Article

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Abstract

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Reference

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Sendai Virus C Proteins Counteract the Interferon-Mediated Induction of an Antiviral State

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We have studied the relationship between the Sendai virus (SeV) C proteins (a nested set of four proteins initiated at different start codons) and the interferon (IFN)-mediated antiviral response in IFN-competent cells in culture. SeV strains containing wild-type or various mutant C proteins were examined for their ability (i) to induce an antiviral state (i.e., to prevent the growth of vesicular stomatitis virus [VSV] following a period of SeV infection), (ii) to induce the elevation of Stat1 protein levels, and (iii) to prevent IFN added concomitant with the SeV infection from inducing an antiviral state. We find that expression of the wild-type C gene and, specifically, the AUG^{114}-initiated C protein prevents the establishment of an antiviral state: i.e., cells infected with wild-type SeV exhibited little or no increase in Stat1 levels and were permissive for VSV replication, even in the presence of exogenous IFN. In contrast, in cells infected with SeV lacking the AUG^{114}-initiated C protein or containing a single amino acid substitution in the C protein, the level of Stat1 increased and VSV replication was inhibited. The prevention of the cellular IFN-mediated antiviral response appears to be a key determinant of SeV pathogenicity.

Interferons (IFNs) are a family of cytokines originally identified by their ability to confer cellular resistance to viral infection (14) and which are also involved in cell growth regulation and immune activation (30, 33). Infection of cells with a wide variety of viruses directly induces the transcription and synthesis of some IFNs (e.g., IFN-α4 and IFN-β) (24, 34). These IFNs are secreted and interact with constitutively expressed cell surface receptors in an autocrine or paracrine manner, transducing signals via the JAK-STAT pathway to activate >50 IFN-stimulated genes, which include the type I IFNs (6). Some of these IFN-stimulated genes are responsible for the versatile biological effect of IFNs, including their antiviral activity. In addition to establishing an antiviral state in uninfected cells, the IFN system helps eliminate virally infected cells (38). The IFN system is thus thought to be essential for the survival of higher vertebrates, because it provides an early line of defense, days before the onset of the specific immune response (33).

Sendai virus (SeV), a model paramyxovirus routinely used to induce type I IFNs in cell culture, is a naturally occurring respiratory pathogen of laboratory mice (15). SeV strains exist which differ markedly in their pathogenicity for mice, but the determinants of their virulence are largely unknown. Our reference SeV strain, Z, was originally isolated in the early 1950s in Japan and adapted to grow in embryonated hen's eggs. This adaptation and continuous passage in eggs undoubtedly reduce its virulence for mice, because the 50% lethal dose (LD_{50}) of SeV^Z is ca. 6×10^3, whereas that of the more recently isolated Ohita field strain (from a lethal animal house epidemic and which was passaged only in mice [SeV^M]) is ca. 4×10^1 (16). SeV^M, however, grows poorly in cell culture, and its adaptation for growth in LLC-MK2 (monkey kidney) cells led to virus clones that exhibited various degrees of reduced pathogenicity for mice. One of these clones, MVC11 (SeV^{MVC}) appeared to be totally avirulent (LD_{50} of $>8 \times 10^5$ [i.e., none of the mice died at the highest possible dose]).

Remarkably, SeV^{MVC} contained only two amino acid substitutions relative to the parental SeV^M, namely, F170S in the C proteins, and E2050A in the L protein (Fig. 1) (16). These two mutations presumably account for the relative inability of SeV^{MVC} to replicate in the mouse respiratory tract and cause serious disease. In a previous study, we partially examined which of the two substitutions was responsible for the phenotypes described above by exchanging the C gene of SeV^Z with that of itself, SeV^M, or SeV^{MVC} in turn (generating the recombinants $rSeV^Z$-C^Z, $rSeV^Z$-C^M, and $rSeV^Z$-C^{MVC}, respectively [Fig. 1]) (12). Wild-type $rSeV^Z$-C^Z (recovered from DNA) has the same virulence for mice as the natural Z virus (LD_{50} of ca. 6×10^3), and the replacement of the resident C^Z gene with that of strain M did not affect this virulence. Replacement of the resident C^Z gene with C^{MVC} in two independently isolated viruses, however, increased the LD_{50} of $rSeV^Z$-C^{MVC} by >2 logs, such that the viruses were now as avirulent as SeV^{MVC}. Because the only known difference between $rSeV^Z$-C^M and $rSeV^Z$-C^{MVC} is the C^{F170S} mutation, the normal function of the C proteins appears to be required for virulence in mice. The wild-type SeV C proteins are also known to act as promoter-specific inhibitors of viral RNA synthesis (1, 28, 32). The C^{F170S} mutation is similarly associated with the loss of this function and is at least partially responsible for the enhanced replication of these viruses in MK2 cells.

