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Sendai virus defective-interfering genomes and the activation of interferon-beta

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Abstract

The ability of some Sendai virus stocks to strongly activate IFNβ has long been known to be associated with defective-interfering (DI) genomes. We have compared SeV stocks containing various copyback and internal deletion DI genomes (and those containing only nondefective (ND) genomes) for their ability to activate reporter genes driven by the IFNβ promoter. We found that this property was primarily due to the presence of copyback DI genomes and correlated with their ability to self-anneal and form dsRNA. The level of IFNβ activation was found to be proportional to that of DI genome replication and to the ratio of DI to ND genomes during infection. Over-expression of the viral V and C proteins was as effective in blocking the copyback DI-induced activation of the IFNβ promoter as it was in reducing poly-I/C-induced activation, providing evidence that these DI infections activate IFNβ via dsRNA. Infection with an SeV stock that is highly contaminated with copyback DI genomes is thus a very particular way of potently activating IFNβ, presumably by providing plentiful dsRNA under conditions of reduced expression of viral products which block the host antiviral response.

Keywords: Sendai virus; Defective-Interfering genome; Interferon beta; Innate immunity

Our understanding of how animal cells recognize and mount an innate antiviral response to intracellular RNA virus replication has recently made great progress. Two DexD/H box helicases with CARD domains, RIG-I and Mda5/Helicard, were found to participate in the detection of cytoplasmic dsRNA (Andrejeva et al., 2004; Yoneyama et al., 2004). dsRNA is thought to be a common product of RNA virus infections that acts as a pathogen-associated molecular pattern (PAMP) responsible for initiating the innate antiviral response. RIG-I and Mda5 initiate antiviral responses by coordinately activating several transcription factors, including NF-κB and IRF-3, that bind to the IFNβ promoter forming an enhanceosome that activates this primary host-response gene (McWhirter et al., 2005). Upon binding dsRNA, the CARD domains of these helicases are thought to be freed for interaction with the synonymous domain of MAVS/VISA/cardif/IPS-1, a mitochondrial protein which in turn is required for recruiting the kinases that activate these transcription factors (Xu et al., 2005; Meylan et al., 2005; Seth et al., 2005; Kawai et al., 2005). Autocrine interaction of secreted IFNβ with its cell surface receptor then closes the "innate immunity loop", leading to increased IFN-stimulated gene (ISG) products, such as other IFNs and the intracellular effectors of the antiviral state (Sen, 2001).

The role of Mda5 in dsRNA signaling to IFNβ was uncovered because Mda5 binds to the paramyxovirus SV5 V protein (Andrejeva et al., 2004). This interaction, and that of other paramyxovirus V proteins, blocks dsRNA signaling, and this property of V maps to the highly conserved cys-rich domain at their C-termini (Poole et al., 2002; Andrejeva et al., 2004). V proteins of different paramyxoviruses, however, are very different at their N-terminal portions, which accounts for their otherwise very different properties (Lamb and Kolakofsky, 2001). Rubulavirus V proteins (e.g., SV5, PIV2) are associated with intracellular and virion nucleocapsids (NCs) and are important in promoting virus growth. PIV2 which cannot express the entire V protein is highly debilitated even in the most permissive cell culture (Nishio et al., 2005). Respirovirus (e.g., Sendai virus (SeV)) V proteins, in contrast, are nonstructural proteins, are not associated with NCs and their expression

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inhibits genome replication (Curran et al., 1991). Moreover, although V expression is not required for replication in cell culture, SeV V expression is essential for virulence in mice (Kato et al., 1997; Delenda et al., 1998). This requirement is presumably due to its ability to block dsRNA-induced activation of the IFNβ promoter (Poole et al., 2002) as infection with SeV that cannot specifically express the V protein strongly activates IFNβ (Strahle et al., 2003). Finally, in contrast to rubulaviruses, respirovirus V mRNAs express a nested set of C proteins from an overlapping ORF, and these C proteins also block the innate antiviral response, in several ways (e.g., by blocking IFN signaling) (Gotoh et al., 1999; Garcin et al., 1999).

It is noteworthy that infection with wild-type SeV or SV5 does not normally activate IFNβ. In contrast, infections with SV5 VΔC (which produces a C-terminally truncated V protein) or infections with SeV with mutations in either the leader region, two regions of the C protein or the V protein all lead to enhanced levels of IFNβ mRNA (He et al., 2002; Strahle et al., 2003). In the case of SeV, these viral genes also appear to be involved in preventing activation of inflammatory cytokines such as IL-8. For SeV, all the viral products that are not known to play essential roles in the replication process itself (the V and C proteins, and leader and trailer RNAs) appear to function in countering the innate antiviral response. In the context of a normal, wild-type infection of cells in culture, the effect of these various viral anti-host-response products is apparently sufficient to prevent, or severely limit, IFNβ activation.

The IFNβ promoter is normally activated in cells treated with dsRNA (poly I:poly C, or poly-I/C) or infected with virus. Sendai virus infection is often used in this respect, and virus stocks which strongly activate IFNβ are also available commercially. However, it has long been known that the remarkable ability of some SeV stocks to induce IFN secretion in macrophage and other cell lines is related to the presence of defective-interfering (DI) particles (Johnston, 1981; Poole et al., 2002), but the nature of the IFN-inducing agent in these stocks was not examined. Moreover, for VSV, another mononegavirus, DI particles containing "snap-back" DI genomes (see below) were found to be very potent inducers of IFN, even in the absence of co-infecting nondefective (ND) helper virus (Marcus and Sekellick, 1977; Sekellick and Marcus, 1982). DI particles contain deleted viral genomes which are generated spontaneously as by-products of ND genome replication. DI genomes have, sine qua non, gained the ability to successfully compete with their helper ND genomes for the viral replication substrates provided by the latter; hence, they are also "interfering" (Perrault, 1981; Lazzarini et al., 1981). Because of their replicative advantage over ND genomes, DI genomes invariably accumulate in SeV stocks that are repeatedly passed in eggs, unless steps to prevent this accumulation are taken.

Nonsegmented negative-strand RNA viruses (NNV) DI genomes can be of two types, internal deletion or copyback (Fig. 1). The replicative advantage of internal deletion DI genomes over ND genomes is not well understood (Garcin et al., 1994), but that of copyback DI genomes is well studied. Copyback DI genomes have always replaced the weaker genomic replication promoter at the 3′ ends of their minus strands with the stronger antigenomic promoter, and thus both DI genomes and antigenomes initiate from the same strong antigenomic promoter. Paramyxovirus replication promoters are contained within the 3′ terminal 91–96 nt of the genomic RNA (narrow boxes, Fig. 1), and all natural copyback DI genomes have copied back 91 nt or more while carrying out this promoter exchange (see Fig. 1). Although these RNAs contain termini that are perfectly complementary for ca. 100 nt, DI genomes are normally present within nucleocapsids (NCs), where their RNA ends are not free to anneal. However, when SDS is used to gently dissociate the N protein from RNA, copyback DI genomes (and antigenomes) rapidly form ssRNA circles with dsRNA panhandles, as seen in the EM and on biochemical analysis (Kolakofsky, 1976). Several copyback DI genomes from independent virus stocks were characterized initially. They all contained complementary termini of ca. 100 nt in length, and as expected, the rate at which they circularized on SDS treatment was inversely proportional to their length.

