

Review

Studies on the paramyxovirus accessory genes by reverse genetics in the Sendai virus–mouse system

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Abstract: Nucleotide sequencing of the entire genomes was completed in the 1980s for most members of the *Paramyxoviridae*. It then became a new common task with challenge for researchers in the field to establish a system to recover the virus entirely from cDNA, thereby allowing reverse genetics (free manipulation of the viral genome). Using Sendai virus, we established a system of incomparable virus recovery efficiency early on. This technology was then fully exploited in answering a series of long-held questions. In particular, two accessory genes whose functions had remained enigmatic were demonstrated to encode special functions critical in viral *in vivo* pathogenesis producing fatal pneumonia in mice, although dispensable in virus replication at the *in vitro* cellular level. Their *in vivo* functions were found to counteract the two respective facets of the antiviral state induced by interferons and an interferon regulatory factor 3-dependent but yet unknown effector. These achievements appear to have facilitated a scientific trend where the accessory genes are a focus of active investigation in studies on other paramyxoviruses and opened up a new common ground shared between virology and immunology.

Keywords: paramyxovirus, Sendai virus, accessory genes, reverse-genetics, interferon, innate immunity

Introduction

SeV, also known as murine parainfluenza virus type 1 or hemagglutinating virus of Japan, is an enveloped virus with a negative-strand RNA genome. SeV is a member of the family *Paramyxoviridae* in the order *Mononegavirales*, which is comprised of a wide range of “classic” viruses of medical or veterinary significance that include MeV, MuV and NDV, and “new” deadly emergent

agents such as NiV and HeV. Three other families (*Rhabdo*-, *Filo*- and *Bornaviridae*) in the *Mononegavirales* also include both classic and new deadly agents such as rabies, Ebola and Marburg viruses.¹⁾

Reverse genetics has been established for SeV and most of its relatives (mononegaviruses), thus allowing the viral genome to be engineered at will and the outcomes assessed not only in terms of virus proliferation at the cellular level but also in the context of the viral pathogenesis in the entire host organism. This technological innovation has played a prominent role in settling outstanding issues and resolving long-held enigmas that had been impenetrable using conventional virology or forward genetics. Viral accessory genes are generally dispensable (non-essential) at cellular level proliferation and can be knocked out. Their functions can be best understood by comparing the phenotypes of the knockout viruses and the parental virus at the cellular level and in the susceptible

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host organism. Thus, accessory genes have become one of the most valuable research-subjects in reverse genetics.

The following two conditions need to be fulfilled for the successful application of reverse genetics in studying accessory genes. First, the efficiency of virus recovery from engineered cDNA needs to be sufficiently high, and that is because even if non-essential at the cellular level, accessory gene products often play great auxiliary roles and if they are deleted, it can cause, while not complete, profound loss of infectivity. Second, an authentic host animal system must be available, in which the outcomes of accessory gene deletion and manipulation can be readily assessed.

The efficiency of SeV recovery in our initial system already proved to be significantly higher (50~100-fold) than those for other paramyxoviruses established by other parties,²⁾ with the efficiency of our current system being even higher (10~100-fold) than the initial one.³⁾ The susceptible host for SeV is the mouse, in which the virus causes severe acute pneumonia. The outcomes of SeV engineering can be assessed with relative ease using laboratory mice with a large variety of genetic background. In contrast, *in vivo* studies of most other paramyxoviruses are considerably more difficult because their susceptible hosts are often humans, non-human primates or other large animals and also because the mouse- or other small animal models do not always satisfactorily mimic natural infections and diseases. Hence the SeV-mouse system represents a good experimental paradigm for use of reverse genetics in understanding the roles of accessory genes. The success of reverse genetics being applied

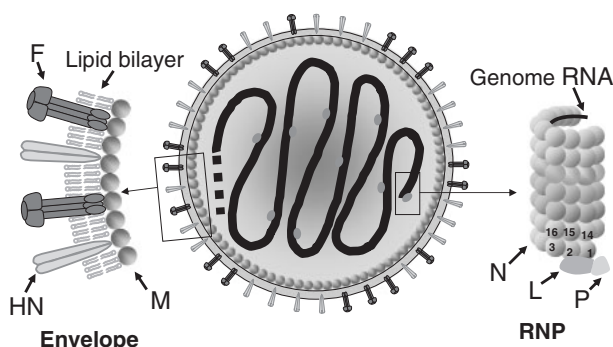


Fig. 1. Schematic diagram of an SeV particle. (Adapted from Ref. 7 with permission.)

in understanding the roles of accessory genes in the SeV-mouse experimental paradigm will now be described.

Fundamentals of SeV structure and biology

The SeV particle (virion) is spherical, with a diameter of approximately 200 nm. It consists of an inner RNP complex and outer envelope (Fig. 1). The RNP has a left-handed helical structure 1.1 μm in length and 18 nm in diameter, with a pitch of 5 nm, in which the 15,384-base RNA genome is encapsidated with the nucleocapsid (N) proteins. It has 13 N proteins per turn. Because one N protein binds to every 6 RNA bases, the entire RNP complex can be calculated as containing 2,564 ($= 15,384/6$) of N protein units. The large (L) and phospho- (P) proteins, the subunits of the RNA polymerase complex involved in genome transcription and replication, attach to the 3'-terminal site as well as occasionally to other sites in the RNP complex.

