,C., Itoh,M., Homma,M., Roux,L., Curran,J. and Kolakofsky,D. (1996) The Sendai paramyxovirus accessory C proteins inhibit viral genome amplification in a promoter-specific fashion. *J. Virol.*, **70**, 5067–5074.
- Curran,J. and Kolakofsky,D. (1988) Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. *EMBO J.*, **7**, 245–251.
- Curran,J., Boeck,R. and Kolakofsky,D. (1991a) The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. *EMBO J.*, **10**, 3079–3085.
- Curran,J., de Melo,M., Moyer,S. and Kolakofsky,D. (1991b) Characterization of the Sendai virus V protein with an anti-peptide antiserum. *Virology*, **184**, 108–116.
- Curran,J., Pelet,T. and Kolakofsky,D. (1994) An acidic activation-like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. *Virology*, **202**, 875–884.
- Fuerst,T.R., Niles,E.G., Studier,F.W. and Moss,B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl Acad. Sci. USA*, **83**, 8122–8126.
- Galinski,M.S., Troy,R.M. and Banerjee,A.K. (1992) RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. *Virology*, **186**, 543–550.
- Hamaguchi,M., Yoshida,T., Nishikawa,K., Naruse,H. and Nagai,Y. (1983) Transcriptional complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology*, **128**, 105–117.
- Harty,N.R. and Palese,P. (1995) Measles virus phosphoprotein (P) requires the NH₂- and COOH-terminal domains for interactions with the nucleoprotein (N) but only the COOH terminus for interactions with itself. *J. Gen. Virol.*, **76**, 2863–2867.
- Ito,Y. and Hosaka,Y. (1983) Component(s) of Sendai virus that can induce interferon in mouse spleen cells. *Infect Immun.*, **39**, 1019–1023.
- Ito,Y., Nishiyama,Y., Shimokata,K., Takeyama,H. and Kanii,A. (1978) Active component of HVJ (Sendai virus) for interferon induction in mice. *Nature*, **274**, 801–802.
- Kato,A., Fujino,M., Nakamura,T., Ishihama,A. and Otaki,Y. (1995) Gene organization of chicken anemia virus. *Virology*, **209**, 480–488.
- Kato,A., Sakai,Y., Shioda,T., Kondo,T., Nakanishi,M. and Nagai,Y. (1996) Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes to Cells*, **1**, 569–579.
- Kestler,H.W., III, Ringler,D.J., Mori,K., Panicali,D.L., Sehgal,P.K., Daniel,M.D. and Desrosiers,R.C. (1991) Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. *Cell*, **65**, 651–662.
- Kiyotani,K., Takao,S., Sakaguchi,T. and Yoshida,T. (1990) Immediate protection of mice from lethal wild-type Sendai virus (HVJ) infections by a temperature-sensitive mutant, HVJpi, possessing homologous interfering capacity. *Virology*, **177**, 65–74.
- Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lamb,R.A. and Kolakofsky,D. (1996) Paramyxoviridae: the viruses and their replication. In Fields,B.N., Knipe,D.M., Howley,P.M. (eds), *Fields Virology*. 3rd edn. Raven Press, New York, pp. 1177–1204.
- Liston,P. and Briedis,D.J. (1994) Measles virus V protein binds zinc. *Virology*, **198**, 399–404.
- Matsuoka,Y., Curran,J., Pelet,T., Kolakofsky,D., Ray,R. and Compans,R.W. (1991) The P gene of human parainfluenza virus type 1 encodes P and C proteins but not a cysteine-rich V protein. *J. Virol.*, **65**, 3406–3410.
- Moyer,S.A., Baker,S.C. and Lessard,J.L. (1986) Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNA. *Proc. Natl Acad. Sci. USA*, **83**, 5405–5409.
- Park,K.H. and Krystal,M. (1992) *In vivo* model for pseudo-templated transcription in Sendai virus. *J. Virol.*, **66**, 7033–7039.
- Paterson,R.G. and Lamb,R.A. (1990) RNA editing by G-nucleotide insertion in mumps virus P gene mRNA transcripts. *J. Virol.*, **64**, 4137–4145.
- Paterson,R.G., Leser,G.P., Shaughnessy,M.A. and Lamb,R.A. (1995) The paramyxovirus SV5 V protein bind two atoms of zinc and is a structural component of virions. *Virology*, **208**, 121–131.
- Pelet,T., Curran,J. and Kolakofsky,D. (1991) The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. *EMBO J.*, **10**, 443–448.
- Power,U.F., Ryan,K.W. and Portner,A. (1992) The P genes of human parainfluenza virus type 1 clinical isolates are polycistronic and microheterogeneous. *Virology*, **189**, 340–343.
- Radecke,F. and Billeter,M.A. (1996) The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cell. *Virology*, **217**, 418–421.
- Rochat,S., Komada,H. and Kolakofsky,D. (1992) Loss of V protein expression in human parainfluenza virus type 1 is not a recent event. *Virus Res.*, **24**, 137–144.
- Roizman,B. and Sears,A.E. (1996) Herpes simplex viruses and their replication. In Fields,B.N., Knipe,D.M., Howley,P.M. (eds), *Fields Virology*. 3rd edn. Raven Press, New York, pp. 2231–2295.
- Ryan,K.W., Morgan,E.M. and Portner,A. (1991) Two non-contiguous regions of Sendai virus P protein combine to form a single nucleocapsid binding domain. *Virology*, **180**, 126–134.
- Shioda,T., Hidaka,Y., Kanda,T., Shibuta,H., Nomota,A. and Iwasaki,K. (1983) Sequence of 3687 nt from the 3' end of Sendai virus genome RNA and the predicted amino acid sequences of viral NP, P and C proteins. *Nucleic Acids Res.*, **11**, 7317–7330.
- Smallwood,S., Ryan,K.W. and Moyer,S.A. (1994) Deletion analysis defines a carboxyl-proximal region of Sendai virus P protein that binds to the polymerase L protein. *Virology*, **202**, 154–163.
- Steward,M., Samson,A.C., Errington,W. and Emmerson,P.T. (1995) The Newcastle disease virus V protein binds zinc. *Arch. Virol.*, **140**, 1321–1328.
- Tao,T. and Ryan,K.W. (1996) Host range restriction of parainfluenza virus growth occurs at the level of virus genome replication. *Virology*, **220**, 69–77.

- Thomas,S.M., Lamb,R.A. and Paterson,R.G. (1988) Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminial proteins P and V of the paramyxovirus SV5. *Cell*, **54**, 891–902.
- Vidal,S., Curran,J. and Kolakofsky,D. (1990a) Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. *J. Virol.*, **64**, 239–246.
- Vidal,S., Curran,J. and Kolakofsky,D. (1990b) A stuttering model for paramyxovirus P mRNA editing. *EMBO J.*, **9**, 2017–2022.
- Wardrop,E.A. and Briedis,D.J. (1991) Characterization of V protein in measles virus-infected cells. *J. Virol.*, **65**, 3421–3428.

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