Although $rSeV^Z$-C^{MVC} was avirulent, it appeared to grow normally in the mouse respiratory tract during the first day of the infection. However, whereas virus titers in $rSeV^Z$-C^M or $rSeV^Z$-C^Z-infected mice lungs increased daily for ca. 5 days, virus titers in $rSeV^Z$-C^{MVC}-infected mice increased only during the first day, and $rSeV^Z$-C^{MVC} was then quickly cleared from the lungs (12). A similar early restriction of the mouse infection was found for rSeV strains which specifically cannot express their AUG^{114}-initiated C protein (22) or which cannot edit their P gene mRNA and therefore do not express their V proteins (7, 8, 19). The rapidity of the host antiviral response in

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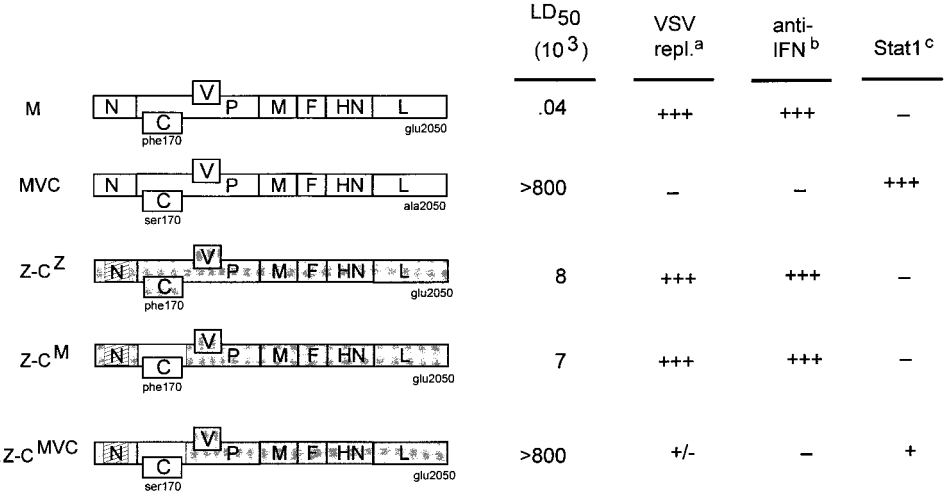

FIG. 1. Genotypes and phenotypes of the SeV strains examined. The genotypes of the various SeV strains examined in this study are represented as rectangles on the left, in which the linear arrays of genes (not drawn to scale) are shown as boxes. The relative positions of the C and V ORFs, which overlap the P ORF, are also indicated. M strain sequences are white, Z strain sequences are gray, and H strain sequences are hatched. These rSeV^{Z} strains all carry a tagged N gene, from which this protein migrates differently on SDS-PAGE than that of strain Z, to distinguish these viruses from natural SeV^{Z}. The amino acids at positions 170 of the C gene and 2050 of the L gene are shown below the genomes. The phenotypes of these virus infections of mice (LD_{50}) are taken from references 12 and 16. Those of BF cells, including the ability of VSV to replicate (repl.) following a period of SeV infection (a) and to prevent IFN from establishing an antiviral state (b) and the resulting intracellular levels of the Stat1 proteins (c) were determined in this study.

immunologically naive mice in limiting the ensuing mutant SeV infections suggested that some aspect of host innate immunity might be involved, presumably due to the loss of V and C protein function. In this view, one function of the SeV C and V proteins would be to modulate the innate immune response, in order to sustain multiple cycles of virus replication in the mouse respiratory tract (19). In this paper, we report that the SeV C proteins play a role in counteracting the IFN-mediated cellular antiviral response.

MATERIALS AND METHODS

SeV stocks and infection. The generation of rSeV strains expressing alternate C (and P) proteins has been described previously (11–13, 22). All SeV stocks were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. Viruses from allantoic fluid stocks were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining after pelleting, and titers were determined by plaquing in LLC-MK2 cells.

The vesicular stomatitis virus (VSV) stock (Mudd-Summers, Indiana) was grown in BHK cells. Virus released into the culture medium was clarified by centrifugation at 10,000 × *g* to remove cell debris, and the titer was determined by plaquing in LLC-MK2 cells. Experiments were performed by infecting (7×10^6) mouse BF cells (36) with various SeV stocks at a multiplicity of infection (MOI) of 10 to 20 in a total volume of 3 ml of minimal essential medium plus 8% fetal calf serum in the presence or absence of 100 U of recombinant mouse IFN-β per ml (Calbiochem). At different times postinfection, cells were superinfected with VSV at an MOI of 50. Five hours later, cells were harvested and then lysed in TNE (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM EDTA) plus 0.5% Nonidet P-40. Proteins were analyzed by immunoblotting, and RNA was extracted with Triazol (Gibco) and analyzed by primer extension.

Immunoblotting. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes by semidry transfer. The primary antibody antibodies used included a rabbit polyclonal antiserum to VSV P protein (provided by J. Perrault and D. Summers), a rabbit polyclonal antiserum to SeV P protein isolated from an SDS gel (anti-P^{SDS}, provided by L. Roux), a mouse monoclonal antibody to SeV N (N 877) (27), a mouse monoclonal antibody to Stat1 (C terminus) (Transduction Laboratories [S21120]), rabbit polyclonal antiserum to actin (provided by G. Gabbiani, Geneva, Switzerland), and rabbit polyclonal antiserum to SeV C protein (provided by Y. Nagai, Tokyo, Japan).

The secondary antibodies used were alkaline phosphatase-conjugated goat antibodies specific for either rabbit or mouse immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Bio-Rad) and quantified by using the Bio-Rad Fluor-S multimager.

Primer extension. Total intracellular RNA was isolated with Triazol, and the genomes present were analyzed by primer extension with Moloney murine leukemia virus reverse transcriptase and 200,000 cpm of ^{32}P-labeled 5′-GAAGCT CCGCGGTACC-3′ (nucleotides 15270 to 15285), which had been purified on a sequencing gel.