Abbreviations used:

[Virus species]

HeV, Hendra virus
hPIV2, human parainfluenza virus type 2
MeV, measles virus
MuV, mumps virus
NiV, Nipah virus
NDV, Newcastle disease virus
SeV, Sendai virus
SV5, simian virus 5

[Miscellanea]

CIU, cell infectious units
DI, defective interfering
ESCRT, endosome-associated complexes required for transport
IFN, interferon
IL, interleukin
iNOs, inducible nitric-oxide synthase

IRF, interferon regulatory factor

ISG, interferon-stimulated gene

Jak, Janus kinase

MDA5, melanoma differentiation-associated gene 5

m.o.i, multiplicity of infection

MyD88, myeloid differentiation factor 88

NK, natural killer

NKT, natural killer T

nt, nucleotide positions

nu, nude

ORF, open reading frame

PKR, protein kinase R

RNP, ribonucleoprotein

SH, small hydrophobic

STAT, signal transducer and activator of transcription

TRIF, Toll/interleukin-1 receptor domain-containing adaptor inducing interferon- β

TNF α , tumor necrosis factor alpha

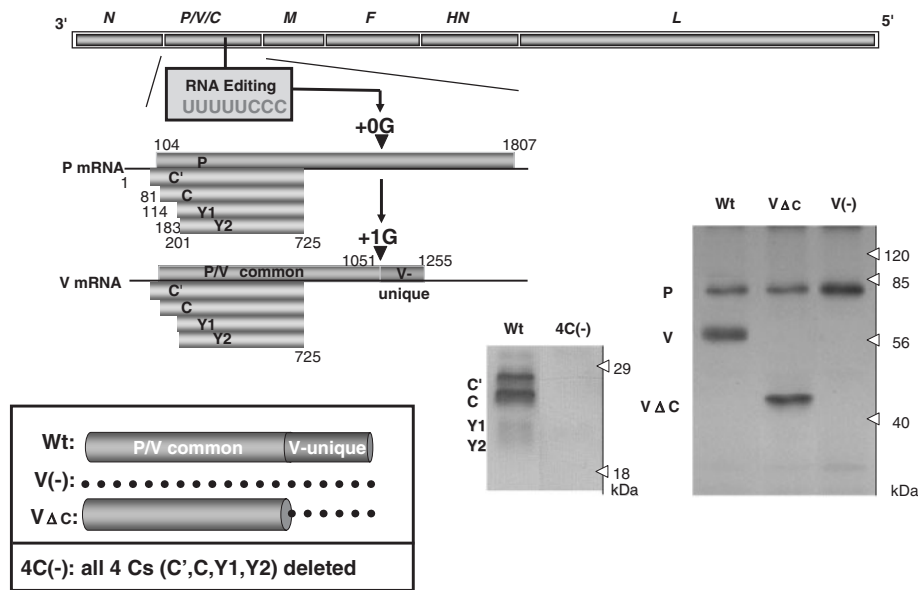


Fig. 2. Expression of C and V proteins from SeV *P* gene and recovery of knockout viruses; V(-) with the entire V protein deleted, VΔC with the V-unique region deleted and 4C(-) with all four C proteins (C', C, Y1 and Y2) deleted. For details see the text. Deletion of the respective proteins was verified by Western blotting of cells infected with the recovered knockout viruses. Wt, cells infected with wild-type SeV. The dotted line in the insert indicates deletion. (Adapted from Ref. 7 with permission.)

The envelope contains two types of glycoproteins protruding in the form of spikes that are anchored within a lipid bilayer derived from the plasma membrane of host cells (Fig. 1). One of the spike glycoproteins has hemagglutinin (HA) and neuraminidase (NA) activities and is named HN. The HN protein recognizes terminal sialic acids in the sugar chains as the receptor on the target cell surface. Its NA activity is essential for the release of progeny virus from the cell surface. The other spike glycoprotein is named F because it induces fusion of the virus envelope with the host cell plasma membrane, thereby introducing viral RNP into the cytoplasm. The F protein is produced as an inactive precursor molecule F₀, which is processed by host proteases into the biologically active (fusion-competent) form, F₁ and F₂, which remain disulfide bridged. The HN protein is a tetramer, and the F protein a trimer.

The matrix (M) protein is located between the envelope and the RNP (Fig. 1). The M protein serves as an anchor to stabilize the spike glycoprotein molecules floating in the lipid bilayer by binding to their cytoplasmic tails and also binds to RNP, thus cross-linking the inner and outer structures. The M protein is indispensable for

structural proteins to assemble into virus particles as well as for viral budding from the plasma membrane. The genes coding for the above six structural proteins lie in the order of 3'-N-P-M-F-HN-L-5' (Fig. 2). For reviews see Refs. 4, 5, 6 and 7.

Coding strategies of the putative accessory genes of SeV

It has been suggested that the SeV *P* gene produces two putative accessory proteins, V and C, in addition to the basal gene product P (Fig. 2). The V and C proteins are widespread among the members of the subfamily *Paramyxovirinae* of the family *Paramyxoviridae* but are not as ubiquitous as the six major structural proteins, and therefore have been regarded as non-essential "accessory" gene products. However, the central question of how they contribute to actual viral replication and pathogenesis has remained unanswered since they were first described three decades ago (reviewed in Ref. 8). Even the non-essentiality of these proteins was yet to be established.

