Sendai virus and the innate antiviral defence

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Abstract
Depuis longtemps, les virus ont développé diverses stratégies afin de contrecarrer l'Interféron, un élément essentiel de la réponse innée cellulaire, produite lors d'une infection virale. L'ARN double brin ainsi que le simple brin portant un 5'triphosphate sont des produits potentiellement responsables de l'initiation de la réponse innée antivirale. Dans ce travail, nous démontrons que le virus de Sendai (SeV) cible de différentes façons les réponses inflammatoires et innées, ainsi que l'état antiviral de la cellule. Nous montrons également que la présence de génomes "défectifs" dans certains stocks de SeV est capable d'activer fortement l'IFNβ. De plus, cette activation peut être inhibée par la surexpression des protéines virales C et V. Finalement nous démontrons que dans deux différentes infections de SeV, l'induction de l'IFN est principalement dépendante de RIG-I et qu'une fonction de la protéine C est de contrecarrer la stimulation de l'IFN dépendante de RIG-I.

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SENDAI VIRUS
AND
THE INNATE ANTIVIRAL DEFENCE

THESE
présentée à la Faculté des sciences de l’Université de Genève
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Summary

SeV genome is characterized by a non-segmented negative stranded RNA of negative polarity, which is tightly associated with the viral nucleoprotein N, forming a very stable helicoidal structure called the nucleocapsid (NC). This genome is replicated via an intermediate RNA of positive polarity, the antigenome, representing the full copy of the genome. SeV is composed of 6 genes, which are flanked by control regions essential for transcription and replication, namely the leader and the trailer. The leader and the trailer are part of the genomic promoter (G/Pr) and the antigenomic promoter (AG/Pr), respectively. The G/Pr (located at the 3’ extremity of the genome) is involved in the control of replication and transcription initiation, whereas the AG/Pr (located at the 3’ end of the antigenome) is only implicated in the replication.

An important component of the host’s innate immune response in viral infection is the production of type I interferons (IFNs). Efforts to understand the molecular mechanisms by which viruses and also double-stranded RNA (dsRNA) trigger the induction of IFN have led to the identification of cellular sensors of viral infection. One type of sensor is the family of cytosolic receptor proteins known as the retinoic acid inducible gene I (RIG-I)-like receptors. This family includes RIG-I and Mda5, two DexD/H box helicases with CARD domains that were found to participate in the detection of cytoplasmic RNA. dsRNA as well as single-stranded viral RNA bearing 5’ triphosphates (5’pppRNA) are thought to be products of RNA virus infections that acts as pathogen-associated molecular patterns (PAMPs) responsible for initiating the innate antiviral defence. 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, which in turn activates the IFNβ gene. IFNβ is secreted and feeds back onto cells in a paracrine manner to prime neighbouring cells for possible infection and in an autocrine manner to induce multiple IFN stimulated genes (ISGs) leading to the antiviral state of the cell. Many viruses including SeV have developed strategies for counteracting the host type interferon I response and this at different levels. For that purpose SeV mainly uses the non-structural C and V proteins to act at the level of the IFN induction pathway by blocking RIG-I activation and also at the level of the IFN feedback loop where C blocks Stat-1 signaling pathway.

In the first study, we have used cDNA arrays to compare the activation of various cellular genes in response to infection with SeV that contain specific mutations. Mutations that disrupt four distinct elements in the SeV genome (the leader RNA, two regions of the C
protein, and the V protein) all lead to enhanced levels of IFN-β mRNA, and at least three of these viral genes also appear to be involved in preventing activation of IL-8. Our results suggest that SeV targets the inflammatory and adaptive immune responses as well as the IFN-induced intracellular antiviral state by using a multifaceted approach.

SeV stocks available commercially are known to strongly induce IFNβ and are commonly used by many laboratories. Plus, these stocks are known for a long time to contain DI genomes. The paramyxoviruses DI genomes can be of two types: internal deletion or copyback DI genomes. Copyback DIs have the capacity to form dsRNA by at least two ways: 1) When the level of the N protein is not sufficient, DI genomes and antigenomes can self-anneal, and 2) Because some DI genomes contain termini that are perfectly complementary, they are free to form dsRNA. Moreover, DIs (especially those from the copyback variety) interfere robustly with the ND helper genome by competing for replication and consequently reducing the production of viral proteins involved in the antiviral state. The second paper shows evidence that the strong induction of IFNβ activation upon SeV infection (SeV stock containing DI genomes) is mainly due to the presence of copyback DI genomes. The level of IFNβ activation was found to be proportional to that of DI genome replication. Moreover, this activation can be inhibited by the overexpression of the C and V proteins, whose concentrations are reduced in DI infected cells, because of the strong interference of the DI versus the ND genome.

In the last paper the contribution of RIG-I (and Mda-5) in the detection of SeV infection is examined. Because 5’-triphosphorylated products have become new potential targets of RIG-I, we decided to test whether SeV infections induced IFNβ activation by producing pppRNAs as well as dsRNA. The involvement of both helicases was also analysed. We used two different Sendai virus infections to study virus-induced IFNβ activation; 1) SeV-DI-H4, which is composed mostly of small, copyback DI genomes, and whose infection is likely to over-produce short 5’ tri-phosphorylated (ppp) trailer RNAs and under-produces the viral V and C proteins, and 2) SeV-GFP(+/-), a co-infection that produces WT amounts of viral gene products but also produces both GFP mRNA and its complement, which can form dsRNA with capped 5’ ends. We found that 1) virus-induced signaling to IFNβ depended predominantly on RIG-I (as opposed to mda-5) for both SeV infections, i.e., that RIG-I senses both pppRNAs and dsRNA without 5’ tri-phosphorylated ends, and 2) that it is the viral C protein (and not V) that is primarily responsible for countering RIG-I dependent signaling to IFNβ.
Introduction générale

Le virus de Sendai (SeV) a été découvert en 1953 au Japon et isolé à partir d’un nouveau né présentant des troubles respiratoires. Ce virus n’est pas pathogène pour l’homme mais il est extrêmement contagieux et virulent chez le rat et la souris de laboratoire. La transmission se fait par contact direct et est suivie par une infection des voies respiratoires. SeV appartient au genre *Respirovirus* de la famille des *Paramyxovirus*. Cette famille avec celle des *Rhabdovirus*, des *Filovirus* et des *Bornavirus* constituent l’ensemble de l’ordre des *Mononegavirus*.

Il existe plusieurs pathogènes humains appartenant à cette famille, comme par exemple le virus de la rougeole, le virus des oreillons ou encore le virus Nipah, un virus apparu récemment. Ces virus sont encore très présents dans les pays en voie de développement. Effectivement le virus de la rougeole à lui seul tue encore plus d’un million de personnes par année. Ainsi, SeV est considéré comme un bon modèle d’étude permettant de mieux comprendre les mécanismes moléculaires de cette famille et de trouver des thérapies.