RESULTS

As mentioned above, SeV^{MVC} was selected for its ability to replicate more efficiently than SeV^{M} in LLC-MK2 cells, and it was found to accumulate ca. 20-fold more viral protein and mRNA than SeV^{M}-infected cells (reference 16 and data not shown). In BF cells, a cell line that is fully IFN competent (i.e., that secretes IFN in response to a viral infection and responds to IFN by establishing an antiviral state [36]); however, SeV^{M} actually grows slightly better (three- to fivefold) than SeV^{MVC}, as estimated by the intracellular levels of viral proteins or genomes (Fig. 2A to D, lanes 1, 3, and 4). To examine whether IFN might be involved in this reversed SeV^{M}-SeV^{MVC} replication efficiency, parallel BF cultures were pretreated with 100 U of IFN-β for 24 h prior to SeV infection, and the accumulation of viral proteins and genomes was determined. SeV^{MVC} was found to be highly IFN sensitive, because IFN pretreatment reduced the accumulation of viral products >20-fold, (Fig. 2B and D, lanes 2, 5, and 6). SeV^{M}, in contrast, was clearly less sensitive, because IFN pretreatment reduced the accumulation of viral products less than fivefold (Fig. 2A and C, lanes 2, 5, and 6).

If endogenously generated IFN is involved in the reversed SeV^{M}-SeV^{MVC} replication efficiency in BF cells, infection with SeV^{MVC} should induce a general antiviral state more strongly during this infection than infection with SeV^{M}. The rhabdovirus VSV is highly IFN sensitive, and its inability to grow in cell culture is considered a reliable indicator of this state. Some BF cultures were therefore pretreated with IFN-β for 24 h prior to SeV^{M} or SeV^{MVC} infection and then superinfected with VSV at 48 h postinfection (hpi). We found that 48 h of SeV^{MVC} infection was sufficient to prevent VSV proteins from accumulating during the VSV superinfection, even when these cultures had not been pretreated with IFN (Fig. 2F, lanes 3 to 6). In contrast, VSV proteins accumulated normally during the superinfection of cells infected with SeV^{M} for 48 h (Fig. 2E, lanes

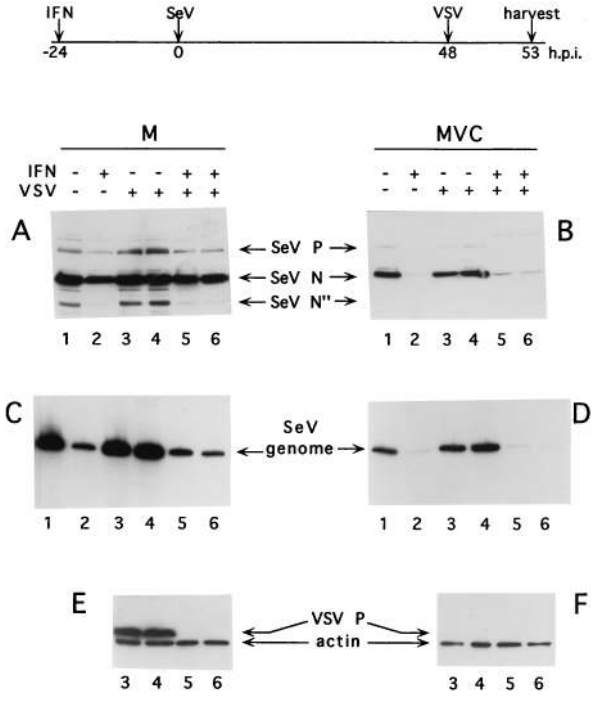

FIG. 2. SeV^{MVC} infection of BF cells, in contrast to SeV^{M} infection, induces an anti-VSV state. Parallel cultures of BF cells were treated (or not) with 100 U of recombinant IFN-β, infected 24 h later with 10 to 20 PFU of either SeV^{M} or SeV^{MVC} per cell, and then superinfected with 50 PFU of VSV per cell at 48 h post-SeV infection, as indicated. Lanes 3 and 4 and 5 and 6 represent independent duplicate infections. All cultures were harvested at 53 hpi, cytoplasmic extracts were prepared, and equal samples (2% of the 10-cm-diameter dish) were (i) separated by SDS-PAGE and immunoblotted with a mixture of anti-SeV P and anti-SeV N antibodies (A and B) or a mixture of anti-VSV P and antiactin antibodies (E and F), and (ii) 10% of a 10-cm-diameter dish was used to isolate total RNA, and the relative amounts of genome RNA present were determined by primer extension. SeV N″ indicates a natural breakdown product of the N protein. A timeline of the experiment is shown above.