An ORF, which is +1 shifted from that of the P protein, gives rise to four proteins: C', C, Y1, and Y2, collectively referred to as the C proteins. This diversity is accomplished by the use of translation

initiation codons starting at different points (nt 81, 114, 183 and 201) (Fig. 2). The frames for the C proteins therefore are accessed thorough "ribosomal choice". The ORFs terminate in the same position (nt 725). C is the major species of the four C proteins as it is expressed in infected cells at a molar ratio several-fold higher than those of the other three. The number of expressed C proteins differs among the paramyxoviruses, ranging from 1 to 4, and their primary structures are poorly conserved. However, they are, being common to all, basic and relatively short with approximately 200 amino acid residues (reviewed in Refs. 6 and 8).

The V protein, on the other hand, is produced by a transcriptional frameshift ("co-transcriptional RNA editing") (Fig. 2). The P mRNA is a faithful copy of the *P* gene, accounting for about three-fourths of the total transcripts and gives rise to the P protein, the smaller subunit of the RNA polymerase complex. The remaining transcripts (one-fourth) are V mRNA, and carry a single guanine residue (+1G) (at nt 1052) inserted by the polymerase stuttering at a specific editing site (UUUUUCCC) located midway down the *P* gene, which then causes the frameshift (Fig. 2). The V protein therefore consists of a P/V common region (the N-terminal approximately three-quarters before the frameshift) and the V unique region (the C-terminal approximately one-quarter after the frameshift) (Fig. 2). The V unique region is highly conserved in the amino acid sequence among paramyxoviruses with a perfect preservation of seven cysteine residues that form a zinc finger-like motif, and indeed binding two atoms of Zn^{2+} (reviewed in Refs. 6 and 8).⁹⁾

Establishing the non-essentiality of the putative accessory genes of SeV

Without affecting the P ORF, mutations were either introduced into the editing site (UUUUUCCC \rightarrow UUCUUUCC) or a stop codon was introduced just downstream of the editing site. Mutants with the total V protein deleted [V(-)] and only the V unique C terminal region deleted (V Δ C) were respectively recovered without any difficulty (Fig. 2).^{10),11)} These mutants replicated in cells as efficiently as the parental SeV, thus establishing that the *V* gene is nonessential.

Through a rather difficult process, a viable clone, 4C(-) SeV expressing none of the four C

proteins (C', C, Y1, and Y2) was recovered by disrupting the respective ORFs, thus indicating that SeV C proteins also fall into the category of nonessential accessory gene products (Fig. 2).¹²⁾ The difficulty of recovery was the result of reducing the 4C(-) virus infectivity by approximately 100-fold, while the success of the virus rescue was attributable to the high efficiency of the SeV rescue system.²⁾ The characterization of the 4C(-) virus and a series of other C mutants with one or two C proteins deleted along with the plasmid-based expression studies of the C proteins demonstrated the extreme versatility of the C protein.

They were found to block the induction of the anti-viral state with type I IFN, IFN- α/β ¹³⁾ and type II IFN, IFN- γ .¹⁴⁾ The inhibitory magnitude of virus growth was much greater with IFN- α/β than with IFN- γ . Subsequent studies therefore focused on the blocking of the action of IFN- α/β . The blocking was caused by inhibition of STAT2 phosphorylation at the cellular level.^{15),16)} STAT2 is a key component and its phosphorylation a key event in the IFN- α/β signaling pathway. The Y2 (175 residues), the smallest of the 4 C proteins, was sufficient to exhibit the capacity to counteract the antiviral action of IFN- α/β .¹⁴⁾ The region responsible for this IFN antagonism was narrowed down to the 106-residue-long C terminal half.¹⁷⁾ The SeV C proteins further inhibited IFN- β production.¹⁸⁾

Although abundantly expressed in cells, the C proteins are nonstructural proteins that are not incorporated into virus particles. They nevertheless were found to make a large contribution to the viral morphogenesis by budding from the plasma membrane,¹⁹⁾ possibly by escorting ESCRT to the site of that budding.²⁰⁾ ESCRT is thought to be required in pinching off of budding plasma membrane patches, the immediate precursors of a viral envelope.

The SeV C protein was initially recognized as a down-modulator of viral RNA synthesis.^{21),22)} This was supported through use of 4C(-) SeV.^{12),19)} It was also previously shown that the inhibition was stronger on an internally deleted DI genome than on a copy-back DI genome, suggesting some promoter specificity.^{22),23)} Using 4C(-) and other C mutants it has recently been established that the C proteins suppress the leader (antigenomic) promoter but not the trailer (genomic) promoter late in the life cycle, thereby facilitating accumulation of the genomic RNA of negative polarity so that the