Similar copyback DI genomes with limited terminal complementarity are common for VSV. However, VSV, unlike SeV, also generates an extreme form of copyback DI genome whose sequences are complementary over their entire length of ca. 2 kb (snapback DIs) and which form long dsRNA "hairpins" (of ca. 1000 bp) rather than ss circles upon SDS treatment (Lazzarini et al., 1981; Perrault, 1981). It is these VSV snapback DI genomes, like DI 011, that were reported to strongly induce IFN by themselves, in aged chick embryo fibroblasts (CEFs) and mouse L cells (Marcus and Gaccione, 1989; Marcus and Sekellick, 1977). These reports have remained controversial, however, because this IFN induction was independent of co-infecting helper virus, whereas Youngner and colleagues found that it correlated with contaminating ND virus in L cells. These latter workers, moreover, were unable to find a correlation between the snapback content of their DIs and IFN induction (Frey et al., 1979). Sekellick and Marcus also reported that snapback DI induction of IFN was unaffected by heat treatment that would inactivate its RNA polymerase, or UV treatment that would prevent its genome from being copied, and concluded that this IFN induction was due to a pre-existing molecule that did not require any synthetic events for its formation (Sekellick and Marcus, 1982; Marcus and Gaccione, 1989). Disassembly of DI 011 NCs intracellularly would then appear to be the only explanation for dsRNA formation. However, as the infection of a single DI 011 particle per CEF was sufficient to induce a quantum (maximum) yield of IFN, the manner in which the DI 011 NC is presumably disassembled so efficiently in vivo (to permit dsRNA formation) remains an enigma as NCs are generally very stable in vivo.

Given the growing appreciation that dsRNA may be a common product of RNA virus infection that participates in the induction of the innate antiviral response, we have reexamined the requirement of SeV DI genomes for the activation of the IFNβ promoter.

Results

Three SeV stocks containing DI genomes (Figs. 1, 2a), as well as a stock containing only ND genomes, were examined for

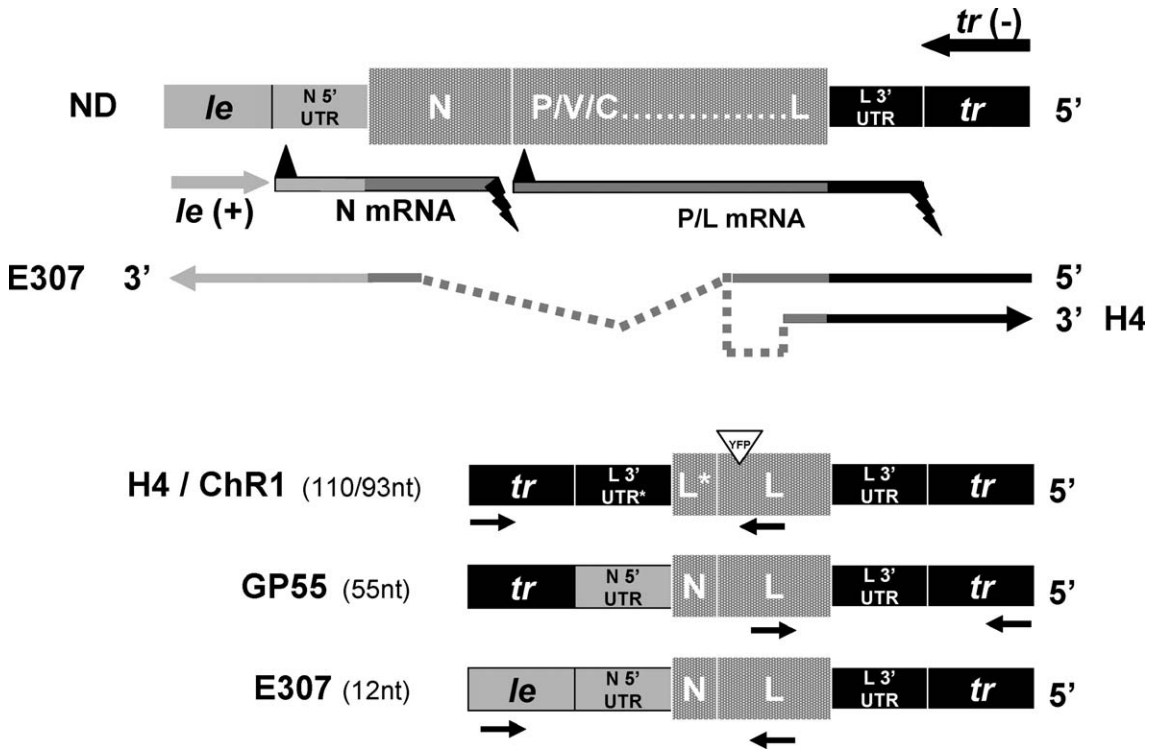

Fig. 1. A schematic view of SeV RNA synthesis and DI genomes. The ND (−) genome is shown above, with its 3′ end genomic replication promoter (on the left) and the complement of the antigenomic replication promoter (at the 5′ end), both contained within the terminal 96 nt, shown as thin boxes. The central protein coding region, from the beginning of the N ORF to the end of the L ORF, is shown as thicker boxes; these regions are not drawn to scale. Only the N/P junction is shown, the remaining genes are lumped together as a P/V/C … L. The (+) RNAs transcribed from the ND genome (*le* RNA and the 6 mRNAs) are shown below the ND genome. The sole transcript of the ND-antigenome, *tr* RNA, is shown above. The lines below the ND genome indicate the progress of the replicase when generating internal deletion (E307) or copyback (H4/ChR1) DI genomes. The structure of the DI (−) genomes is shown below; the dark shading on the left indicates the extent of the terminal complementarity, which is also indicated in brackets on the left. The inverted triangle indicates the yfp sequence used to tag H4. The arrows below the DI genomes show the PCR amplification strategy used to clone the DI RNA of the Charles River SeV stock.

their ability to activate a luciferase reporter gene driven by the IFNβ promoter. The DI-H4 stock is composed predominantly of a natural 1410 nt long copyback DI genome, whose termini are perfectly complementary for 110 nt (Calain et al., 1992). The DI-S104 stock, like H4, was generated by passage in eggs, but this stock is composed of 5 major DI species. Only the smallest DI of this stock has been cloned as DNA and found to be an internal deletion DI of 1794 nt (called E307) (Engelhorn et al., 1993). Their termini are complementary for only 12 nt, like those of ND genomes. However, Northern analysis with a leader/N gene probe, which anneals specifically to internal deletion DI genomes, shows that this stock contains 3 internal deletion as well as 2 copyback DI genomes (the latter of ca. 2500 and 3500 nt in length) (Fig. 2a). DI-GP55, in contrast to the others, was produced and rescued by recombinant means (Garcin et al., 1995) and is identical to E307 except that it contains the 55 nt trailer region at the (−) DI genome 3′ end, in place of the leader region. DI-GP55 is thus a copyback DI genome whose termini are complementary for 55 nt (Fig. 1). As mentioned above, copyback DI genomes with more extensive terminal complementarity, such VSV DI 011, have not been reported for SeV, and we have no stable SeV stocks that contain only internal deletion DI genomes.