SeV contient une enveloppe sphérique provenant de la membrane plasmique de l’hôte dans laquelle sont ancrées les glycoprotéines virales de fusion (F) et l’hémagglutinine-neuraminidase (HN), toutes deux impliquées dans l’attachement, la fusion et le relâchement des particules virales. Les protéines de la matrice (M) se trouvent contre la surface interne de la membrane et jouent un rôle important dans la structure et l’assemblage de la particule virale. À l’intérieur de cette particule se trouve le génome à ARN(-) de SeV étroitement associé aux protéines de la capsido (N), formant une structure hélicoïdale très stable appelée la nucléocapside (NC) ou le complexe ribonucléoprotéique. Attachées à ce complexe, les protéines virales P et L forment la polymérase à ARN, qui est responsable de la transcription et de la réplication du virus.

Le génome de SeV est long de 15'384 nucléotides et se caractérise par un simple brin d’ARN, non-segmenté et de polarité négative. SeV est composé de six gènes : N, P, M, F, HN et L. L’ordre de ces gènes est extrêmement bien conservé parmi les paramyxovirus. Chaque gène commence avec une courte séquence régulatrice de transcription de dix nucléotides appelée “gene start” et se termine par une séquence de terminaison nommée « gene end ». Entre ces deux séquences se trouve une région intergénique (IG) non transcrite de trois nucléotides. Les extrémités flanquant les 6 gènes comportent des régions extracistoniques essentielles pour la transcription et la réplication, nommées le leader à l’extrémité 3’ et le trailer à l’extrémité 5’.

**Le cycle viral et la synthèse d’ARN viral**

Lors d’une infection, le virus est adsorbé par les récepteurs cellulaires situés à la surface de la cellule, ce qui conduit à la fusion entre l’enveloppe du virus et la membrane plasmique. Suite à la fusion, la nucléocapside hélicoïdale se trouve libérée dans le cytoplasme de la cellule hôte, lieu de toutes les étapes du cycle de multiplication virale. La nucléocapside contenant l’ARN génomique est la matrice pour toutes les synthèses d’ARN. Deux fonctions sont assurées par le génome viral : la transcription des ARN messagers et la réplication de l’ARN viral. Les protéines N, P/C/V, M, HN et L protéines sont synthétisées par le système de traduction de la cellule, et finalement suit l’assemblage du génome viral avec les protéines de la capside. La protéine M se place à la surface interne de la membrane plasmique alors que les protéines de surface F et HN se trouvent au niveau des patchs créés par la protéine M, excluant les protéines cellulaire. Une fois que les nucléocapsides sont associées à la protéine M, les nouvelles particules se forment et sortent de la cellule en emportant avec elles une partie de la membrane plasmique cellulaire.

Le génome viral encapsidé sert de matrice à la polymérase virale pour synthétiser dans un premier temps les ARN messagers nécessaires pour produire les protéines virales qui sont impliquées dans la réplication même du virus. La polymérase virale entre sur le génome à l’extrémité 3’ et transcrit en premier le leader qui est un ARN non codant, puis commence la transcription des six gènes en six messagers à ARN et ce d’une manière séquentielle et polaire. Occasionnellement la polymérase oublie de réinitier le messager suivant la jonction, en attendant par conséquent la transcription des gènes en aval. Ainsi un gradient de messagers synthétisés peut être observé, qui est inversement proportionnel à la distance du gène par rapport à l’extrémité 3’ du génome. La protéine N est la protéine la plus synthétisée et la concentration intracellulaire de sa forme « non-assemblée » est un moyen de contrôler le taux de transcription et de réplication à partir de la matrice génomique. Lorsque la quantité de protéines N « non assemblées » est suffisante, la synthèse d’ARN viral est couplée avec l’encapsidation concomitante de la chaîne naissante. Dans ces conditions la polymérase ignore toutes les jonctions, formant ainsi une copie complète du génome de polarité positive entièrement encapsidée. Ce dernier est appelé antigénome et servira à son tour à la synthèse...
d’une nouvelle copie d’ARN génomique, qui à nouveau sera utilisée comme matrice ou alors qui sera envoyée et assemblée dans une particule virale naissante.

**Les protéines accessoires**

Les protéines C et V de SeV sont désignées comme des protéines « accessoires », car elles ne sont pas présentes dans tous les virus de la famille des *Paramyxoviridae*. Effectivement certains virus possèdent les deux protéines alors que d’autres ne possèdent que l’une ou l’autre. Les protéines C et V sont exprimées à partir du gène P qui contient 5 codons d’initiation ainsi qu’un site d’editing en son milieu, ce qui lui permet de coder pour 8 protéines : P, V, W, C’, C, Y1, Y2 et X.

Les protéines C’, C, Y1 et Y2 sont collectivement nommées les « protéines C ». La participation des protéines virales C et V dans la contremesure des réponses innées de la cellule, a été intensément étudiée ces 10 dernières années. Apparemment, les Paramyxovirus (incluant SeV) utilisent la protéine C pour cette fonction, alors que les autres membres de la famille (Rubula-, Morbili- et Henipa-virus) utilisent la protéine V. Les protéines C de SeV sont des protéines non-essentielles à la multiplication du virus *in vitro*, mais sont nécessaires à la réplication du virus dans les souris. Cette particularité reste cependant dépendante du type cellulaire étudié. Les protéines C contiennent une séquence qui les localise à la membrane plasmatique de la cellule mais elles sont également retrouvées dans le cytoplasme. Toutes les protéines C interagissent physiquement avec Stat1 (Signal transducer and activator of transcription 1), dont le rôle est de transduire le signal de l’IFN pour permettre l’expression de protéines antivirales. Cette interactions (C/Stat1) empêche la signalisation de l’IFN par la voie de JAK/Stat et en même temps bloque l’établissement d’un état antiviral de la cellule. Les quatre protéines C (C’, C, Y1, Y2) partagent la même région C-terminal alors que seul les longues protéines C (C’ et C) partagent la même région N-terminale. La partie C-terminal de la protéine C est nécessaire et suffisante pour bloquer le signal de l’IFN. Par conséquent les protéines Y1 et Y2 peuvent à elle seules garantir cette fonction. Par contre les longues protéines de C (C’,C) provoquent l’instabilité de Stat1, réduisant le niveau de Stat1 tout en augmentant celui de Stat1 sous sa forme phosphorylé. Ainsi la partie N-terminal de la protéine C est associée avec la dégradation de Stat1 qui permet de renverser l’état antiviral induit par l’IFN. Il a aussi été démontré que la partie C’1-23 (nucléotides 1-23 de la partie N-terminal) était suffisante pour réduire la quantité de Stat1 et agissait comme un signal ciblant la membrane plasmique. De plus la localisation des longues protéines C à la membrane plasmique est apparemment nécessaire à leur activité. Finalement, il a été montré également
que la protéine C pouvait inhiber la signalisation de RIG-I, un détecteur cytoplasmique d’ARN viral. Les protéines Y1 et Y2 (partie C terminale) seraient responsables de cette inhibition, mais aucun résultat n’a encore été clairement montré.