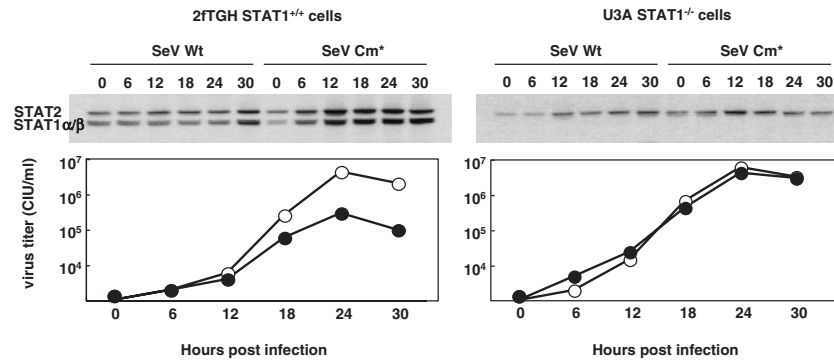


Fig. 3. Replication of SeV Cm* in wild-type cells and in STAT1 deficient cells. Parental SeV Wt (wild type) and SeV Cm* were inoculated to the parental STAT1^{+/+} 2fTGH cells (left) and to its STAT1 deficient (STAT1^{-/-}) derivative, U3A cells (right) at an m.o.i. of 5. Cells were collected at the hours indicated. The cell lysates were immunoblotted with a mixture of anti STAT1 and anti STAT2 antibodies (top). Titers of SeV Wt (open circle) and SeV Cm* (closed circle) in the culture fluid are shown (bottom) (Modified from Ref. 26).

released viruses mainly contain negative-sense RNA genomes.²⁴⁾ It was surprising that an accessory gene plays such a fundamental role as guaranteeing that SeV is a “negative-strand” RNA virus. It would be intriguing to study how genome polarity is determined in other negative strand RNA viruses. Furthermore, C proteins appear to encode anti-apoptosis function.²⁵⁾

Pivotal roles of SeV accessory proteins in viral growth and pathogenesis in mice

(i) The C protein as an antagonist of IFN signaling. As described above, SeV causes fatal pneumonia in mice. When intranasally inoculated under our experimental conditions, the virus titers in the lung increase exponentially during the initial one or two days. This is followed by a period of high viral load of up to 7–9 days, during which the lung lesion (consolidation) score increases (see Fig. 4). All mice eventually die of severe hemorrhagic pneumonia.

As noted above, SeV C proteins are multifunctional. We identified some amino acids in the C protein crucial to the different functions and were able to generate a mutant Cm* that remained incapable of blocking IFN- α/β signaling but that did restore the other two functions (promoter-dependent RNA synthesis regulation and contribution to morphogenesis) to almost normal levels.²⁶⁾

After binding to the receptor, IFN- α/β signaling is transduced through the Jak/STAT pathway. STAT1 is one of the key molecules in this signal

transduction. The properties of the wild-type and Cm* SeV were studied using STAT1^{+/+} normal human 2fTGH cells and 2fTGH derived STAT1 deficient (STAT1^{-/-}) cells (Fig. 3).²⁶⁾ The lack of STAT1 expression in the latter cells was confirmed using immunoblotting. STAT1 and STAT2 are products induced by IFN- α/β . Both wild-type and Cm* SeV stimulate IFN- α/β production. In STAT1^{+/+} normal cells infected with SeV Cm*, the induction of STAT1 and STAT2 becomes detectable at 6 hr post infection (p.i.) and continues up to 30 hr, whereas little induction was observed in the same cells infected with wild-type SeV (Fig. 3). These results confirmed that the intact C expressed from wild-type SeV had the capacity to block IFN- α/β signaling, whereas the Cm* mutant had lost the IFN antagonism. Accordingly, the growth of SeV Cm* was self limiting in normal cells and comparable to that of wild-type SeV in STAT1^{-/-} cells (Fig. 3). It remains to be elucidated whether or not SeV Cm* retains the anti-apoptosis and anti-IFN synthesis functions.

Using the Cm* virus the attempt was made to verify whether anti IFN- α/β capacity of SeV C protein would indeed play a role in *in vivo* pathogenesis.²⁶⁾ In the lungs of STAT1^{+/+} normal mice, SeV Cm* did not grow at all and produced no lung lesions, in striking contrast to the wild-type SeV that grew vigorously with increasing consolidation scores and killed all the mice (Fig. 4). In STAT1^{-/-} mice, however, SeV Cm* grew far better than in STAT1^{+/+} mice. And although the virus did not

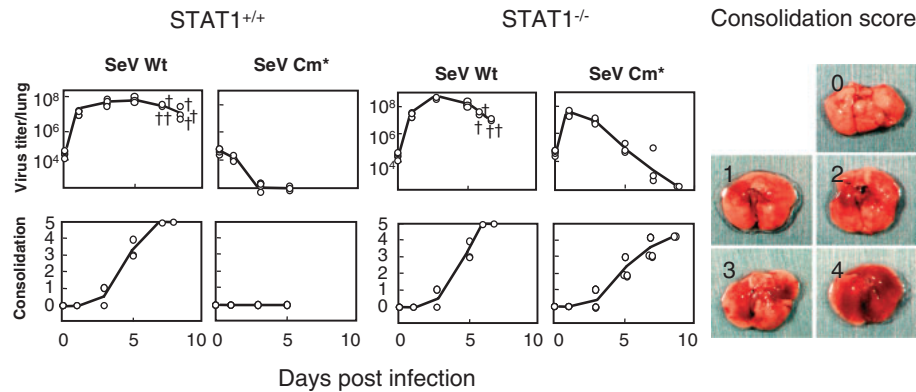


Fig. 4. Growth and pathogenicity of SeV Cm* in wild-type and STAT1^{-/-} mice. Five-week-old wild-type STAT1^{+/+} 129S6 and STAT1 deficient (STAT1^{-/-}) 129S6 mice were infected intranasally with 10⁷ CIU of SeV Wt or SeV Cm*. Two or three mice from each group were sacrificed at the intervals indicated and examined for virus infectivity in the lung (top) and lung consolidation (bottom). The symbol “†” indicates a dead mouse. Actual macroscopic views of the lung corresponding to the consolidation scores of 0 (no lesion) through 4 (maximum) are presented as references (right). When a mouse died, one point was added to the consolidation score (score 5) (Modified from Ref. 26).