Infection of 293T cells with all 3 DI stocks was found to activate the IFNβ reporter, but to very different extents (Fig. 2c). H4 was the most potent, followed by GP55, and S104 was the least potent. In contrast, infection with ND genomes alone barely activated the reporter gene, even though the ND infection accumulated as much or more viral proteins than the DI infections (Fig. 2b). In these and other experiments, there is often an inverse correlation between the accumulation of viral proteins and the extent of IFNβ activation, as might be expected if activation is due to the presence of DI genomes. More importantly, plaque purification of the H4 stock yields a virus preparation that does not contain DI genomes and which does not activate the IFNβ promoter during infection (ND-H4, Fig. 3). The ability of the DI-H4 stock to activate IFNβ is thus not due to mutations within the ND genome, which could have arisen because of the presence of the DI genomes.

IFNβ activation requires modification of IRF-3, which is hyper-phosphorylated in response to viral infection, or dsRNA (Fitzgerald et al., 2003; Sharma et al., 2003). Activated IRF-3 dimerizes and migrates to the nucleus where it binds to the PRD I and III elements of the IFNβ promoter, as part of an enhanceosome (Maniatis et al., 1998). The activation of the IFNβ promoter by SeV DI infection appears to require IRF-3 since this activation is largely ablated by co-expression of a dominant-negative form of IRF-3 (data not shown). When the extent of IRF-3 dimerization was examined, a significant fraction of the IRF-3 was found as dimers (on non-denaturing

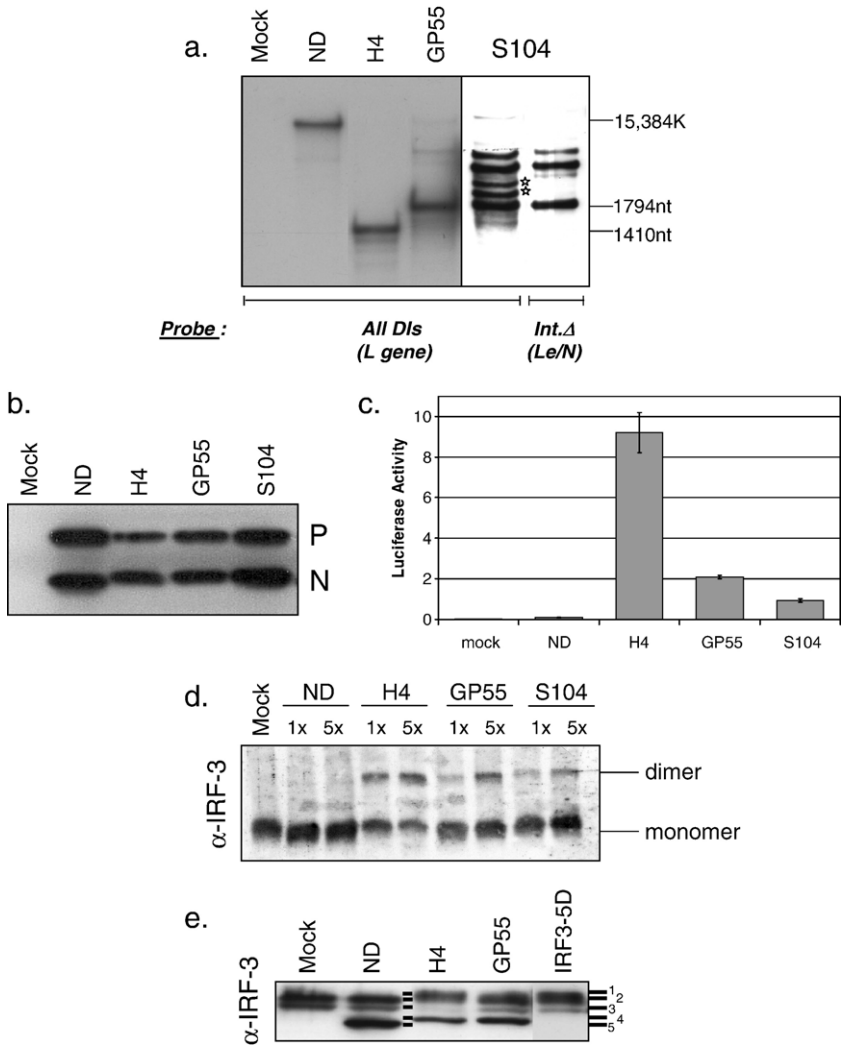

Fig. 2. Only DI infections activate IFNβ in 293T cells. (a) Northern blot of encapsidated (CsCl banded) RNAs from the various infections as indicated, using a (+) riboprobe from the end of the L gene (which detects all DI genomes) or a (+) riboprobe from the leader/N gene region, which detects only internal deletion DI genomes. The DI-H4 and GP-55 genomes do not anneal with the leader/N gene probe. (b) A Western blot of equal amounts of cell extracts (total proteins) of the various infections, using anti-P and anti-N antibodies. (c) 293T cells were transfected with a luciferase reporter plasmid under the control of the IFNβ promoter (and control plasmid) for 24 h then infected (or not) with the various SeV stocks for 20 h. Cell extracts were prepared, and levels of the renilla and firefly luciferases were determined. The cumulative results of 3 experiments are shown. (d) Two different amounts (1× and 5×) of total cell proteins from extracts of the various infections were separated on nondenaturing gels and Western blotted with anti-IRF-3. (e) Equal amounts of cell extracts of the various infections (total proteins) were separated on SDS gels and Western blotted with anti-IRF-3. The right-hand lane shows uninfected cells that express transfected IRF-3 5D. The 5 isoforms, numbered 1 to 5, are indicated.

gels) in extracts of all 3 DI infections, and the extent of dimer formation was roughly in proportion to the degree of IFNβ activation (Fig. 2d). In contrast, there was no evidence of dimers in the ND extract. We also examined the electrophoretic mobility of IRF-3 on SDS-PAGE as this mobility is sensitive to IRF-3 phosphorylation status (Hiscott et al., 2003; Hasegawa et al., 1992; Yoneyama et al., 2002). We could distinguish 5 electrophoretic forms of IRF-3 in our extracts (Fig. 2e). Mock-infected extracts predominantly contained forms 2 and 3. Infection with ND SeV led to the strong appearance of faster form 5, without loss of 2 and 3. In contrast, infection with DI-H4 and GP55 led to the appearance of the slightly slower form 4, reduction in the intensity of forms 2 and 3, and the appearance of the slowest form 1, which co-migrates with phospho-mimetic IRF-3 5D that is constitutively active (Lin et al., 1999). IRF-3 is thus being modified in response to the ND infection, but these modifications do not lead to dimerization or the activation of the IFNβ promoter. The presence of DI genomes during infection somehow causes IRF-3 to be modified differently, leading to its dimerization and IFNβ promoter activation.