Leader et Trailer
Chez les paramyxovirus le promoteur génomique et le promoteur antigénomique (G/Pr et AG/Pr) se trouvent à l’extrémité 3’ du génome et à l’extrémité 5’ de l’antigénome, respectivement. Chez SeV ces promoteurs ont tous deux une longueur de 96 nucléotides. Le leader fait partie du G/Pr, incluant les 55 premiers nucléotides à l’extrémité 3’ du génome (-). Cette séquence contient les signaux d’initiation pour la synthèse d’ARN (transcription et réplication) par la polymérase virale. Le trailer comporte les 57 premiers nucléotides à l’extrémité 3’ de l’antigénome. Cette séquence est essentielle pour la réplication virale. Les transcrits du leader et du trailer sont tous deux exempt de régions codantes et ne sont ni coiffés, ni polyadenylés. Par contre ils portent chacun à leur extrémité 5’ un triphosphate, qui a été récemment déterminé comme une nouvelle signature virale détectée spécifiquement par des sentinelles cytoplasmiques de la cellule. L’AG/Pr, responsable de la synthèse du génome est plus fort que le G/Pr. Effectivement, l’AG/Pr a une plus grande affinité pour la polymérase et la production de génome est dix fois supérieure à celle de l’antigénome. De plus, il a été montré que le « gène start » réduisait la force du promoteur de réplication du G/Pr.

Les génomes défectifs et interférants: les DIs
Des formes incomplètes de génome viral générées lors du processus de réplication du virus standard ont été observées dans quasiment tous les virus à ARN et ADN et ont été nommées les “génomes défectifs et interférants”. Ce phénomène apparaît lorsque le virus est passé à haute multiplicité d’infection et dépend de l’ARN polymérase virale. La fréquence des ces événements est faible et se manifeste pendant la transcription ou la réplication du génome non-défectif (ND). Plusieurs DIs peuvent être générés, mais seul quelques uns sont sélectionnés selon leur capacité à interférer avec le génome ND. La notion d’interférence est importante car elle permet aux génomes DI de se répliquer et de s’amplifier au dépend du génome ND, qui doit rentrer en compétition avec ces derniers afin de générer les protéines virales, elles-mêmes impliquées dans la réplication et la maturation du virus. En général, la plupart des DIs ne sont pas capables de transcrire, ni de traduire. Ainsi, ils sont entièrement dépendants du ND pour leur réplication. Une autre particularité de ces génomes DI est qu’ils sont requis dans l’établissement et la maintenance d’infections persistantes.
On peut observer principalement deux types de DI: le DI de « délétion interne » et le DI d’extrémités symétriques ou « Copyback ». Dans le premier cas, la polymérase à ARN commence à synthétiser le génome et à un certain moment saute en avant sur sa matrice pour continuer la synthèse. Le DI à « délétion interne » conserve les extrémités 3’ et 5’ mais une partie plus ou moins grande du génome manque ou est absente. Dans le cas du DI « copyback », la polymérase commence la synthèse normalement, mais à un certain moment se détache de sa matrice et au lieu de continuer en avant, elle commence à copier dans le sens inverse, utilisant la chaîne naissante comme matrice. Par conséquent, dans le DI « copyback », il manque une grande partie 3’ du génome ND, mais on trouve le même AG/Pr à chaque extrémité. Cette particularité du DI « copyback » lui permet d’avoir une meilleure réplication et d’interférer ainsi avec le virus ND. Le fait que les DIs « copyback » n’expriment pas de protéines ne veut pas dire qu’ils n’ont aucun rôle. Effectivement, le fait qu’ils possèdent deux AG/Pr implique aussi la présence de deux trailers, qui ont un rôle antiapoptotique. Ceci expliquerait en partie la mise en place d’une infection persistante, lors d’une infection avec un stock de SeV contenant des DIs de type « copyback ».

Le système de l’Interféron

Lors de leur évolution, les cellules ont développé des défenses efficaces afin de contrecarrer les infections virales. La première ligne de défense d’un organisme contre l’invasion d’un pathogène se traduit principalement par la sécrétion d’interférons (IFNs). Les IFNs font partie de la famille des cytokines identifiées par leur capacité à induire une forte résistance cellulaire suite à une infection virale. Leur action a comme effets d’induire un état antiviral aux cellules et tissus alentours à l’infection et d’utiliser différents moyens biologiques pour interférer avec la réplication virale, moduler la réponse immune et réguler l’apoptose.

L’ARN double brin est connu pour être un fort inducteur de l’IFN et est potentiellement généré lors du processus de la transcription et de la réplication du génome viral. Il existe trois types d’IFNs : 1) L’IFN de type I, incluant l’IFNα et IFNβ. 2) L’IFN de type II (également appelé IFNγ) comprend les IFNε,κ and ω qui ont été récemment définis. 3) L’IFN de type III ou IFNλ est une nouvelle cytokine similaire à IL10. Les IFNs de types I et II sont tous deux impliqués dans l’activité antivirale, mais l’IFN de type I joue un rôle plus important dans l’immunité innée alors que l’IFNγ est plus impliqué dans l’immunité adaptative. L’IFN de type III possède des activités et des fonctions similaires aux IFNs de type I, bien qu’il utilise un autre complexe de récepteurs de surface. Les trois types d’IFNs induisent des réponses
transcriptionnelles à travers la voie de signalisation de JAK-Stat qui résultent en l’activation de multiples gènes. La régulation de l’expression des IFNs est bien caractérisée et demande la participation de différents complexes de facteurs de transcription qui sont déjà présents dans la cellule et qui sont activés lors d’une infection virale. Il existe quatre facteurs de transcription connus pour lier l’enhancer activatrice de l’IFNβ: ATF-2/ C-Jun, NF-κB et deux facteurs de transcriptions (IRFs), IRF-3 and IRF-7. La famille des protéines IRF contient 9 membres, qui sont extrêmement importants pour un grand nombre de processus incluant la réponse immune, la signalisation de cytokines et la croissance cellulaire ainsi que l’hématopoïèse. IRF-3 et IRF-7 résident dans le cytoplasme des cellules non infectées et qui, à la suite d’une infection virale, sont transloquées dans le noyau. IRF-3 and IRF-7 sont essentiels pour l’induction maximale de l’expression d’IFNα/β. Alors qu’IRF-3 est constitutivement exprimé, IRF-7 est principalement dépendant de son induction par l’IFN. À la suite d’une infection, IRF3 est activé et s’installe dans le noyau où il initie la synthèse d’IFNα/β en se liant aux régions enhancer activatrice. Les IFNα/β sont alors sécrétés et se lient à nouveau de façon paracrine sur les cellules voisines afin de les alerter d’une possible infection ou alors de façon autocrine pour induire plusieurs gènes ISGs (IFN stimulated genes) dans la cellule même. De cette façon la première vague de défense est mise en place résultant en un état antiviral des cellules.