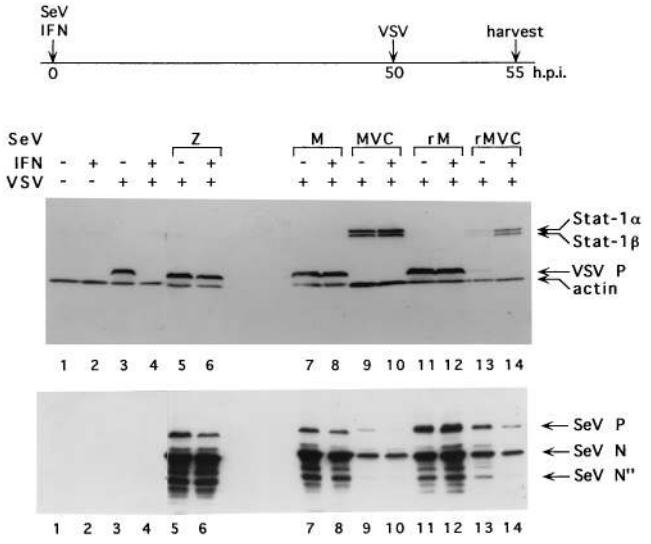

FIG. 3. SeV^{M}, $rSeV^{Z}$-C^{M}, and $rSeV^{Z}$-C^{Z} infection of BF cells, in contrast to SeV^{MVC} and $rSeV^{Z}$-C^{MVC} infection, interferes with the IFN-mediated induction of an anti-VSV state. Parallel cultures of BF cells were treated (or not) with 100 U of IFN-β and infected at the same time with 10 to 20 PFU of either $rSeV^{Z}$-C^{Z} (Z), SeV^{M} (M), SeV^{MVC} (MVC), $rSeV^{Z}$-C^{M} (rM), or $rSeV^{Z}$-C^{MVC} (rMVC) per cell and then superinfected with 50 PFU of VSV per cell at 50 h post-SeV infection, as indicated above. All cultures were harvested at 55 hpi, cytoplasmic extracts were prepared, and equal samples (2% of a dish) were separated by SDS-PAGE and immunoblotted with a mixture of anti-VSV P, anti-Stat1, and antiactin antibodies (top panel) or a mixture of anti-SeV P and anti-SeV N antibodies (bottom panel). A timeline of the experiment is shown above.

3 and 4). Thus, infection of BF cells with SeV^{MVC} induces an anti-VSV state (after 24 to 48 h [data not shown]), whereas infection with SeV^{M} does not induce this state, even at 72 hpi (data not shown). In all of the experiments reported here, the appearance of normal levels of VSV P protein on superinfection correlated with strong cytopathic effects and cell death, whereas the absence of VSV P protein correlated with the absence of cytopathic effects and cell survival.

SeV^{M} infection, but not that of SeV^{MVC}, interferes with the IFN-mediated induction of an antiviral state. The absence of an anti-VSV state following SeV^{M} infection could be because SeV^{M} avoids triggering the IFN system. Alternatively, SeV^{M} infection may interfere with the establishment of an antiviral state, even though it does not suppress the anti-VSV state induced by 24 h of IFN pretreatment (Fig. 2E, lanes 5 and 6). BF cultures were therefore treated with IFN at the same time as they were infected, so that the IFN-mediated induction of the antiviral state would take place concomitantly with the SeV infection and not have a 24-h head start as in Fig. 2. These cultures were then superinfected with VSV at 50 h post-SeV infection, and the accumulation of VSV (and SeV) proteins was determined by immunoblotting at 55 hpi. The intracellular levels of the 91-kDa Stat1α and 84-kDa Stat1β proteins in these extracts were also determined by immunoblotting. Stat1 is a key component of the signaling cascade which ensues with activation of the IFN receptor (6, 9, 26), and its elevated expression leads to programmed cell death (PCD), another cellular response to virus infection (3, 20, 29).

As shown in Fig. 3, 50 h of infection with SeV^{M} (lanes 7 and 8) did not prevent the VSV P protein from accumulating normally (lane 3) on VSV superinfection, nor did it increase Stat1 levels over background levels. This infection, moreover, totally prevented IFN from inducing an anti-VSV state, as judged by the normal intracellular accumulation of the VSV P protein. In contrast, 50 h of infection with SeV^{MVC} (lanes 9 and 10) strongly induced an anti-VSV state even in the absence of IFN treatment and very dramatically increased Stat1 levels (up to 100-fold). SeV^{M} infection thus appears to interfere with the establishment of an IFN-mediated antiviral state, as well as the elevated expression of Stat1.

The role of the C protein. SeV^{M} and SeV^{MVC} differ by two amino acid substitutions, C^{F170S} and L^{E2050A} (Fig. 1). To examine the role of the C gene mutation in suppressing the ability of SeV^{M} to interfere with the establishment of an IFN-mediated antiviral state, we examined the infection of BF cells with $rSeV^{Z}$-C^{M} and $rSeV^{Z}$-C^{MVC}. These are chimeric viruses in which the resident C gene of SeV^{Z} (and the overlapping portion of the P gene) was replaced with those of SeV^{M} and SeV^{MVC}. (The P genes of the M and Z strains are 85% identical.) $rSeV^{Z}$-C^{M} and $rSeV^{Z}$-C^{MVC} thus differ only by the C^{F170S} mutation. $rSeV^{Z}$-C^{Z}, in which the C gene was exchanged with itself as a cloning control (12), served as the wild-type $rSeV^{Z}$. As shown in Fig. 3, $rSeV^{Z}$ (lanes 5 and 6) and $rSeV^{Z}$-C^{M} (lanes 11 and 12) behaved similarly to wild-type SeV^{M} (lanes 7 and 8). Fifty hours of these infections did not prevent the VSV P protein from accumulating normally on VSV superinfection, nor did it increase Stat1 levels over background levels. These infections also prevented IFN from inducing an anti-VSV state. In contrast, 50 h of infection with $rSeV^{Z}$-C^{MVC} (lanes 13 and 14) strongly induced an anti-VSV

state even in the absence of IFN treatment, but led to only a very modest increase in Stat1 levels over background levels (lane 13). This slight increase, however, is superior to that induced with IFN alone (lane 2), and IFN treatment plus $rSeV^{Z}$-C^{MVC} infection appeared to act synergistically on Stat1 levels (lane 14). Thus, viruses that contain a wild-type C gene (SeV^{M}, $rSeV^{Z}$-C^{M}, or $rSeV^{Z}$-C^{Z} [all C^{F170}]) appear to interfere efficiently with the establishment of an IFN-mediated antiviral state, in contrast to those with a mutant C gene (SeV^{MVC} and $rSeV^{Z}$-C^{MVC} [both C^{S170}]). The C proteins thus appear to be important in determining whether SeV infection will induce an antiviral state.