kill the STAT1^{-/-} mice, the consolidation scores reached as high as 4 at day 9 (Fig. 4). These results clearly indicate that the IFN- α/β inducible antiviral state is indeed a key host defense mechanism at the forefront when coping with infecting SeV and that the SeV C protein plays a key role in the viral evasion of the antiviral state, thereby facilitating virus growth in the lung and causing severe pneumonia.

Recently SeV C proteins were found to inhibit PKR.²⁷⁾ This may play an additional role in counteracting the antiviral state induced by IFN- α/β .

(ii) The V protein as an antagonist of innate virus clearance through IRF3 activation, but not via IFNs. Both V(-) and V Δ C proliferated in the lungs of normal mice that included IRF3^{+/+} mice as efficiently as the parental wild-type SeV until day 1 post infection. However, they were then rapidly cleared and produced only moderate consolidation scores, in contrast to the wild-type SeV, which maintained both a high viral load and high consolidation scores up to 7–9 days (Fig. 5A). The wild-type SeV was usually lethal for most mouse strains so far tested, whereas V(-) and V Δ C were never lethal.^{10),11)} Similar attenuation was observed with several mutants with the zinc finger-like motif disrupted and no capacity to bind zinc.^{28),29)} The V protein, which is fully dispensable in the viral life cycle, was therefore found to encode a special

function required for pathogenesis. The V unique region, particularly its zinc binding capacity, appeared to be crucial for this function.

Of the various mice of high genetic diversity so far tested, IRF3^{-/-} mice were found to be highly and almost equally susceptible to V(-), V Δ C and wild-type viruses (Fig. 5A).³⁰⁾ The unique, rapid clearance of V(-) and V Δ C viruses could no longer be observed. Instead, they grew as efficiently as the wild type and caused severe pneumonia and death almost as rapidly as the wild type. IFN- β is one of the well-known IRF3 inducible gene products, and IFN- α/β is subsequently amplified via IRF7 activation.³¹⁾ It is therefore a reasonable assumption that the SeV V protein is also a type I IFN (IFN- α/β) antagonist.

As shown in Fig. 5B, however, no such active growth beyond 1 day after infection as seen in IRF3^{-/-} mice was found for the V(-) virus in IFN- α/β -receptor^{-/-} mice and STAT1^{-/-} mice, where IFN- α/β mediated antiviral-state is not inducible. V(-) appeared to be cleared in these mice as efficiently as in normal mice.³⁰⁾ The V(-) virus was also efficiently cleared in IFN- γ ^{-/-} mice, indicating that type II IFN is not involved, either. It was also rapidly cleared in ISG15 (another IRF3 dependent product) deficient mice and various other mouse strains deficient in different facets of innate and adaptive immunity. The latter included NK cell deficient beige mice and T cell deficient nu/nu

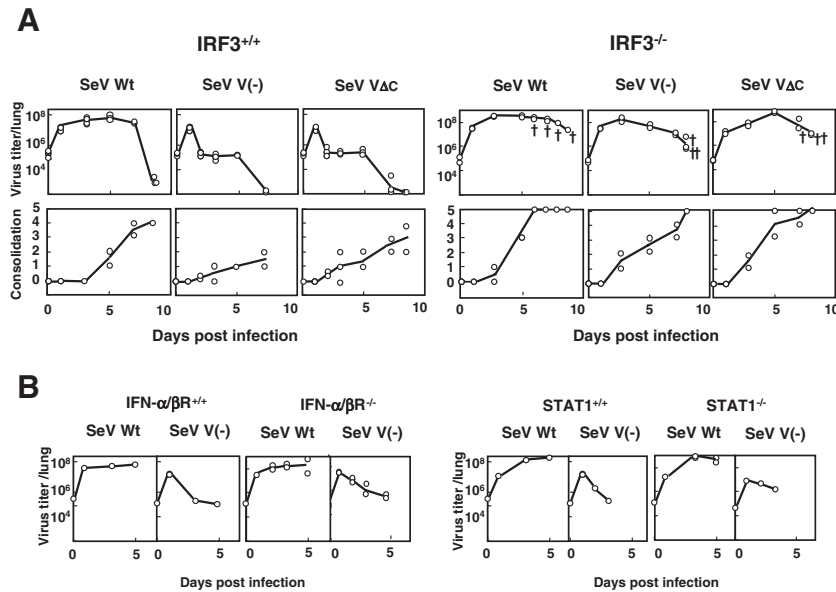


Fig. 5. Replication and pathogenicity of SeV V(-) and SeV VΔC in IRF3^{-/-} mice. (A) Five-week-old IRF3^{+/+} C57BL/6J and IRF3 deficient (IRF3^{-/-}) C57BL/6J mice were infected intranasally with 10⁷ CIU of SeV Wt, SeV V(-) or SeV VΔC. Two or three mice from each group were sacrificed at the indicated intervals and examined for virus infectivity in the lung and lung consolidation. The symbol "†" indicates a dead mouse. (B) Six- to seven-week-old IFN-α/βR^{+/+} A129/Sv and IFN-α/β receptor deficient (IFN-α/βR^{-/-}) A129/Sv mice were infected intranasally with 10⁷ CIU of SeV Wt or SeV V(-) (left). Five-week-old STAT1^{+/+} 129S6 and STAT1 deficient (STAT1^{-/-}) 129S6 mice were infected intranasally with 10⁷ CIU of SeV Wt or SeV V(-) (right). Viral infectivity in the lung was measured at the indicated time intervals after infection (Modified from Ref. 30).