Les détecteurs d’infections de virus à ARN
Plusieurs voies de signalisation conduisant à l’induction des IFNα/β ont été découvertes récemment. Elles incluent différents récepteurs cellulaires qui ont la particularité de détecter la présence du virus en reconnaissant les signatures moléculaires virales. Ces signatures virales font partie des PAMPs (pathogen associated molecular pattern) qui contiennent beaucoup de signatures potentielles provenant de différents pathogènes, incluant les virus, les bactéries et les champignons. Ces PAMPs sont reconnus par un large spectre de récepteurs nommée PRRs (pattern recognition receptors) qui comprennent les « R-proteins » (pathogen-resistance protein) chez les plantes, les récepteurs Toll-like (TLRs), les récepteurs NOD-like (NLRs) ainsi que les récepteurs Rig-like (RLRs) chez les animaux. Les TLRs se trouvent essentiellement associées à la membrane plasmique, soit à la surface cellulaire soit dans la membrane des endosomes. Les NLRs et les RLRs quant à eux, sont des protéines solubles qui surveillent le cytoplasme et qui sont prêts à détecter la présence de pathogènes à l’intérieur de la cellule. Jusqu’à maintenant, la reconnaissance des bactéries dans la cellule reste
spécifique aux NLRs, alors que celle des virus reste restreinte aux RLRs. Finalement il semblerait que la coopération entre ces PRRs constitue un véritable bouclier contre les pathogènes envahissants.

Il existe trois membres de la famille des RLRs : retinoic-acid-inducible gene 1 (RIG-1), melanoma differentiation-associated gene 5 (MDA-5) et laboratory of genetics and physiology 2 (LPG2). RIG-I et Mda-5 sont des hérlicases, dont le rôle est de distinguer les ARNs du soi de la cellule et les ARNs du non soi provenant des virus, et de réguler ainsi le signal de transduction en aval. Par contre, LPG2 joue un rôle de régulateur-inhibiteur de RIG-I et de Mda-5. Des études ont révélé que RIG-I était essentiel à la reconnaissance d’un set spécifique de virus incluant les Paramyxoviruses, Flaviviruses, Orthomyxoviruses et Rhabdoviruses, alors que Mda-5 était essentiel à la reconnaissance d’un différent set de virus, incluant les Picornaviruses et les Alphaviruses. Il a été également démontré que RIG-I était activé par de l’ARN double brin ainsi que par des ARN non-cappés, portant un triphosphate à leur extrémité 5’. De ce fait, il semblerait que différentes voies d’activation peuvent être activées selon le PRRs impliqué dans la reconnaissance de la signature virale et selon le type de virus. Malgré ces différences, RIG-I et MDA-5 gardent la même voie de signalisation en aval. Ils sont tous deux exprimés ubiquitairement dans la plupart des tissus et font partie des ISGs. Il a été montré que la reconnaissance des PAMPs induisait un changement de conformation de RIG-I et Mda-5, les rendant capables de se lier à une protéine liée à la surface externe de la membrane mitochondriale, nommée Cardif. Cette interaction conduit indirectement à l’activation de différentes kinases qui induisent l’activation de IRF-3 et NF-kB, ce qui finalement résulte dans l’induction de l’IFN de type I et à la mise en place de l’état antiviral de la cellule.

Papier 1 :
Le virus de Sendai cible de multiples façons les réponses inflammatoires ainsi que l’état antiviral induit par l’IFN
Pendant leur évolution, les virus ont développé plusieurs stratégies afin de réguler et contrecarrer les réponses innées des cellules hôtes, en particulier la production des IFNs. Pour cela, le virus de SeV utilise ses protéines C pour combattre les réponses à l’IFN. Le premier papier se base sur des microarrays d’ADN complémentaires afin de comparer l’activation de différents gènes cellulaires en réponse aux infections de SeV contenant des mutations spécifiques dans le gène C ou dans les régions promotrices du génome (qui ont apparemment un rôle dans la prévention de l’apoptose et dans l’infection persistante). Ces analyses ont
permis d’observer que le niveau d’activation d’environ 20 ARNm augmentait significativement lors d’infection avec les virus SeV mutants, comparé aux infections de SeV WT. Trois différents groupes de gènes cellulaires ont pu être mis en évidence selon les mutations que porte SeV. Certains des gènes sont connus comme des gènes stimulés par l’IFN (ISGs) d’autres, comme IL-6 ou IL-8 ne sont pas directement induits par l’IFN. Le gène de l’IFNβ, qui est essentiel pour initier l’état antiviral fut également activé lors d’infection de SeV portant des mutations. Ce travail met en avant le fait que SeV portant des mutations spécifiques dans le gène de C, par opposition au SeV WT, active l’expression d’IL-6 et IL-8 ainsi que quelques autres ISGs. Ainsi les protéines accessoires C et V et la présence du leader ont un rôle dans la prévention de l’expression de ces gènes cellulaires qui sont essentiels autant pour les réponses inflammatoires, que pour les réponses adaptatives et innées de la cellule hôte.

Papier 2: 
Les génomes défectifs de SeV (DIs) et l’activation de l’Interféron-beta
Le promoteur de l’IFNβ est normalement activé lorsque les cellules sont traitées avec de l’ARN double brin synthétique (polyI/C) ou lorsqu’elles sont infectées par des virus. SeV est généralement utilisé à cet effet, et des stocks de SeV peuvent être facilement obtenus dans le commerce. Cette remarquable capacité à induire l’IFN est cependant reliée au fait que ces stocks contiennent des génomes défectifs (DIs). Dans ce travail nous avons tout d’abord comparé la capacité d’induire l’IFN de plusieurs stocks de SeV, contenant ou non différents génomes défectifs. Plusieurs niveaux d’induction de l’IFN ont pu être observés selon le type de stock utilisé. Apparemment, cette propriété est particulièrement due à la présence de DI copyback et corrèle a priori avec leur capacité à s’hybrider et à former de l’ARN double brin. Le niveau d’activation de l’IFN semble également être proportionnel à la réplication même du génome défectif et au ratio génomes défectifs/génomes ND lors de l’infection. Dans cette étude, on démontre d’autre part que les protéines C et V de SeV sont aptes à bloquer l’induction à l’IFN lors d’infection avec les DIs ou lors de traitement avec le polyI/C. Ainsi on conclut que les infections de SeV contenant des DIs sont particulièrement de puissants inducteurs de l’IFNβ. Effectivement il est probable qu’ils fournissent une grande quantité de double brin et qu’ils réduisent en même temps l’expression virale des protéines C et V (normalement responsables de contrecarrer la réponse antivirale de l’hôte) favorisant de ce fait l’induction à l’IFN.
Papier 3:

Lors d'infections non-naturelles de SeV la voie d’activation de l'IFNβ requiert RIG-I et est inhibée par les protéines virales C

Sachant que SeV-WT n’active que très faiblement l'IFNβ et afin d’étudier l’activation de l’IFNβ dans les cellules MEFs, nous avons utilisé deux différentes sorte d’infection de SeV:

1) Le stock de SeV-DI-H4 est composée en majorité de génomes non-défectifs de type “copyback” et lors d’un infection, il produit en grande quantité les ARNs trailer contenant trois phosphates en extrémité 5’ et, en quantité moindre, les protéines virales C et V.