DI-H4-induced activation of IFNβ is proportional to DI genome replication

The relative amounts of DI and ND genomes in cell extracts can be examined by Northern blotting (Figs. 2 and 3), but this analysis is linear only over a relatively narrow range and often unequally estimates RNAs that vary significantly in length (>10-fold in this case). RT/PCR is better suited to this task, but this method cannot differentiate between DI-H4 and ND

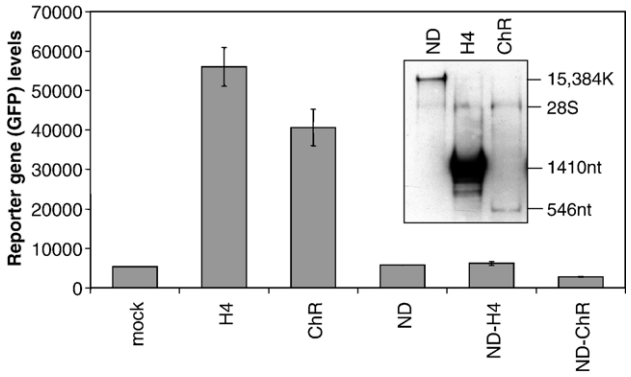

Fig. 3. Allantoic fluid stocks prepared from plaque-purified SeV do not activate IFNβ. The DI-H4 and ChR stocks were titered on LLC-MK2 cells in the absence of serum. Single plaques from the highest positive dilution (10^{-7}) were picked, and 3/10 of each plaque was inoculated into hen's eggs that were incubated at 33 °C for 3 days. The allantoic fluid stocks resulting from the plaque-purified virus (ca. 10^9 pfu/ml) were compared with the original stocks containing the same amount of viral proteins (as determined by Coomassie blue staining of SDS gels) for their ability to activate pIFNβ-GFP in 293T cells. A Northern blot of encapsidated (CsCl banded) RNAs from equal amounts of ND-, H4-, and ChR1-infected cells, using a (+) riboprobe from the end of the L gene, is shown in the insert.

genomes unless the complementary termini are included in the amplification. However, the dsRNA panhandles of circular DI genome RNAs are exceedingly stable and cannot be invaded by primers extended by RTase even at elevated temperatures. To circumvent these technical problems, we prepared a tagged version of the DI-H4 genome containing a 162 nt insertion from the YFP gene (Fig. 1), such that the DI and ND genomes could be independently and accurately estimated by RT/PCR, over a large range of values. A second reason for preparing DI-H4+yfp is that, although our H4 stocks are composed predominantly of the 1410 nt long species cloned as DNA, overexposure of Northern blots shows that several other RNAs are present in much lower amounts and which have not otherwise been characterized. As we do not know whether all the DI genomes in the H4 stock contribute equally to inducing IFNβ activation or whether activation is due to a particular (and perhaps uncharacterized) species, we cannot be sure that IFNβ activation is in fact due to the 1410 nt copyback DI genome. Recapitulation of these results with a tagged copy of the DI-H4 genome would settle this issue as well.

DI-H4+yfp genomes were recovered from DNA in BSR T7 cells that were subsequently co-infected with ND SeV. Stocks containing this DI genome were then generated by multiple passages in embyonated chicken eggs (Methods and materials), and each passage was tested for its ability to activate a GFP reporter gene under the control of the IFNβ promoter (pIFNβ-GFP) upon infection of 293T cells. Although some IFNβ activation above background appeared by passage 3, this activation increased slowly and erratically at first and eventually reached activation levels approximately half those of the reference H4 stock by passage 20 (Fig. 4a), During the later passages (16 to 20), the level of intracellular ND genomes steadily decreased (Fig. 4b), leading to a reduction in the levels of viral proteins (Fig. 4c), whereas the levels of DI-H4+yfp genomes steadily increased (Fig. 4b). The ability of SeV DI stocks to induce IFNβ activation thus correlates with the relative levels of DI genomes during infection. As DI and ND genome NCs are relatively stable structures, these levels reflect the rates that DI and ND genomes are synthesized during infection with the various passage levels. The evolution of the DI-H4+yfp stock towards IFNβ activation (during relatively undiluted passage in eggs) thus correlates with the level of DI genome synthesis during infection. The remarkable ability of

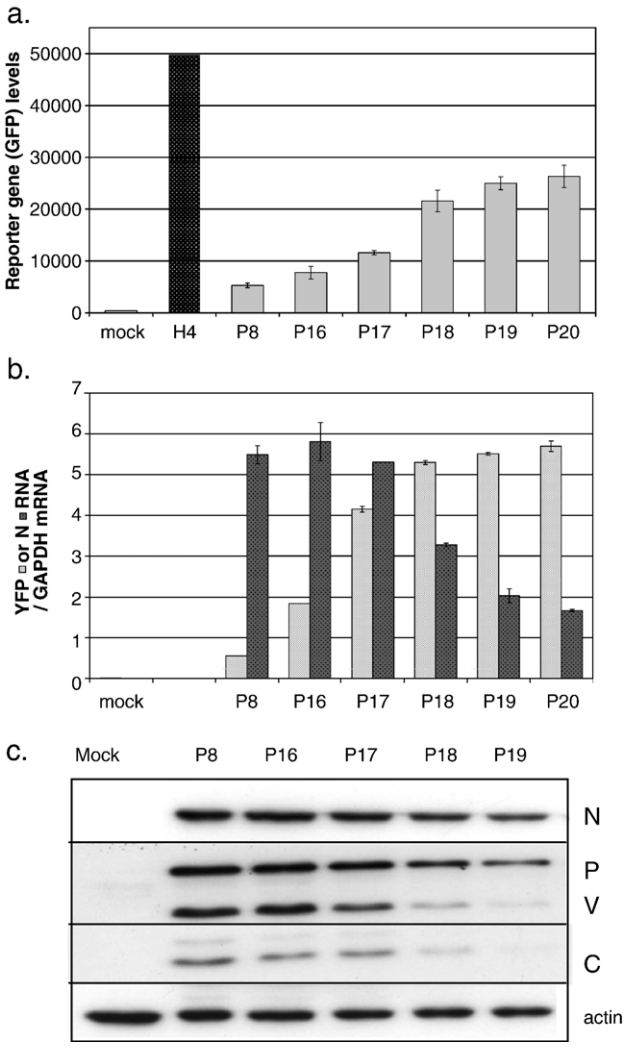

Fig. 4. Evolution of the DI-H4+yfp stock on passage in eggs. (a) The DI-H4+yfp genome was prepared from DNA by reverse genetics (Methods and materials) and rescued by superinfecting the BSR T7 culture with ND SeV (Methods and materials). The culture supernatant was inoculated into eggs, and the resulting allantoic fluid was diluted 1 to 500 for reinoculation, for a total of 20 times. The stocks from each passage level were assayed for their ability to activate a GFP gene under the control of the IFNβ promoter. Only the results from passage levels 8 and 16 to 20 are shown, along with DI-H4 as positive control and mock-infected as a negative control. (b) Total RNA was prepared from cells infected with passage levels 8 and 16 to 20 and examined by RT/PCR for their levels of ND genome (N gene) and DI genome (YFP) relative to that of GADPH mRNAs. Total RNA from a mock-infected culture served as the negative control. (c) Equal amounts of extracts from cells infected with passage levels 8 and 16 to 19 were examined by Western blotting with anti-N and anti-P/V/C and anti-actin as a loading control.

the DI-H4 stock to activate IFNβ can now also be traced to the predominant 1410-nt-long copyback DI genome.