The AUG^{114}-initiated C protein is specifically required to counteract IFN. The SeV C proteins are expressed as a nested set of four proteins (C′, C, Y1, and Y2) initiated at different start codons by a variety of ribosomal gymnastics, including the use of non-AUG codons, leaky scanning, and shunting (5, 23). We have previously described rSeV strains which selectively do not express either their C′, C, or C′, and C proteins (double mutant), due to mutation of their respective start codons. The phenotypes of the C'^{-} and C^{-} viruses were similar. They grew slightly less well than wild-type virus in eggs, and their infections of BHK cells overaccumulated viral macromolecules, consistent with the ability of either C′ or C (but not Y1 and Y2) to inhibit viral RNA synthesis (4). Despite these similar phenotypes in eggs and cell culture, C'^{-} virus was as virulent as $rSeV^{Z}$ for mice, whereas C^{-} virus was avirulent. The double mutant, which in contrast to the single mutants was relatively noncytopathic in cell culture (and displayed a small-plaque phenotype), was also avirulent at the highest doses tested (22).

Figure 4 shows the effect of infection with rSeV strains which do not selectively express their C′ or C proteins on Stat1 levels and subsequent VSV superinfection, in comparison to that of $rSeV^{Z}$-C^{M} and $rSeV^{Z}$-C^{MVC}. Only C'^{-} behaved like its wild-type control ($rSeV^{Z}$) (lanes 1 and 2). Fifty hours of C'^{-} infection (lanes 9 and 10) did not induce an anti-VSV state (lane 9) and prevented the effects of IFN treatment as well (lane 10), and the levels of Stat1 proteins were not elevated over background levels (lanes 9 and 10). C^{-} infection (lanes 7 and 8), in contrast, and the double mutant infection (lanes 11 and 12) to an even greater extent did (at least partially) induce an anti-VSV state and significantly elevated Stat1 levels over background levels. Thus, the AUG^{114}-initiated C protein (but not C′, Y1, or Y2) is preferentially required to prevent SeV infection from inducing the IFN system in cell culture, consistent with its specific requirement for sustained viral replication in the mouse respiratory tract (22).

Suppression of the IFN-mediated anti-VSV state is dominant. Although SeV^{MVC} grows ca. 20-fold better than SeV^{M} in MK2 cells, this virus actually grows ca. 5-fold less well than SeV^{M} in eggs (as well as in BF cells), and these relative replication efficiencies also apply to $rSeV^{Z}$-C^{MVC} versus $rSeV^{Z}$-C^{M}. All of our rSeV strains are isolated in hen's eggs, and generally stocks at the 2nd or 3rd passage level (p2 or p3) are used to infect cell cultures. Six $rSeV^{Z}$-C^{MVC} stocks were originally isolated from DNA independently, and all behaved as shown (Fig. 3) and grew relatively poorly in eggs. On further passage, however, some $rSeV^{Z}$-C^{MVC} stocks clearly grew better (5- to 10-fold) than others. Because the F170S mutation apparently confers reduced growth in eggs (and the loss of an *Xmn*I site), the relevant region of the viral genomes in two stocks which grew well (p4MVC-118 and p5MVC-1.8) and two which grew poorly (p4MVC-119 and p3MVC-9.9) was amplified and examined (Fig. 5). At the same time, BF cells were infected with the same stocks and superinfected with VSV at