2) L’infection SeV-GFP(+/−) est une coinfection, qui génère (en plus des produits de gènes viraux en quantité équivalente à une infection SeV-WT) des ARN messagers GFP ainsi que leurs compléments pouvant former des ARNs double brin synthétiques (polyI/C) comportant des extrémités 5’ cappées. Ce travail nous a permis de découvrir 1) que l’activation de l’IFN, induit par les deux types de virus, dépendait principalement de RIG-I (et non de Mda-5). Plus précisément, que RIG-I détectait les pppARN simple brin ainsi que les ARN double brins ne comportant pas les trois phosphates en extrémité 5’ ; 2) que c’est la protéine C (et non la V) qui est responsable en premier lieu de contrecarrer l’induction de l’IFN dépendante de RIG-I. SeV n’exprimant pas spécifiquement les protéines C, ne peut pas prévenir l’activation de l’IFN induite par du polyI/C ou par les pppARN simple brin. Cette activation se trouve même amplifiée. D’autre part, de SeV n’exprimant pas de protéine V se comporte comme un SeV-WT et contrecarre les effets du polyI/C ou des pppARN simple brin.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AG/Pr</td>
<td>Antigenomic promoter</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>DI</td>
<td>Defective interfering</td>
</tr>
<tr>
<td>G/Pr</td>
<td>Genomic promoter</td>
</tr>
<tr>
<td>HPIV 1,2 and 3</td>
<td>Human parainfluenza virus type 1,2 and 3</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>le</td>
<td>Leader</td>
</tr>
<tr>
<td>Mda-5</td>
<td>Melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MeV</td>
<td>Measles virus</td>
</tr>
<tr>
<td>MV</td>
<td>Mumps virus</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>ND</td>
<td>Non defective</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
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<tr>
<td>NNV</td>
<td>Nonsegmented negative stranded RNA virus</td>
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<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
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<tr>
<td>RV</td>
<td>Rabies virus</td>
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<tr>
<td>SeV</td>
<td>Sendai virus</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SV5</td>
<td>Simian virus 5</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoic-acid-inducible gene 1</td>
</tr>
<tr>
<td>tr</td>
<td>Trailer</td>
</tr>
<tr>
<td>vRNAP</td>
<td>Viral RNA polymerase</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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I. General Introduction

Sendai virus (SeV) was discovered in Japan in 1953. It was isolated in the Tohoku University Hospital from a newborn patient presenting pneumonia syndromes. SeV is also referred to as murine parainfluenza type I virus as it was found to infect respiratory tract of mice, to cause pneumonia and to spread to uninfected animals. SeV is currently an important respiratory pathogen of laboratory rodents, causing terrible epidemics with high mortality during the acute phase. It is extremely contagious and transmission occurs via contact and aerosol infection of the respiratory tract.

SeV is an enveloped nonsegmented negative stranded RNA virus (NNV) of the *Paramyxoviridae* family, subfamily *Paramyxovirinae* and genus *Paramyxovirus* (or Respirovirus). It is considered to be a good model to study the *Paramyxoviridae* family because it includes significant human pathogens of infants and children, such as Mumps virus (MV), Measles virus (MeV), respiratory syncytial virus (RSV) and Nipah virus. Some of these viruses are still importantly present in undeveloped countries (e.g. MeV still causes a million deaths/year) and some others have only recently emerged. Thus, studies on SeV can offer important information for understanding the molecular mechanisms of this virus family and consequently offer medical treatment and therapy.

The *Paramyxoviridae* family along with the *Rhabdoviridae*, the *Filoviridae* and the *Bornaviridae* families, are all part of the *Mononegalvirales* order. It is subdivided into two subfamilies: the *Paramyxovirinae* containing the Respiro-, the Rubula-, the Morbilli-, the Avula- and the Henipa- viruses; and the *Pneumovirinae*, containing the Pneumo- and the Metapneumo-viruses. Emergence of new paramyxoviruses, such as Hendra and Nipah, causing respiratory and neurological disease in cattle and human, has been observed recently (Table 1).

The classification of these different viruses is based on morphologic criteria, the organisation of the genome, the biological activities of the proteins, and the sequence relationship of the encoded proteins. The Mononegalviruses share a number of fundamental characteristics: (1) Their genome is a single negative stranded RNA, packaged in a helical nucleocapsid (NC); (2) nucleocapsids are enclosed within an envelope derived from the plasma membrane of the cell; (3) a virus-coded RNA polymerase packaged in the virion synthesizes the viral mRNAs by transcribing the RNA as part of the intact NC after it enters the cell; (4) the RNA polymerase begins transcribing at the 3’end of the genome RNA and sequentially transcribes 5-10 genes, terminating and releasing each mRNA before starting the next one.
Family **Paramyxoviridae**

**Subfamily Paramyxovirinae**

**Genus Respirovirus (Paramyxovirus)**
- Sendai virus (mouse parainfluenza virus type 1)
- Human parainfluenza virus type 1 and 3
- Bovine parainfluenza virus type 3
- Simian virus 10

**Genus Rubulavirus**
- Simian virus 5 (Canine parainfluenza virus type 5)
- Mumps virus
- Human parainfluenza virus type 2, type 4a and 4b (hPIV2/4a/4b)
- Porcine rubulavirus

**Genus Morbillivirus**
- Measles virus
- Dolphin morbillivirus
- Canine distemper virus
- Peste-des-petits-ruminants virus
- Phocine distemper virus
- Rinderpest virus
- Cetacean morbillivirus

**Genus Avulavirus**
- Newcastle disease virus (avian paramyxovirus 1)

**Genus Henipavirus**
- Hendra virus
- Nipah virus

**Subfamily Pneumovirinae**

**Genus Pneumovirus**
- Human respiratory syncytial virus
- Bovin respiratory syncytial virus
- Murine pneumonia virus (Pneumonia virus of mice)

**Genus Metapneumovirus**
- Avian pneumovirus

**Unclassified paramyxoviruses**
- Tupaia Paramyxovirus

*Table 1: Classification of the Paramyxoviridae family ([www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm](http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm))
B. Virion structure of Sendai Virus

SeV virions are surrounded by a lipid bilayer envelope that is derived from the plasma membrane of the host cell in which the virus has grown (Choppin et al., 1975). The envelope contains two surface glycoproteins, F and HN, which mediate the entry and exit of the virus from its host cell. The nucleocapsid (NC), which is composed of the viral RNA genome tightly surrounded by hundreds of copies of the viral nucleoprotein (N), forms the active viral genome inside the envelope. The phosphoprotein (P) and the large (L) protein form the viral polymerase complex, which initiate intracellular virus replication. Between the envelope and the core lies the viral matrix (M) protein, which interacts with itself, with the lipid bilayer, with the NC, and with the cytoplasmic tails of the HN and F proteins (Fig. 1).