Examination of the UV inactivation kinetics of DI-induced IFNβ activation, as compared to the UV inactivation kinetics of ND and DI genome replication, can provide broad information on the nature of this IFNβ activation. DI-H4+yfp (P(passage) 17, insert, Fig. 5) was irradiated with 256 nm UV light for various times (0.5 to 8 min) and used to infect 293T cells containing pIFNβ-GFP. Intracellular RNA was isolated at 20 hpi, and the levels of ND genome RNA and DI-H4+yfp genome RNA were measured by RT/PCR. GFP expression was monitored by FACS (Fig. 5a). DI-H4+yfp (1572 nt) is 1/10 the length of the ND genome (15,384 nt) and thus proportionately less sensitive to UV inactivation. This difference in the loss of ND and DI genome levels is most apparent at the shortest times of UV irradiation and is lost at the higher doses, presumably because DI genome replication ultimately depends on ND genomes to provide all the replication substrates (N, P and L proteins). The reduction of GFP expression levels upon increasing UV irradiation parallels that of ND and DI genomes and most closely follows the loss of the DI genomes at the lowest doses of UV. More importantly, as the reduction of GFP expression levels closely follows that of DI genomes over a range of 2 logs, IFNβ activation is clearly proportional to the level of copyback DI genome replication (in 293T cells) for a given stock as well.

The manner in which SeV DI genomes presumably generate dsRNA that induces IFN is thus quite different from that of VSV DI 011. SeV DI genomes not only require co-infection with ND helper virus, IFN induction here (293T cells) is strictly proportional to the level of DI genome replication, in contrast to VSV snapback DI IFN induction (in aged CEFs) where viral RNA synthesis is not required.

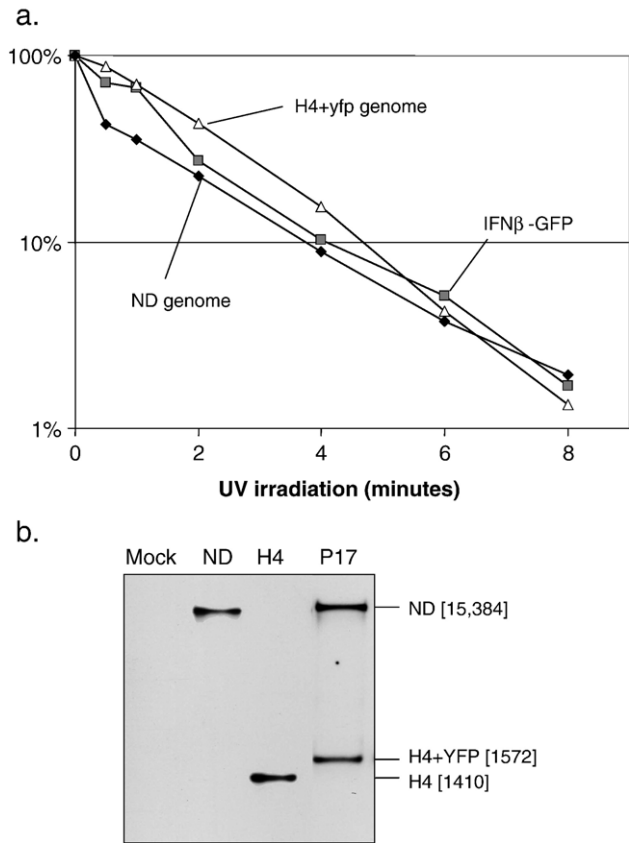

Fig. 5. UV inactivation kinetics of DI-H4+yfp infections. (a) The allantoic fluid of DI-H4+yfp passage level 17 was irradiated with 254 nm light (110 μW/cm^2) from 0.5 to 8 min and then used to infect 293T cells that had been transfected with pIFNβ-GFP. Total RNA was prepared at 24 hpi, and the levels of DI-H4+yfp genome and ND genome were determined by RT/PCR. GFP expression levels were monitored by FACS. The results are shown relative to RNA from cells infected with non-irradiated allantoic fluid. The cumulative results of 2 experiments are shown. The error bars, always <20%, are not shown for clarity. (b) A Northern blot of encapsidated RNAs from ND, DI-H4 and DI-H4+yfp P17 infections, using a (+) probe from the end of the L gene.

The SeV stock of Charles Rivers Laboratory

As mentioned above, SeV stocks (Cantell strain) whose infection of cultured cells strongly activates IFNβ, are available from Charles River Laboratory. A fresh allantoic fluid stock of this virus preparation was found to activate IFNβ to levels similar to those of DI-H4 (Fig. 3), and this stock was found to contain a very small DI genome (of ~600 nt) by Northern analysis (insert, Fig. 3). When the ND virus of this preparation was plaque purified on LLC-MK2 cells, allantoic fluid stocks prepared from the purified virus had lost the ability to activate IFNβ (ND-ChR1, Fig. 3). Thus, similar to DI-H4, the ability of the Charles River virus preparation to activate IFNβ appears to be due to the presence of the DI genome(s), and not to mutations within its ND genome.

To determine the nature of this DI genome, we cloned the DI genome as DNA, as illustrated in Fig. 1 (small horizontal arrows are primers). We used one set of primers to amplify the common right end of all DIs (arrows under GP55, Fig. 1) and 3 sets of primers that were specific to the left end of either internal deletion (arrows under E307, Fig. 1) or copyback DIs (arrows under H4, Fig. 1). The common right end primer set and the 3 copyback-specific left-end primer sets all yielded a PCR product of the expected size, whereas the 3 internal-deletion-specific left-end primers failed to produce visible DNA (not shown). When these amplified DNA fragments were sequenced, DI-ChR1 was deduced to be a simple copyback DI genome of 546 nt (453 nt are co-linear with the 5′ end of the ND (−) genome), with terminal complementarity over 93 nt. To our knowledge, this is the smallest natural SeV DI genome described to date, and this property may be related to its ability to activate IFNβ so strongly.