mice.³⁰⁾ More recently grouped in the category that is characterized by early clearance of V(-) virus were mouse strains deficient in NKT, TNFα, IL6, iNOS, TRIF and MyD88 (Kiyotani *et al.*, manuscript in preparation).

These results predict the presence of a hitherto unknown (novel), antiviral innate-immunity mechanism induced following IRF3 activation.

The SeV V protein appears to interact with MDA5, thereby inhibiting IRF3 activation and IFN production.³²⁾ However, this IFN synthesis inhibition did not appear to be profound; quantitative differences in IFN in the mouse lung were about only 2.5-fold between infections with V(-) and the wild-type SeV during the early period when their viral loads were similar.³⁰⁾ The wild-type SeV displayed no appreciable difference in growth capacity in normal and MDA5^{-/-} mice. The growth of V(-) virus showed no appreciable difference, either, in normal and MDA5^{-/-} mice. The V protein interfering with the virus-sensing MDA5 may therefore not have an appreciable influence upon *in vivo* virus growth (Kiyotani *et al.*, manuscript in preparation).

Accessory genes of other paramyxoviruses

The subfamily *Paramyxovirinae* consists of five genera (*Respiro*-, *Morbilli*-, *Henipa*-, *Avula*- and *Rubulavirus*). All the members of these five genera encode the V protein, whereas the C protein is only encoded by the members of three genera, *Respiro*-, *Morbilli*- and *Henipavirus*. The way they express the respective accessory genes are schematically presented using a virus species representative of each genus in Fig. 6A. Mainly using the data obtained with these representative species, the nature of the V and C proteins in terms of dispensability, functions assigned *in vitro* cell culture and their verification *in vivo* will now be discussed.

(i) Dispensability. The dispensability of both the V and C proteins appears to have been established for the *Respirovirus* and *Morbillivirus* including for SeV and MeV, respectively (reviewed in Ref. 8). Reverse genetics was relatively recently established for the *Henipavirus*,³³⁾ and the data are not yet available for the dispensability or indispensability of the V and C proteins of this genus.

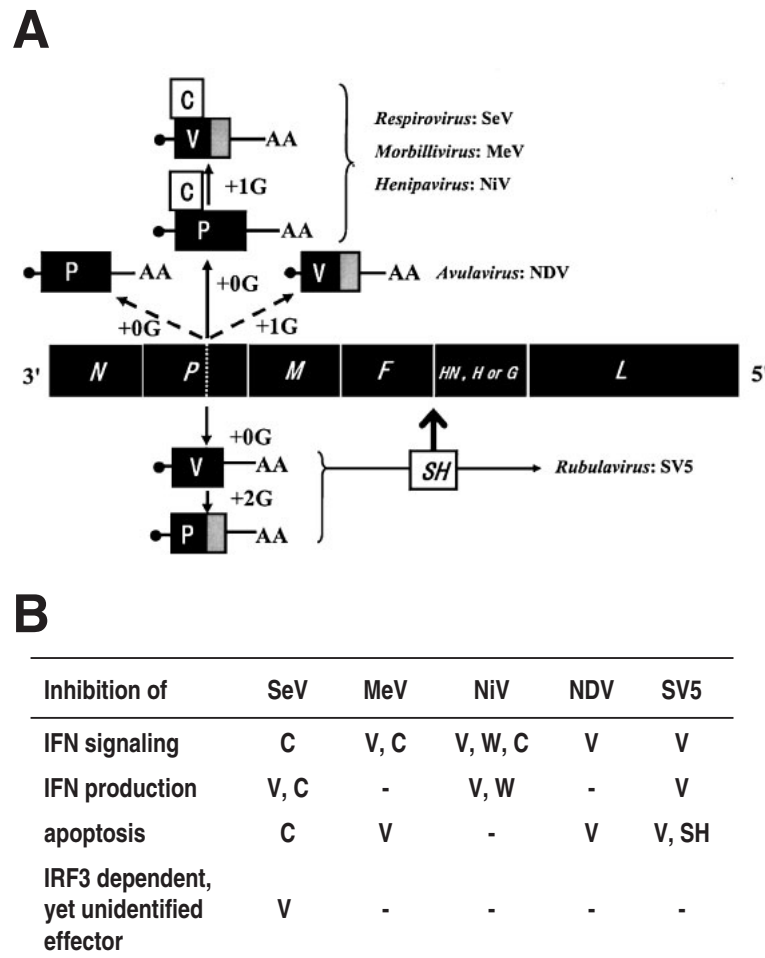


Fig. 6. (A) Expression of accessory genes from members of the subfamily *Paramyxovirinae*. The fifth gene encoding the receptor binding protein is named HN for *Respiro*-, *Avula*- and *Rubulavirus*, H for *Morbillivirus* and G for *Henipavirus*. Dotted line in the P gene, editing site; ●, cap of mRNA; AA, 3' polyadenylic acid of mRNA. SH, inserted between the F and HN genes (Modified from Ref. 8). (B) The coding assignment of the individual inhibitory functions. -, data not available; W, encoded by the mRNA generated by +2G insertion. For details refer to the text and Refs. 6, 8 and 39.