![Figure 1: Schematic representation of SeV particle (From P. Le Mercier)](image-url)
**C. Genome & encoded proteins**

The genome of SeV is a nonsegmented, single stranded RNA genome of negative polarity, which replicates entirely in the cytoplasm, and contains 15’384 nucleotides. SeV is composed of 6 genes, the N, P/C/V, M, F, HN and the L, which are flanked by control regions essential for transcription and replication. The 3’ extracistronic region is known as the leader (le) and the 5’ extracistronic region is known as the trailer (tr). SeV genes are monocistronic except for the P gene, which can produce multiple protein species (P,V,W,C’,C,Y1,Y2 and X) via overlapping reading frames and mRNA editing. Each mRNA begins with a short transcription regulatory sequence of ten nucleotides named the gene start (UCCCANUUNC) and terminates with a gene end sequence (UNAUUCU₃). There are also intergenic regions located between these sequences gene boundaries that are precisely three nucleotides long containing GAA, except for the junction between HN and L containing GGG (Fig. 2).

![Genomic organisation of SeV](image)

**Figure 2**: Genomic organisation of SeV (From (Lamb and Kolakofsky, 2001).

**The N protein**

SeV N protein, also named the nucleoprotein, and genome RNA are assembled in a helicoidal helix forming the nucleocapsid (NC), with a stoichiometry of one N protomer for six nucleotides. This precise hexamer arrangement is required for an efficient replication and is called “the rule of six”, that is also applied to the other Paramyxoviruses of the Paramyxovirinae subfamily. In this rule, nucleocapsid assembly presumably begins with the
first nucleotide at the 5’ end of the nascent chain, and continues by assembling six nucleotides at a time until the 3’ end is reached (Calain and Roux, 1993; Kolakofsky et al., 1998). The NC core is composed of 2564 N proteins and approximately 300 P proteins and 50 L proteins (Lamb et al., 1976). It is remarkably stable, as it withstands the high salt and gravity forces of cesium chloride density gradient centrifugation. Within the NC, the RNA is also resistant to nuclease attack at any salt concentration (Lamb and Kolakofsky, 1996). For paramyxoviruses, SeV N protein is divided into two regions; the well conserved N\textsubscript{CORE} (1-400aa) and the hypervariable N\textsubscript{TAIL} (401-524aa) (Houben et al., 2007). The N protein associates with the P-L polymerase during replication. In the current model for encapsidation, there is an initial sequence specific binding of N to viral leader RNA followed by the cooperative assembly of N on the growing chain, presumably through N-N and non-specific N-RNA interactions. Several paramyxoviruses N proteins, including those of SeV, MeV, Newcastle disease virus (NDV) and parainfluenza virus (PIV) 2, have been shown to self assemble onto cellular RNA to form NC–like particles when they are expressed alone, thus providing support for a N-N interaction (Buchholz et al., 1993; Errington and Emmerson, 1997; Nishio et al., 1999).

**Sendai Virus RNA polymerase: The P and L proteins**

The P protein and the L protein form the core of the viral RNA dependent RNA polymerase (vRNAP). The vRNAP is involved in the transcription and the replication, which are two important steps in the life-cycle of the virus. For transcription, the polymerase complex is constituted only by the P and L proteins, but for efficient replication, the N protein is required in addition to the L and P proteins. Several studies indicate that there are two different possible states (multiprotein complex) of the polymerase depending on whether it is transcribing or replicating. For example, it has been recently shown that the polymerase of Vesicular Stomatitis virus (VSV) founds itself in a replicase or in a transcriptase state, depending on its protein composition (Qanungo et al., 2004). There is, however, no indication that this could be the case for the Paramyxovirus RNA polymerase.

The polymerase cofactor P protein of SeV forms a tetramer and is named for its highly phosphorylated nature. It is a modular protein with distinct functional domains (Deshpande and Portner, 1984). It is composed of N-terminal and a C-terminal domain separated by hypervariable hinge (Curran and Kolakofsky, 1990). The N-terminal part is a chaperone for unassembled N proteins (N\textsuperscript{°}), preventing it from binding to nonviral RNA in the infected cell (Curran et al., 1995) and forming a complex (P-N\textsuperscript{°}) whose intracellular concentration is
believed to regulate rates of transcription and replication from genomic template (Curran et al., 1995; Masters and Banerjee, 1988). The C-terminal part is only functional as an oligomer and forms, along with L, the polymerase complex. It has already been shown for some other paramyxoviruses that the association of the N and P proteins has an effect on the N conformation (MeV) and on the virus assembly (VSV) (Das and Pattnaik, 2005; Kingston et al., 2004).

The L protein is the largest and the least abundant protein of the structural proteins. Its gene is also the most promoter-distal in the transcriptional map. It binds to the N:RNA template via the P protein and contains all vRNAP catalytic activities like synthesis, capping/polyadenylation and methylation of the nascent viral mRNA (Ogino et al., 2005). The L protein is highly unstable when expressed alone and needs to bind P to confer a good stability and a proper conformation, as co-expression of P and L is necessary for the formation of an active polymerase complex (Curran et al., 1995; Horikami and Moyer, 1982; Horikami et al., 1997). Until today, no crystal structure of the mononegalvirales L proteins is available. However, primary structure conservation among the RNA polymerases suggests similar protein architecture. Sequence comparisons of NNV-L proteins have identified 6 conserved regions, interrupted by variable sequences. These regions have been proposed to correspond to functional domains of the protein (Poch et al., 1990; Sidhu et al., 1993). There is also new evidence that the L protein could directly interact with itself and that this interaction would help RNA synthesis (Smallwood and Moyer, 2004).

**The M protein**

The matrix (M) protein is the most abundant protein in SeV virion. It is a quite basic and hydrophobic protein. The M protein is considered to be the central organizer in paramyxovirus budding and virus morphogenesis (Mottet et al., 1996; Sakaguchi et al., 1994b). The M protein self-associates and interacts with membranes, forming patches at the inner surface of the plasma membrane (Stricker et al., 1994). It also interacts with the cytoplasmic tails of integral membrane proteins such as the F and HN proteins, the lipid bilayer and the NC (Ali and Nayak, 2000; Yoshida et al., 1979). Moreover, the M protein forms vesicles and self-releases from cells when singly expressed from cDNA (Takimoto et al., 2001).
The HN & F proteins

The fusion of SeV requires co-expression of both HN (hemagglutinin-neuraminidase) and F (fusion) proteins. The HN and the F proteins are integral membrane glycoproteins and are essential for regulating morphogenesis and budding (Fouillot-Coriou and Roux, 2000; Takimoto et al., 1998). The HN protein is involved in cell attachment and is responsible for the adsorption of the virus to sialic acid-containing cell-surface molecules. In addition, it mediates enzymatic cleavage of sialic acid, namely neuraminidase activity, from the surface of virions and of infected cells. This activity prevents self-aggregation of viral particles during budding at the plasma membrane. The F protein mediates viral penetration by fusion between the virion envelope and the host cell plasma membrane. The F protein is synthesised as a precursor F0 which must be proteolytically cleaved to F1 and F2 for fusion activity (Morrison, 2003). The fusion occurs directly at the cell surface in an endosome-independent way, suggesting that infection does not require the acid pH of endosomes to activate fusion. The F protein has a self-release activity when expressed alone (Takimoto et al., 2001). After infection, the F proteins expressed at the plasma membrane of infected cells can mediate fusion with neighbouring cells to form syncytia, a cytopathic effect that can lead to tissue necrosis in vivo and might be a mechanism of virus spread.