The SeV V and/or C proteins inhibit DI-H4-induced IFNβ activation

Although our 293T cells produce IFN in response to DI-H4 infection, they do not respond to added IFN. 293T cells are thus useful in studying IFNβ activation in isolation because the activation is not also driven by positive feedback via ISGs. However, to study the broader aspects of the

DI-H4 infection, we used 2fTGH cells, which both produce IFN in response to infection and respond as well to the secreted IFN. When 2fTGH cells containing a pIFNβ-luc reporter are treated with poly-I/C (either added to the medium or via transfection (*)) or IFNα, poly-I/C* and H4 infection strongly activate IFNβ promoter, whereas IFNα has no effect and poly-I/C treatment has little effect (IFNβ is not an ISG, and TLR3 may be poorly expressed in these fibroblasts). Moreover, whereas poly-I/C* and IFNα clearly increased ISG levels (STAT1, RIG-I, ISG15 and ISG56), H4 infection failed to increase these ISG levels above the untreated control. The SeV V protein blocks poly-I/C-induced IFNβ activation (Poole et al., 2002) and presumably should also block that induced by DI-H4 infection. We reasoned that, if DI-H4 infections generated abnormally large amounts of dsRNA, the amount of V expressed from the ND genome during DI infections might be insufficient to block dsRNA signaling via RIG-I and Mda5. We therefore over-expressed the V and C proteins by plasmid transfection in 2fTGH cells containing pIFNβ-luc and re-examined the effects of the various treatments. As shown in Fig. 6a, over-expression of the V and C proteins was as effective in blocking the DI-H4-induced activation of the IFNβ promoter as it was in reducing the poly-I/C*-induced activation. This result is consistent with the notion that DI-H4 infection induces IFNβ activation, at least in part, via dsRNA. Over-expression of the V and C proteins also partially blocked the poly-I/C*- and IFNα-induced increase in ISG levels, including that of RIG-I.

To examine whether DI-H4 infection was indeed able to block IFN signaling, we similarly treated 2fTGH cells containing a pIRSE-luc reporter and examined the effects on the reporter gene and ISG protein levels (Fig. 6b). In contrast to IFNα and poly-I/C* treatment which activated this promoter and increased ISG levels, DI-H4 infection did not activate the ISRE reporter over the untreated control, and the levels of STAT1, RIG-I, ISG15 and ISG56 were not increased in these extracts. Thus, there appears to be sufficient viral proteins expressed during DI-H4 infection to block IFN signaling.

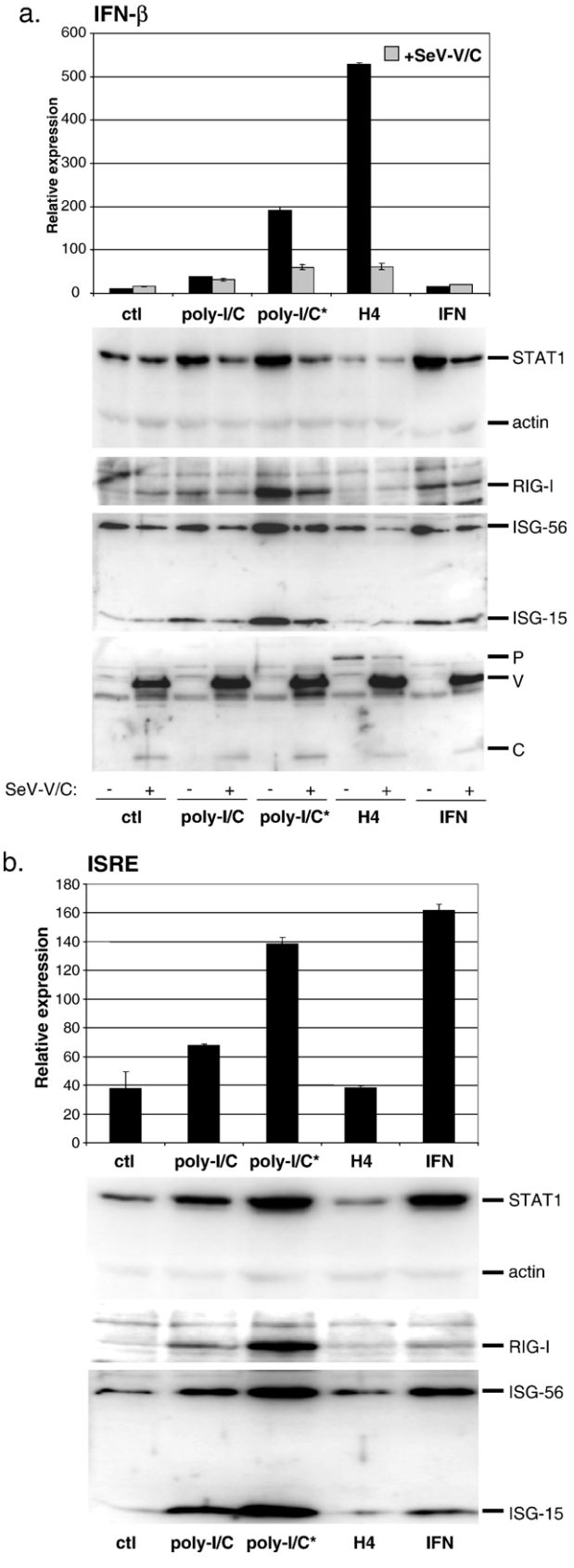

Fig. 6. (a) The effect of poly I/C, DI-H4 infection and IFNα treatment on IFNβ and ISGs in 2fTGH cells, in the presence and absence of the SeV V and C proteins. 2fTGH cells were transfected with a luciferase reporter plasmid under the control of the IFNβ promoter (and the TK-renilla control plasmid) and pSeV-V/C that expresses V and C proteins from the same mRNA (or and empty plasmid) for 24 h. Parallel cultures were then either treated with 100 μg/ml of poly I/C in MEM (poly-I/C), transfected with 1 μg of poly I/C (poly I/C*), infected with DI-H4 or treated with 1000 IU of IFNα. Cell extracts were prepared 20 h later, and the levels of the renilla and firefly luciferase activities were determined. The cumulative results of 2 experiments are shown. Equal amounts of total protein of each extract were also examined for their levels of STAT1 (and actin), RIG-I, SeV P, V and C proteins and ISG-15 and ISG-56 by Western blotting with specific antibodies (Methods and materials). (b) The effect of poly I/C, DI-H4 infection and IFNα treatment on ISGs in 2fTGH cells. 2fTGH cells were transfected with a luciferase reporter plasmid under the control of an ISRE promoter (and the TK-renilla control plasmid) for 24 h. Parallel cultures were then treated as in panel A. Cell extracts were prepared 20 h later, and the levels of the renilla and firefly luciferase activities were determined. The cumulative results of 2 experiments are shown. Equal amounts of total protein of each extract were also examined for their levels of STAT1 (and actin), RIG-I and ISG-15 and ISG-56 by Western blotting.