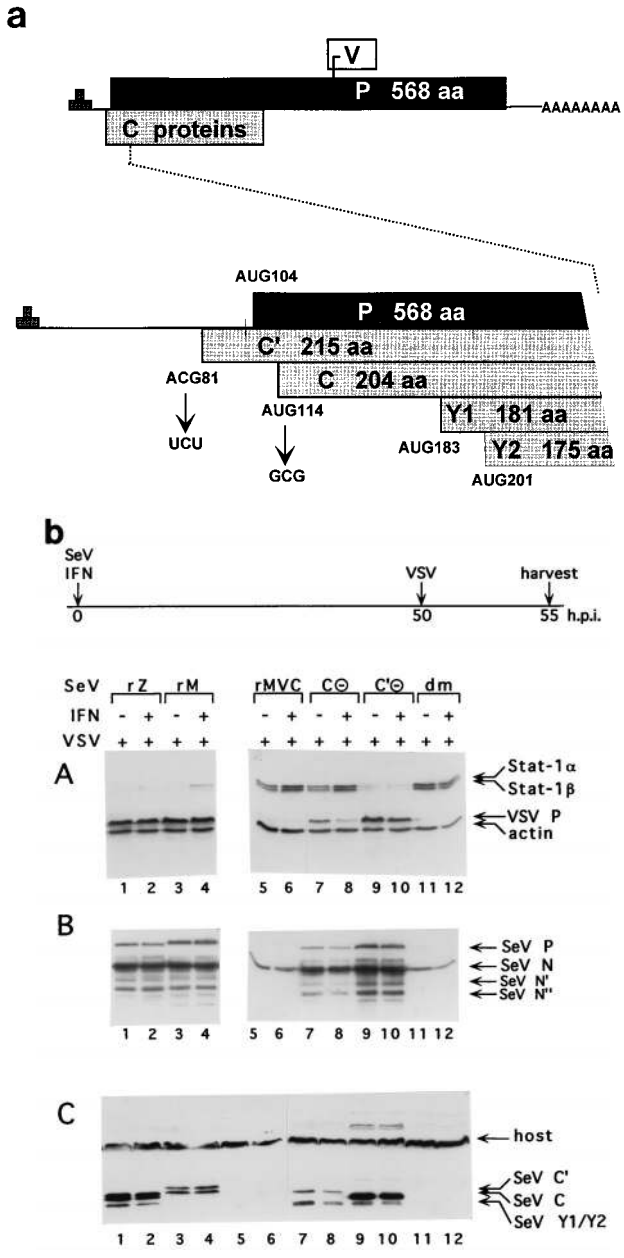

FIG. 4. (a) ORF organization and expression of the SeV P gene. The three ORFs expressed as proteins (P, C, and V) are shown as horizontal boxes, drawn roughly to scale (above). An expanded diagram of the 5′ end of the mRNA is shown in the middle, and the five ribosomal start sites in this region are indicated. The mutations used to eliminate expression from the C′ and C protein start codons are shown. Numbers refer to positions from the 5′ end of the mRNA and to the first base of the start codon. (b) The AUG^{114}-initiated C protein is preferentially required to prevent the IFN-mediated induction of the antiviral state. Parallel cultures of BF cells were treated (or not) with 100 U of IFN-β and infected at the same time with 5 PFU of either $rSeV^{Z}$-C^{Z} (rZ; lanes 1 and 2), $rSeV^{Z}$-C^{M} (rM; lanes 3 and 4), $rSeV^{Z}$-C^{MVC} (rMVC; lanes 5 and 6), $rSeV^{Z}$-[C^{-}] ($C\ominus$; lanes 7 and 8), $rSeV^{Z}$-[C'^{-}] ($C'\ominus$; lanes 9 and 10), or $rSeV^{Z}$-[C'/C^{-}] (dm [double mutant]; lanes 11 and 12) per cell. The cultures were then superinfected with 50 PFU of VSV per cell at 50 h post-SeV infection, as indicated above. All cultures were harvested at 55 hpi. Cytoplasmic extracts were prepared, and equal samples (2% of a dish) were separated by SDS-PAGE and immunoblotted with a mixture of anti-VSV P, anti-Stat1, and antiactin antibodies (A); a mixture of anti-SeV P and anti-SeV N antibodies (B); or antibodies to the SeV C protein (C). In panel C, note that the C^{M} proteins (lanes 3 and 4) migrate slightly slower than the C^{Z} proteins (lanes 1 and 2 and 7 to 10), even though they are of the same length. The C^{MVC} (lanes 5 and 6) and C^{dm} proteins (lanes 11 and 12) are not detected in these samples, because these viruses grow relatively poorly in BF cells (B). A timeline of the experiment is shown above.

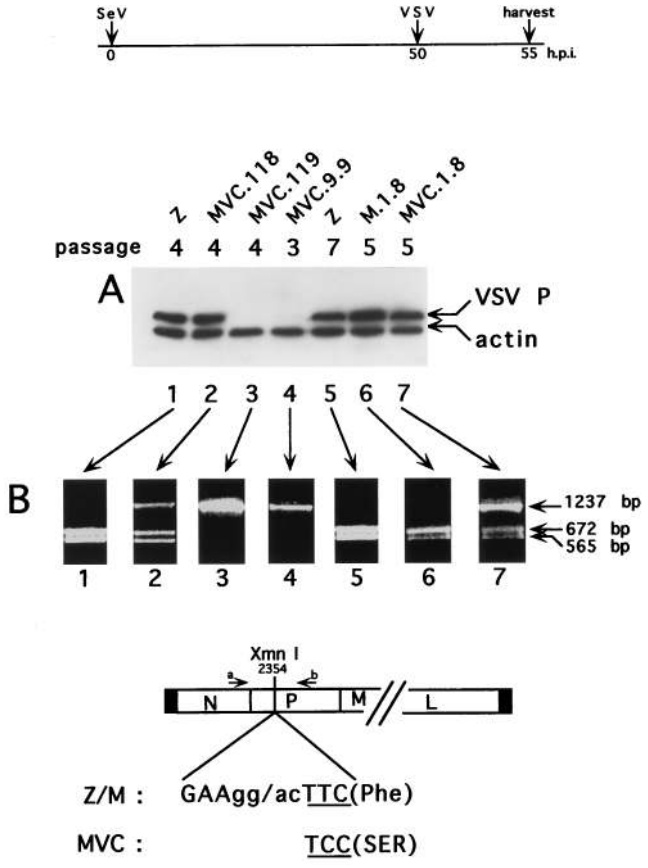

FIG. 5. Reversion of rSeV^{Z}-C^{MVC} to rSeV^{Z}-C^{M} by passage in hen's eggs. Four independently generated rSeV^{Z}-C^{MVC} stocks, including two which continued to grow poorly in eggs (MVC.119 and MVC.9.9; lanes 3 and 4) and two which began to grow normally in eggs (MVC.118 and MVC.1.8; lanes 2 and 7), along with two stocks of rSeV^{Z}-C^{Z} (lanes 1 and 5) and one of rSeV^{Z}-C^{M} (M1.8; lane 6), at the egg passage levels indicated, were used to infect BF cultures at an MOI of 10 to 20. The cultures were superinfected with VSV at 50 h post-SeV infection, and the relative amounts of intracellular VSV P protein at 55 hpi were determined by immunoblotting (A). RNA was isolated from these allantoic fluid stocks, and 1237 bp of the relevant region of the viral genome (T/C^{2354}) was amplified by reverse transcription-PCR as shown below. The PCR product was digested with *Xmn*I, separated on an agarose gel, and stained with ethidium bromide (B).