D. Viral life cycle

All aspects of the replication of SeV happen in the cytoplasm. In cell culture, single-cycle growth generally last for 24 hours. As the infection takes place, the virus is adsorbed to the receptors found at the cell surface, and fusion occurs between the viral membrane and the cellular plasma membrane. This leads to the release of the helical NCs in the cytoplasm. This NC containing the viral RNA genome is the template for all RNA synthesis (Fig.3). Two functions are provided by the viral RNA genome: the mRNAs transcription and the viral RNA replication. The N, P/C/V, M, HN and L proteins are synthesised by the cellular ribosomes. The assembly of the genomes and the N proteins takes place in the cytoplasm. The M protein lies in the inner surface of the cytoplasmic membrane whereas the HN and the F float at the membrane and concentrate to the M patches, excluding other cellular proteins. Finally, the NCs associate with the M proteins and the new viral particles bud out of the cell taking a portion of the plasma membrane (Lamb and Kolakofsky, 2001).
**RNA synthesis**

Intracellular replication begins with the transcription of the viral genome into capped and polyadenylated mRNAs by the vRNAP. The vRNAP first transcribes the leader RNA at the 3’end of the genome, and then begins the transcription of the genes into six individual mRNAs in a sequential and polar manner. This polymerase occasionally fails to reinitiate the downstream mRNA at each junction, leading to the loss of transcription of further-downstream genes, consequently a gradient of mRNA synthesis that is inversely proportional to the distance of the gene from the 3’end of the genome is observed. The N protein is the most abundant of the structural proteins being synthesized, and the intracellular concentration of its unassembled state (N°) is a way of controlling the relative rates of transcription and the replication from the genome template. When sufficient amounts of N° are present, viral RNA synthesis becomes coupled to the concomitant encapsidation of the nascent (+) RNA chain. Under these conditions, vRNAP ignores all the junctions, to produce an exact complementary
antigenome (+) chain, in a fully assembled NCs. The antigenome will then be used for the synthesis of a new RNA genome, which will be used again as a template or assembled into a nascent viral particle. The vRNAP can also initiate RNA synthesis at the 3’end of the antigenome in the absence of sufficient N, but only a trailer RNA is made in this case (Fig.4) (Lamb and Kolakofsky, 2001).

Figure 4: RNA synthesis of SeV. The viral polymerase copies the genome into the leader (le) and six individual mRNAs from the genomic promoter (G/Pr). When the N° is sufficient, the viral polymerase starts replicating the genome into full-length antigenomes, which serve as intermediates in genome replication. The viral polymerase synthesises from the antigenomic promoter (AG/Pr) new genomes and other small RNA products, the trailer (tr). Both the genome and antigenomes are tightly encapsidated with the N proteins. (From (Lamb and Kolakofsky, 2001)
E. Accessory Proteins

The P gene of SeV expresses multiple species of proteins by means of using overlapping open reading frames (ORFs) (Fig. 5). This gene encodes as many as eight polypeptides via these ORFs: the P, V, W, C’, C, Y1, Y2 and X proteins. SeV P gene mRNA contain 5 start codons near its 5’ end, four of which are used for a nested set of “C” proteins that initiate at ACG^{81} (C’), AUG^{114} (C), AUG^{183} (Y1) and AUG^{201} (Y2) and terminate at UAA^{726}. Among the four C proteins, the C is the major species expressed in infected cells, at a molar ratio several fold higher than that of the other three proteins (Kurotani et al., 1998). The second start codon, AUG^{104}, initiates 3 proteins (P, V and W) as a consequence of cotranscriptional mRNA editing (Lamb and Kolakofsky, 2001). The start site (AUG^{104}) for translation of the P protein is in a favourable context (kozak) for recognition by the ribosome; and since it is placed right after ACG^{81} (which is normally not a favourable start site) it is more often used. For SeV, AUG^{104}, and AUG^{114} are initiated by leaky scanning, whereas AUG^{183} and AUG^{201} are initiated by ribosomal shunting (Curran and Kolakofsky, 1988; Gupta and Patwardhan, 1988; Latorre et al., 1998b).
SeV P gene contains an editing site in the middle of its reading frame. At this sequence, the vRNAP recognises the 3′-UUU UUU CCC stretch on the template and occasionally stutters. This stuttering most likely occurs when the vRNA pauses, and the growing RNA chain slips backward on the RNA template by one (or more) nucleotides (Hausmann et al., 1999; Pelet et al., 1991; Vidal et al., 1992; Hausmann et al., 1999). The vRNAP then resumes elongation.

When this happens in the run of three G’s, an extra G is added in the growing chain changing the reading frame. Addition of one G at the editing site produces an mRNA that encodes the V protein, whereas addition of two Gs leads to the W protein. The frequency of V and W production can vary depending on the kind of virus (Lamb and Kolakofsky, 2001).

F. **Role of the C and V proteins**

The C proteins are relatively small (175-215 residues), highly basic proteins and non-essential for the virus multiplication *in vitro*. Together with the V protein, they are referred to as “accessory” proteins (Tapparel et al., 1997), because viruses that do not express them are still viable in cell culture. SeV like many other members of the paramyxovirinae subfamily uses one or more products of its P/V/C gene to modulate viral RNA synthesis and to antagonize innate immunity. During the last decade, the involvement of the paramyxoviruses C and V proteins in counteracting the innate immune response was intensively studied. The Paramyxoviruses are likely to use their C proteins for this function by opposition to the Rubula-, Morbili-, and Henipa-viruses that use their V protein.

**SeV C proteins**

The localisation of the C proteins is likely to be at the membrane for two reasons: First, the C proteins were found to interact with a host protein involved in apoptosis and endosomal membrane trafficking, called Alix (Sakaguchi et al., 2005). Secondly, the C proteins contain a specific sequence at the N-terminus that functions as a membrane targeting signal and membrane anchor (Marq et al., 2007).
The C proteins are also required for virus replication because they act as inhibitors of the replication of the antigenomes in a promoter-specific fashion. More precisely, as the C proteins slowly reach a certain concentration during the course of infection, the genomic promoter (G/Pr) gets particularly sensitive to their presence, and the replication of the antigenome is reduced (Cadd et al., 1996a; Tapparel et al., 1997).