Discussion

dsRNA is thought to be a common product or PAMP of RNA virus infections that initiates the innate antiviral response, in part by activating IFNβ. However, the source of this dsRNA is presumably different for different viruses. (+) RNA virus genomes contain highly conserved 2° and 3° structures at their 5′ and 3′ ends that are essential for virus replication (Simmonds et al., 2004), and these highly structured RNA regions by themselves can initiate signaling to IFNβ upon binding to RIG-I (Sumpter et al., 2005). In contrast, NNV genomes are not known to contain conserved 2° structures, and, moreover, NNV genomes function in RNA synthesis not as free RNAs but as assembled NCs, in which the genome RNA cannot normally anneal (Lamb and Kolakofsky, 2001). One possible source of dsRNA during ND SeV replication is the occasional extension of the trailer RNA beyond the trailer/L gene junction (see Fig. 1), producing run-on trailer RNAs whose 3′ sequences can anneal to those of the L mRNA (Vidal and Kolakofsky, 1989). In a similar vein, the transcriptase which synthesizes the L mRNA presumably reads through the L gene-end site at a frequency of ca. 5% (similar to other gene junctions; Le Mercier et al., 2002) and terminates at the genome 5′ end, thus providing read-through L transcripts that can anneal to trailer RNAs. Although the NNV replication strategy appears to minimize dsRNA potential during intracellular replication, this strategy presumably cannot exclude the generation of small amounts of dsRNA. It is reasonable that the levels of V and C expressed during ND genome replication are designed to counteract the small amounts of dsRNA generated. If so, the presence of significant amounts of copyback DI genomes during intracellular replication will certainly change the nature of the SeV infection.

The most important new finding of this study is that not all SeV stocks that are heavily contaminated by DI genomes are equally able to activate IFNβ. The H4, GP55 and S104 stocks all contain the H strain ND genome as helper and can be directly compared. S104 infections accumulate more viral products than H4 infections as the S104 DI genomes appear to interfere less with their helper virus replication than those of H4 (Fig. 2b). At the same time, S104 infections activated IFNβ 10 to 20-fold less strongly than H4 infections in multiple experiments (Fig. 2a and data not shown). Besides the different extents to which S104 and H4 DI genomes interfere with ND genome expression and thus affect the intracellular concentration of the viral V and C proteins, H4 stocks are composed exclusively of copyback DI genomes, whereas S104 stocks are composed predominantly (>70%) of internal deletion DI genomes. Moreover, the two copyback DI genomes in this stock are both longer than DI-H4. DI-GP55 is also longer than DI-H4, its termini are complementary for only half the length as H4 (55 nt), and DI-GP55 interferes with ND genome expression less than DI-H4. Taken together, these data suggest that the ability of SeV DI stocks to activate IFNβ is related both to (i) their ability to interfere with helper genome expression, which leads to lower levels of V and C intracellularly, and (ii) their relative content of copyback DI genomes. The size of the DI genome may also play a role in this latter respect. However, it will be necessary to examine these properties of SeV DI genomes more directly, e.g., by extending the terminal complementarity of DI-GP55 to 110 nt, or by altering the length of DI-H4+yfp, to be more certain of these conclusions.

Copyback DI genomes may be stronger activators of IFNβ than internal deletion DI genomes because they have a stronger potential to form dsRNA. Copyback DI genomes are composed of equal amounts of genomes and antigenomes (rather than a 10-fold excess of genomes), and their termini can self-anneal intramolecularly as well. The question then remains of how this dsRNA potential is expressed as the synthesis of DI genomes, like that of ND genomes, is thought to be coupled to their assembly into NCs. The vast majority of these NCs, once formed, are very stable in vivo and band in CsCl density gradients as fully assembled NCs. However, it is not rare to find small amounts of DI-H4 genomes and antigenomes in extracts of infected cells which pellet through these gradients as free RNAs (<5%, unpublished). It is not clear whether these non-assembled DI genomes were normally made as NCs which subsequently disassembled or were actually made de novo without concurrent assembly with N protein, as reported for some conditions of VSV DI genome replication in vitro (Chanda et al., 1980). Independent of how non-assembled DI-H4 genomes are presumably generated in vivo, their formation appears to be proportional to their synthesis (Fig. 4). Their presence would represent a vast increase in the dsRNA potential of SeV DI vs. ND infections, under conditions where there is less V protein available to dampen dsRNA signaling to IFNβ (Fig. 4c). The relatively short lengths of the H4 and ChR1 DI genomes may play a role in how frequently their non-assembled RNAs are formed in vivo, but this needs to be investigated.

There is one further aspect of SeV copyback DI infections that should be mentioned to explain their ability to induce IFN so efficiently, coupled with the remarkable fact that these DI genomes with dsRNA potential are actually selected for on passage in eggs (e.g., Fig. 4) (Le Mercier et al., 2002). We have previously prepared ambisense ND SeV in which an additional mRNA was expressed from the 3′ end of the antigenome. In contrast to copyback DI genomes, genomes and antigenomes of the ambisense SeV contain the weaker genomic promoter. These ambisense SeV grew poorly in IFN-sensitive cultures and were relatively IFN-sensitive. They were also highly unstable on passage in eggs and reverted to virus that grows well even in IFN-pretreated cells that restrict vesicular stomatitis virus replication, i.e., the wild-type SeV phenotype. Since this reversion was always associated with a point mutation in the ambi-mRNA start site that severely limited its expression, we concluded that the selection of mutants unable to express ambi-mRNA on passage in chicken eggs was presumably due to increased levels of dsRNA during infection (vRdRp read-through of the ambi-mRNA stop site creates a capped transcript that can potentially extend the entire length of the antigenome, whereas extension of the uncapped trailer RNA (wt SeV) is limited by the poor processivity of its vRdRp). If ND ambisense SeV with dsRNA potential are strongly selected against in eggs, then how are DI genomes with dsRNA potential positively selected under the same conditions?

There are two possible explanations for this conundrum. Firstly, the dsRNA potential of ambisense SeV is not associated

with any selective advantage and a single point mutation in the ambi-mRNA promoter will largely eliminate this potential. The ND genomes of SeV stocks containing copyback DI genomes, in contrast, cannot escape their DI genomes by simple mutation, and the dsRNA potential of copyback DI genomes is always associated with a strong selective advantage as copyback DI genomes outcompete their ND genomes for the replication substrates provided by the latter. Secondly, and perhaps more importantly, copyback DI infections are relatively non-cytopathic and often end as persistent infections (Roux et al., 1991). This is in part due to the ectopic expression of trailer RNA (in place of leader RNA) from the copyback DI (−) genome (Garcin et al., 1998) (Fig. 1). Trailer RNA is known to interact with TIAR, a protein with many links to apoptosis, and this interaction is important in suppressing SeV-induced PCD (Iseni et al., 2002). The relative absence of leader RNAs during copyback DI infections may also contribute to this DI phenotype as mutations in the SeV leader region are associated with virulence in mice (Fujii et al., 2002), and the normal expression of leader RNA appears to be required to prevent IFNβ activation (Strahle et al., 2003). The ability of copyback DI infections to delay, and in many cases completely prevent PCD, may compensate for the negative consequences of increased dsRNA during infection, which presumably selects against SeV that express ambi-mRNAs.