For the *Rubulavirus*, the V mRNA is a faithful copy of the P gene, whereas the indispensable P mRNA is generated by +2G insertion editing (reviewed in Refs. 6 and 8) (Fig. 6A). Hence the rescue of recombinant SV5 that would express only P mRNA but not V mRNA may not be as easy as for other genera that express the V protein from edited mRNA. And in fact it has been unsuccessful.³⁴⁾ Thus deletion of the C-terminal V-unique domain was attempted to generate VΔC by changing the sixth and eighth codons downstream of the editing site to stop codons. This mutant grew poorly in cells and interestingly, the two stop codons introduced could not be stably maintained

and were instead converted to sense codons, although not identical to the original ones, thus generating a pseudo-wild type.³⁴⁾ This indicates the presence of strong pressure to repair the truncated V to a full-length V. An analogous VΔC virus created from another rubulavirus hPIV2 was also shown to have critically impaired infectivity in cell cultures with highly anomalous virion morphology.³⁵⁾ The constraint due to V deletion did not appear to be fully attributable to the capacity of the *Rubulavirus* V protein to inhibit IFN signaling because strong constraint and the repair were still observed in Vero cells, which do not produce IFN.³⁴⁾ And hence dispensability or indispensability is yet

to be established for the *Rubulavirus* V protein.

Avulavirus NDV V mRNA is generated by +1G editing (Fig. 6A). Severely impaired infectivity was also observed for its editing-minus mutant as well as V Δ C version.^{36)–38)} However, the constraint was much smaller in the Vero cell line, an IFN non-producer, indicating that it was largely due to increased IFN sensitivity caused by V deletion.³⁸⁾ And hence the *Avulavirus* V protein falls into the category of nonessential gene product.

(ii) IFN antagonism as a common role shared with V and C genes of the *Paramyxovirinae*. Inhibition of IFN- α/β mediated signaling to induce the antiviral state was demonstrated to be a major role in common with the paramyxovirus accessory gene products (reviewed in Refs. 6, 8 and 39) (Fig. 6B). This capacity to evade the antiviral state in host animals is encoded by either the V or C protein or by both, depending on the virus species (Fig. 6B). The inhibition mechanisms are highly diverse. The inhibition is caused through proteasome-dependent degradation of STAT1 or STAT2 by the *Rubulavirus* V protein,^{40),41)} an irrelevant complex formation of the *Morbillivirus* C protein or *Henipavirus* V protein with STAT1 and STAT2,^{42),43)} or inhibition of STAT2 phosphorylation by the *Respirovirus* SeV C protein.^{15),16)}

Furthermore, inhibition of IFN- α/β production appeared to be encoded by the *Respirovirus*, *Henipavirus* and *Rubulavirus*⁴⁴⁾ (Fig. 6B). The mechanisms responsible for the inhibition of IFN production by the accessory proteins are poorly understood. With SV5 and SeV, the interaction of the V protein with virus-sensing MDA5 may be involved.^{32),45)}

(iii) Other functions of accessory genes to evade host defense. Virus infection often causes apoptosis, which limits further virus spread in the body. Probably to evade this host defense, not all but many genera of the *Paramyxovirinae* appear to encode the anti-apoptosis function (Fig. 6B). SV5 encodes anti-apoptosis in not only the V gene but also another accessory gene, namely the SH gene.^{46),47)} The latter is a small gene that evolved through insertion between the F and HN genes (Fig. 6A) in two members (SV5 and MuV) of the *Rubulavirus* and encodes 44- and 57-residue-long SH proteins, respectively (reviewed in Ref. 8).

Mice appeared to be capable of resisting SeV through not only exerting the IFN system but also

inducing the IRF3 dependent, but as-yet-unidentified effector(s). Identification of the effector in this puzzling IRF3 dependent innate immunity is of critical importance. It would also be intriguing to decide whether a similar innate immunity is present in other virus-host systems.

(iv) *In vivo* verification of the *in vitro* assigned functions. Many of the inhibitory mechanisms described above have been suggested by plasmid-based expression of the accessory genes and their variants or by the phenotypes of respective gene knockout viruses at the cellular level, and await *in vivo* verification to decide whether *in vitro* assigned functions indeed have grave consequences on *in vivo* virus growth and pathogenicity.

In this context, at least three different situations were encountered with the SeV-mouse system. First, the *in vitro* assessed property (IFN antagonism by the C protein) indeed brought about a far-reaching consequence *in vivo* as strongly supported by the results obtained with the SeV Cm* in STAT1^{-/-} and normal mice (Fig. 4). Second, an *in vitro* assigned function (of the V protein to interfere with virus sensing MDA5) did not appear to have an appreciable consequence on *in vivo* virus growth as suggested by the results obtained with SeV V(-) in MDA5^{-/-} mice (see above). Third, even if no phenotype was found *in vitro* following deletion of an accessory gene, the consequence of deletion was quite grave as shown by the case of SeV V(-) in normal and IRF3^{-/-} mice (Fig. 5A). These results indicate that the gap between *in vitro* and *in vivo* experimental outcomes can sometimes be significant. Careful consideration and further experimentation are needed to bridge that gap.