48 hpi as before, to determine whether infection with these stocks continued to induce an anti-VSV state.

As shown in Fig. 5, the two stocks that continued to grow poorly (lanes 3 and 4) contained genomes in which the *Xmn*I site remained absent (Ser at position 170), and 48 h of infection with these stocks continued to strongly prevent subsequent VSV growth. In contrast, (i) the two stocks that grew well (lanes 2 and 7) contained genomes which had regained the *Xmn*I site and had therefore reverted to Phe at position 170, as well as those in which this site was absent (170S), and (ii) these stocks had lost the ability to prevent VSV growth, like rSeV^{Z}-C^{M} (lane 6). Influenza viruses that cannot express their NS1 protein grow poorly in eggs because they are more sensitive to the egg's developing IFN system (10). If SeV were also sensitive to the egg's IFN system, there would be pressure to select for mutants with restored ability to suppress IFN action. Because the S170F reversion requires only the single back transition, it would be expected to be present after several passages in eggs.

The infections in Fig. 5 were carried out at 10 to 20 PFU/cell, and cultures infected with p4MVC-118 or p5MVC-1.8 would thus be coinfected with a mix of rSeV^{Z}-C^{MVC} and the revertant rSeV^{Z}-C^{M}. In both of these mixed infections (and other reconstituted mixed infections [data not shown]), the phenotype of the revertant rSeV^{Z}-C^{M} was dominant over rSeV^{Z}-C^{MVC} and the antiviral state was not induced (i.e., VSV replication was not prevented by the rSeV^{Z}-C^{MVC} coinfection). The dominant nature of the suppression of the IFN-mediated anti-VSV state in mixed infections is consistent with this suppression being an active process.

DISCUSSION

To sustain its infection of the mouse respiratory tract, SeV must avoid or counteract the innate immune response of its animal host, which includes natural killer cells, the IFN system, and PCD. The most virulent virus strains, like SeV^{M}, must be extremely efficient at these countermeasures, because they require only ca. 40 PFU to kill half of the mice inoculated intranasally. SeV^{MVC}, an SeV^{M} mutant with only two amino acid substitutions (one in the C gene and the other in the catalytic subunit of the viral polymerase [L]), however, appears to be virtually defenseless against the innate immune system of the mouse, because lung titers increase only during the first day of infection, and virus is then quickly cleared (16). Moreover, similar results were found for mice infected with rSeV^{Z}-C^{MVC}, a chimeric Z strain virus containing the mutant SeV^{MVC} C gene, or rSeV^{Z}-[C^{-}], which contains a wild-type C gene but does not express the AUG^{114}-initiated C protein. The SeV C gene thus plays a critical role in infections of its animal host, presumably by counteracting innate immunity.

Itoh et al. (17) have recently found that SeV^{M} infections do not induce PCD either in MK2 cells or in the mouse respiratory tract, whereas SeV^{MVC} infections are highly apoptogenic in both cases. The present work has found that SeV^{M} infection interferes with the IFN-mediated induction of an antiviral state, in strong contrast to SeV^{MVC}. It is possible that SeV^{M} infections counteract the induction of PCD and the antiviral effects of IFN by separate pathways, but it is more likely that these effects are linked. Cells from mice lacking PKR (35) or the 2-5A-oligoadenylate synthetase dependent RNase L (37) show defects in PCD, suggesting a role for these enzymes in virus-induced, IFN-mediated PCD. Furthermore, several viruses have recently been found to induce PCD via activation of the IFN system (31). The Stat1 proteins themselves also induces genes involved in PCD (3, 20, 29), and SeV^{MVC} infection may be highly apoptogenic because it induces high levels of Stat1.

The molecular basis of the antiviral response to IFN is not fully understood. More than 50 genes are induced by IFN-α/β, but only a few of these gene products display intrinsic antiviral activity, namely, p68 protein kinase (PKR) and 2-5A oligoadenylate synthetase (both dependent on double-stranded RNA for activity) (18), certain Mx family proteins (reviewed in reference 30), and, most recently, the promyelocytic leukemia (PML) protein (2). While IFN-treated cells are resistant to many different viruses, individual overexpression of these intrinsically antiviral proteins confers resistance only against some viruses. Thus, overexpression of PKR or 2-5A oligoadenylate synthetase confers resistance to the picornavirus encephalomyocarditis virus (EMCV), but not to the rhabdovirus VSV, whereas overexpression of human MxA or PML proteins confers resistance to VSV, but not to EMCV (2). The enormous selective pressures imposed by viruses have resulted in a rich and diverse set of antiviral pathways, and the antiviral state induced via the IFN system is thus suitably complex. We

have used two criteria—the ability of VSV to replicate following a period of SeV infection and the intracellular levels of the Stat1 proteins—as indicators of this cellular antiviral state. We have found that two different mutations in the C gene, the F170S substitution and the specific loss of AUG^{114}-initiated C protein (although C′ is normally expressed and the Y1 and Y2 proteins are overexpressed), largely eliminate the ability of the mutant infections to prevent the IFN-mediated antiviral state. The SeV C gene thus plays a critical role in this process. The E2050A mutation in the L gene of SeV^{MVC} also appears to be involved in the attenuation of this virus, because the phenotype of SeV^{MVC} is consistently stronger than that of $rSeV^{Z}$-C^{MVC}, especially in preventing the elevation of Stat1 proteins, thereby eliminating one pathway to the induction of PCD.