In summary, infection with an SeV stock that is highly contaminated with copyback DI genomes is a potent way of activating IFNβ. These DI infections presumably provide plentiful dsRNA, under conditions of reduced expression of viral products which block the host response to dsRNA, and with minimal cytopathic effects that lead to persistent infection. In contrast, infection with an SeV stock that is not contaminated with copyback DI genomes does not activate IFNβ and is highly cytopathic. These are two very different virus infections, and they should not be confused when SeV stocks of unknown composition are used to activate IFNβ.

Methods and materials

Cells, viruses, and antibodies

BSR-T7 cells were grown in BHK-21 Medium (Glasgow MEM, Gibco) supplemented with 5% fetal calf serum (FCS) in the presence of the relevant maintenance drug (G418 at 400 μg/ml). 2fTGH cells and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

SeV stocks were grown in the allantoic cavities of 9-day-old embryonated chicken eggs for 3 days at 33 °C. For ND stocks (10^9 pfu/ml), 0.1 ml of a 10^5 dilution (ca. 1000 pfu) was inoculated per egg. In the case of DI stocks, 0.1 ml of a 10^2 to 10^4 dilution was used. In all cases, the amount of viral proteins present in the resulting allantoic fluid was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining of pelleted virus. Virus titers were determined by plaquing on LLC-MK2 cells. Anti-IRF-3 (Santa Cruz), anti-actin (Chemicon), anti-Stat1 (C-terminus) (Transduction Laboratories), anti-N-877 and anti-PCV, anti-ISG-15 and anti-ISG-56 were provided by Dr. Ganes Sen from The Cleveland Clinic Institute. Anti-Rig-I was provided by Tadaatsu Imaizumi from Hirosaki University School of Medicine.

Plasmids, transient transfections, luciferase assay and FACS

pβ-IFN-fl-lucter, which contains the firefly luciferase gene under the control of the human IFNβ promoter, is described in King and Goodbourn (1994). The IFNα/β-responsive reporter plasmid, p(9–27)4tkD(239)lucter, referred to here as pISRE-fl-lucter, contains four tandem repeats of the IFN-inducible gene 9–27 ISRE fused to the firefly luciferase gene (Didcock et al., 1999). pTK-rl-lucter used as a transfection standard contains the herpes simplex virus TK promoter region upstream of the renilla luciferase gene (Promega). pIRF-3ΔN, which expresses a dominant negative form of IRF-3, and pIRF-3 5D, which is constitutively active, were obtained from John Hiscott and Paula Pitha (Lin et al., 1998).

For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pβ-IFN-fl-lucter or pISRE-fl-lucter, 0.3 μg of pTK-rl-lucter, with or without 1 μg of IRF-3ΔN, or 1 μg of EBS plasmid expressing SeV-V protein (Nishio et al., 2005), and Fugene (Roche) according to the manufacturer's instructions. At 24 h post-transfection, the cells were (or were not) infected with various Sendai virus stocks or treated with 100 μg of poly(I)–poly(C) (Sigma, St. Louis, MO) per ml or transfected with 1 μg of poly(I)–poly(C) using Fugene. Twenty hours later, cells were harvested and assayed for firefly and renilla luciferase activity (dual-luciferase reporter assay system; Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of renilla luciferase. pIFNβ-GFP, which expresses GFP under the control of the IFNβ promoter, was constructed by cloning the IFNβ promoter region from pIFNβ-fl-lucter into pEGFP-N^3 (BD Biosciences Clontech), between the *Ase*I and *Hin*dIII sites. For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pIFNβ-GFP and $CaPO_4$ (Stratagene) according to the manufacturer's instructions. At 24 h post-transfection, the cells were (or were not) infected with Sendai virus or different DI stocks. Twenty hours later, cells were harvested and assayed for GFP expression by FACS analysis.

Preparation of DI-H4+yfp

100,000 BSR-T7 cells were plated in six-well plates 20 h before transfection with a mix containing 0.75 μg of pTM1-L, 1.5 μg of pTM1-N, 1.5 μg of pTM1-P/C^{stop} (which does not express C proteins), 1 μg of the various pDI constructs and Fugene. Six hours later, the transfection mix was discarded and replaced with 2 ml of Glasgow MEM supplemented with 5% FCS. Twenty four hours post-transfection, the cells were infected with ND SeV. Forty eight hours post-infection, the cells were scraped into their medium and injected directly into the allantoic cavity of 9-day embryonated chicken eggs. Three days later, the allantoic fluids were harvested and injected undiluted into eggs. For further passages, the virus stocks were

clarified by centrifugation (10 min at 3000 rpm) and diluted 1/500 before injection. The presence of viruses in the resulting stock was determined by pelleting allantoic fluids (100 μl) through a TNE (10 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA)–25% glycerol cushion for 20 min at 14,000 rpm in an Eppendorf 5417C centrifuge. Virus pellets were resuspended in sample buffer, and the proteins were separated by sodium dodecyl sulfate–10% PAGE and stained with Coomassie brilliant blue, alongside an ND stock of known titer.

Analysis of encapsidated RNAs

Confluent 293T cells in 9 cm ∅ Petri dishes (2×10^7 cells) were infected with 10 pfu/cell of ND stocks, and an equivalent amount of viral protein for DI stocks. Two days post-infection, the cells were collected, and the intracellular viral nucleocapsids (NC) were purified by 20–40% (w/w) CsCl density gradient centrifugation and pelleted. After treatment with SDS and proteinase K, the nucleocapsid RNAs were phenol-extracted and ethanol-precipitated. The resulting RNAs were characterized by Taqman analysis using specifics oligonucleotides and Taqman probes and by Northern blotting using a biotinylated riboprobe generated in vitro by T7 RNA polymerase transcription of plus strands complementary to nucleotides 13,397–14,850 of the (−) ND genome.

Reverse transcription and real-time PCR via Taqman

Total RNA was extracted using Trizol (Invitrogen). Twenty micrograms of total RNA was mixed with 0.5 μg Random Hexamers (Promega) and subjected to a Superscript reverse transcription (RT) reaction as described by the manufacturer (Gibco) in a total volume of 50 μl. Two microliters of each cDNA was then combined with 1 μl of internal control Human GAPDH (Applied Biosystems), 11 μl MasterMix (Eurogentec), 20 pmol (each) of forward and reverse primers and 4.4 pmol of Taqman probe in a total volume of 22 μl. The following primers and probes (Eurogentec or Microsynth) were used: N gene: 5′-GCAATAACGGTGTCGATCACG-3′ (Fwd); 5′-TGCCTGAGCCGATCGG-3′ (Rev); 5′-CGAAGATGACGATACCGCAGCAGTAGC-3′ (Probe). YFP gene: 5′-CCGACAACCACTACCTGAGCTA-3′ (Fwd); 5′-GAACTCCAGCAGGACCATGTG-3′ (Rev); 5′-AAAGACCCCAACGAGAAGCGCGA-3′ (Probe). Real-time PCR was carried out in the 7700 Sequence Detector (Applied Biosystems).

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