In other virus-host systems, *in vivo* verification would be even more difficult. However, several mutants with the accessory genes deleted grew poorly and were less pathogenic in host animals including rhesus macaques^{48)–53)} or grew better in STAT1^{-/-} mice than in normal mice,^{34),46)} agreeing with the concept that the *in vitro* assigned functions are of *in vivo* significance.

Concluding remarks

Genome nucleotide sequencing of the *Paramyxoviridae* was completed in the 1980s for most of the members and then for the newly emergent members. One of the important issues revealed by that sequencing was the identification of ORFs

encoding putative accessory proteins. These accessory genes were then focused upon in active investigation using reverse genetics established in the mid 1990s.

The SeV-mouse system probably assumed the lead role in that line of research because of the extremely high efficiency of the reverse genetics system and the availability of mice of diverse genetic backgrounds as susceptible hosts. Research conducted using this model revealed that in the realm of *in vivo* pathogenesis, accessory genes were not mere accessories or minor players, but rather central players. Mice appear to resist SeV by exerting at least two facets of innate immunity. One is the IFN system and the other is through IRF3 dependent, but as-yet-unidentified effector(s). To counteract these two antiviral mechanisms and persist in nature, SeV appears to have acquired the capacity to express both the C and the V proteins in the evolutionary process. Hence accessory genes should be highly relevant to virus biology in natural settings or virus ecology.

This view appears to be well shared by the members of the subfamily *Paramyxovirinae*. The accessory genes of another subfamily *Pneumovirinae* appear to have evolved in a rather different way (insertion before or between the basal six genes), but play essentially the same roles; i.e. evasion of innate host defense, in particular, the IFN system (reviewed in Refs. 8 and 39).

The *P* gene, except for its V unique region, is the least conserved of the six basal genes in the members of the subfamily *Paramyxovirinae*, and thus might be most subject to change during the evolutionary process. Apparently the same important occupation, inhibiting the IFN signaling, is encoded by the C protein in *Respirovirus* SeV and by the V protein in *Avulavirus* NDV and *Rubulavirus* SV5, whereas *Morbillivirus* MeV and *Henipavirus* NiV encode the same function in both the V and C proteins (Fig. 6B). These differences in coding strategies of the accessory function may be related to the concept that *Morbillivirus* and *Henipavirus* are phylogenetically close and map between the *Respirovirus* and *Rubulavirus* and that *Avulavirus* maps between *Rubulavirus* and the *Morbilli-Henipa* group.^{8),54)}

The P/V common region of NiV was as active as the full-length V in inhibiting IFN signaling.⁵⁵⁾ In the inhibition by MeV, not only the P/V

common region but also the P protein was as active as the V protein.⁵⁶⁾ On the other hand, not the P/V common region but the V unique region is generally essential, although not sufficient, to carry out the IFN antagonism for the *Rubulavirus* (reviewed in Ref. 8). The different coding patterns of IFN antagonism depending on the genus may be related to the different underlying mechanisms as noted above. The different patterns further suggest that there could be considerable options in the way to encode IFN antagonism in the paramyxovirus *P* gene. Although highly speculative, the ancestral *P* gene might encode multiple functions including a role in RNA synthesis, IFN antagonism and others. V ORF might then open and become widespread in taking over the IFN antagonism as its principal occupation and some additional other jobs. In some other members, the *C* gene further evolved to take over some of the numerous functions of *V* gene. *Respirovirus* SeV may have evolved so as to encode the capacities to evade the two different facets of host defense (IFN system and IRF3 dependent, yet unknown function) in two separate ORFs, V and C.

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Profile

Yoshiyuki Nagai was born in Gifu Prefecture, Japan, in 1939. He graduated from the Faculty of Medicine at Nagoya University in 1965, started his research career in the field of virology at the graduate school of the same university and earned his Ph. D. in 1973. He made a research stay at the Institut fuer Virologie, Giessen, Germany from 1974 to 1976 as a Scholarship Investigator of Alexander von Humboldt Foundation. His academic career involves assignment to Professor of the Research Institute for Disease Mechanism and Control, Nagoya University in 1984, Professor of the Institute of Medical Science, the University of Tokyo in 1993 and Director of AIDS Research Center, National Institute of Infectious Diseases in 1998. He further served in the field of public health as Director of Toyama Institute of Health from 2001 to 2005 and returning to Tokyo in 2005, he is taking an active part in constructing Japan's Asian-African research network for infectious diseases as Director of the Center of Research Network for Infectious Diseases (CRNID), RIKEN. For his great deal of scientific contribution, especially to the understanding of molecular basis of paramyxovirus replication and pathogenicity, he was awarded Humboldt Research Award in 1990, Hideyo Noguchi Memorial Award for Medical Sciences in 1994, Chunichi Cultural Award in 1995, Professor Emeritus of Nagoya University and Takeda Prize for Medical Sciences in 2000, the Medal with Purple Ribbon in 2001 and the Japan Academy Prize in 2008. His administrative roles have also been greatly appreciated as exemplified by the assumption to President of the Japanese Society for Virology (2004–2005) and inauguration to Vice President of the International Union of Microbiological Societies this year (2008).