While this paper was under review, Didcock et al. (8a) reported that SeV strain H (SeV^{H}, which is very similar to SeV^{Z}) circumvents the IFN response and does so by interfering with the transcriptional activation of IFN-responsive genes. This study used only wild-type SeV^{H} and another paramyxovirus, SV5. However, it directly examined IFN signaling in BF cells, using transient transfection of plasmids in which a luciferase reporter gene was placed under the control of an IFN-α/β-responsive promoter (or an IFN-β promoter), followed by virus infection. The IFN-α/β-responsive promoter was found to be strongly activated in both mock- and SV5-infected cells treated with IFN, whereas little or no activation of this promoter was observed in SeV-infected cells. In contrast, SeV was a stronger inducer of the IFN-β promoter than SV5. These authors concluded that the failure of SeV^{H}-infected cells to respond to the substantial levels of IFN-α/β they produce was due to the effectiveness of the SeV^{H}-induced block. SeV^{M} and SeV^{MVC} infections of human peripheral blood mononuclear cells are found to produce equivalent (and substantial) amounts of IFN-α (3,395 IU/ml for M and 3,776 IU/ml for MVC) (13b). Thus, all of these wild-type SeV strains (strains M, Z, and H) presumably counteract the IFN-induced antiviral state by interfering with the activation of IFN-stimulated genes.

The dominant nature of $rSeV^{Z}$-C^{M} versus $rSeV^{Z}$-C^{MVC} in coinfections of eggs (Fig. 5) and BF cells [data not shown] suggests that the C gene actively interferes with the induction of the antiviral state rather than simply avoiding the induction, although it might do this as well. We have not as yet examined whether the C gene products can act in BF cells independent of virus infection. The C proteins might well interact with directly (and suppress) some component of the signaling pathway leading to an antiviral state, because many viruses are known to subvert the IFN system by a wide variety of mechanisms (30). The apparent enhancing effect of the polymerase L^{E2050A} mutation in viruses with C^{F170S} (SeV^{MVC}), however, suggests that the C proteins act as well via viral RNA synthesis. C is well placed to do this. The linear paramyxovirus genomes and antigenomes contain promoters for RNA synthesis at their 3′ ends. The C protein acts as an inhibitor of viral RNA synthesis in a promoter-specific fashion (1, 32); i.e., it predominantly inhibits antigenome and mRNA synthesis from the genomic promoter. This inhibition depends on the relative ratio of C protein to genomes, which increases dramatically during the early stages of infection, because C protein is essentially a nonstructural protein.

The SeV V protein is also known to affect viral RNA synthesis (3a). However, in contrast to rSeV-[C^{-}], infection of BF cells with two different rSeV strains which cannot edit their P gene mRNA and do not express V (Δ6A [7] and A5G3 [13a]) was similar to that of wild-type virus. These rSeV-[$edit^{-}$] infections did not induce an anti-VSV state, and they continued to prevent IFN (added concomitant with the infection) from inducing an anti-VSV state (data not shown). The SeV V protein therefore does not appear to be important in counteracting IFN action, although it is also thought to function in counteracting some aspect of innate immunity (19). The SeV C and V proteins have been referred to as "accessory" proteins, in the sense that they are not expressed by all paramyxoviruses. For example, human parainfluenza virus type 1, the virus closest to SeV and a very successful parasite of children, does not contain a V open reading frame (ORF) at all (25), and rubulaviruses (except for Newcastle disease virus) do not contain a C ORF. The overlapping C and V ORFs appear to have been added to the P ORF as a late event of virus evolution, possibly to adapt these viruses to a particular ecological niche (e.g., the mouse respiratory tract). The V gene clearly fits this description, providing a "luxury" function (19), because rSeV strains which do not express V are perfectly competent for growth in cell culture or eggs and are only debilitated for growth in the mouse respiratory tract (7, 8, 19).

The C gene is more complex than the V gene. There are four independently initiated C proteins, and the phenotypes of viruses in which C′ and C have been specifically ablated suggest that the various C proteins must, at least in part, have nonoverlapping functions. While rSeV strains which cannot individually express C′ or C grow well in (IFN-incompetent) BHK cells and accumulate viral macromolecules more rapidly than wild-type virus, the double mutant (which cannot express either C′ or C) grows poorly in BHK cells and accumulates viral macromolecules with delayed kinetics relative to wild-type virus. C′ and C (but not Y1 or Y2) may provide a common function that accelerates the accumulation of viral products intracellularly, so that only the double mutant shows the loss-of-function phenotype (22). C′ and C also appear to have nonoverlapping functions, because although C or Y1 and Y2 can replace C′ for virulence in mice, C′ or Y1 and Y2 cannot replace C for this property (22). Y1 and Y2 presumably also play a role in intracellular virus replication, because rSeV strains which cannot express any of their C proteins have not been isolated (22), and those which at best express only some Y2 protein are at the limit of recovery from DNA (21, 23). Unlike the V gene, the SeV C gene clearly plays an essential role in intracellular virus replication, even in the protected environment of defenseless cells in culture. What may have started out as a luxury function could have evolved to carry out essential functions in virus replication. Multiple forms of C protein expression may then have evolved to better regulate their function.

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