As mentioned before (Fig. 5), all four C proteins (C’, C, Y1, Y2) of SeV share the same C-terminal region and only the long proteins (C’ and C) contain the same N-terminal region. SeV that cannot express any of the C proteins are at the limit of viability. SeV mutants that can only express the short C proteins (Y1 and Y2) block IFN signaling like SeV-WT but are highly debilitated. The four C proteins physically interact with signal transducer and activator of transcription 1 (Stat1), which is a specific intracellular protein whose role is to protect and signal pathogen invasion. Activated Stat1 is phosphorylated on tyrosine 701, and is referred to as p-Stat1. As a consequence, these interaction between C and Stat1 will prevent IFN signaling through the JAK/Stat pathway and at the same time block the establishment of the antiviral state (Stark et al., 1998). Moreover, the SeV C proteins have also a role in disrupting Stat2 phosphorylation (Gotoh et al., 2003; Li et al., 2006). It was shown that the C terminal domain of C is necessary and sufficient for blocking the IFN signaling, suggesting that the Y1 and Y2 proteins are able to guarantee this function on their own. On the other hand, only the longer C proteins (C’, C) provoke the instability of Stat1, by reducing Stat1 levels and inducing p-Stat1 formation in an IFN-independent manner throughout the course of infection (Fig.6) (Garcin et al., 2003). Consequently, the pre-existing IFN-induced antiviral state is reversed and this suggests that the N-terminal domain of the C protein is associated with the degradation of Stat-1 in the cell (Garcin et al., 2002). Furthermore, C1-23 (23 residues at the N-terminal of the C proteins) was shown to be sufficient for reducing Stat1 levels and to act as a membrane targeting signal. Moreover, the activities of the longer C proteins are required for the localisation of C at the plasma membrane (Marq et al., 2007). Finally, recent data suggest that the C protein is able to inhibit the IFNβ signaling of RIG-I, a cytoplasmic viral sensor involved in the induction of IFNβ, by a yet unknown mechanism. The Y protein domain would presumably be responsible for the inhibition of RIG-I but no binding between the C protein and RIG-I has been shown yet.
Figure 6: SeV C proteins counteract the host interferon response by at least two mechanisms. All four C proteins (C’, C, Y1, Y2) of SeV share the same C-terminal region and only the long proteins (C’ and C) contain the same N-terminal region. These regions are associated with different functions. 

a) The C-terminal domain is necessary for the binding to Stat1, preventing its activation in response to IFN. 
b) The N-terminal domain (1-23 residues) is associated with the degradation of Stat-1, in this case only the C’ and C target Stat1 for degradation.

SeV V protein

The V protein seems to display similar functions to the C proteins. Elimination of V by mutating the editing site, or mutations in the C-terminal domain specific to V, reduces the virulence of SeV in mice, indicating that V is essential for efficient virus replication and pathogenesis in mice. Therefore, the SeV V protein seems to interfere with some host mechanisms that reduce virus replication or spread (Sakaguchi et al., 2003). SeV V protein as well as other V proteins of the paramyxoviruses, has been shown to limit IFNβ induction upon synthetic dsRNA poly(rI)-poly(rC) (PolyI/C) treatment (Andrejeva et al., 2004; Childs et al., 2007).

The role of the V protein, in inhibiting the host interferon response, has been discovered only recently and can act differently depending on the virus. Indeed, expression of the Rubulavirus V proteins (MV, SV5 and HPIV2) was demonstrated to induce polyubiquitylation of their target Stat (Stat1, 2 or 3 depending on the virus) resulting in efficient proteasomal degradation.
Nipah and Hendra viruses share the V-dependent IFN signaling evasion properties with other paramyxoviruses, but unlike the Rubulaviruses, they do not induce Stat destabilization. Indeed, they subvert IFN responses by sequestering Stat1 and Stat2 in high molecular weight complexes without inducing their degradation (Rodriguez et al., 2003). Finally, MeV encodes a V protein distinct from both the Rubula- and Henipa-viruses genera. Its expression effectively prevents both IFNα/β and IFNγ-induced transcriptional response. It does not degrade Stat or prevent Stat phosphorylation, but blocks IFN-induced Stat1 and Stat2 nuclear import (Palosaari et al., 2003).

G. Leader and Trailer RNAs

The genomic and antigenomic replication promoters (G/Pr and AG/Pr) of paramyxoviruses are found within the terminal 96 nucleotides of each RNA and are bi-partite in nature (Murphy et al., 1998; Pelet et al., 1996; Tapparel et al., 1998) (Fig 7).

![Diagram of SeV replication promoters](From (Vulliemoz et al., 2005).)

**Figure 7:** Primary structure of the SeV replication promoters.
The 96 nt of the genomic (G/Pr) and antigenomic (AG/Pr) promoters are presented as RNA sequence in ‘hexamers’, numbered 1–16 from the 3’ end (—OH 3’). In the G/Pr, the leader coding sequence is outlined, as well as the N gene transcription start signal (nt 56–65), which constitutes the first transcription start signal (N gs1) seen by the viral polymerase. In the AG/Pr, the trailer coding sequence is shown, as well as the complement of the L gene polyadenylation site (L ge). (From Vulliemoz et al., 2005).
SeV leader and trailer RNAs are short transcripts generated during abortive antigenome and genome synthesis, respectively. They both contain no coding region, and are neither capped nor polyadenylated, and carry triphosphates at their 5’ends that are believed to be potential targets for cellular antiviral genes (Plumet et al., 2007). The leader is part of the G/Pr and includes the first 55 nucleotides at the 3’end of the negative-strand genome RNA. Its sequence contains signals for initiation of RNA synthesis by the vRNAP, and is also thought to contain the encapsidation signal that direct packaging of full-length plus-strand copies of the viral genome in NCs, as it is the case for the leader of VSV (Smallwood and Moyer, 1993).

On the other hand, the trailer is believed to include the last 57 nucleotides at the 3’end of the antigenome RNA. This sequence, very rich in A/U is essential for the viral transcription and replication and also contains signals for genome packaging. Moreover, it has been shown by Iseni et al. in 2002, that a U-rich trailer sequence, nucleotides 31-41, has been found to bind the cellular TIAR protein, involved in the induction of apoptosis. Recombinants of SeV expressing the TIAR binding domain from both the G/Pr and the AG/Pr, which was modified in order to contain the U-rich sequence, exhibited a reduced cytopathic effect, and lead to infected cells survival (Iseni et al., 2002a). This result underlines the fact that, although G/Pr and AG/Pr carry out similar functions, they also have distinct properties. Indeed, the exchange of these sequences has interesting effects on virus infections. One of the main differences between the AG/Pr and the G/P is their strength: It has been shown that the AG/Pr has a stronger affinity with the polymerase and that there is 10 times more genome than antigenome produced (Lamb and Kolakofsky, 2001). Plus, an excess of 5-10 fold of genome over antigenome has also been observed for VSV (Kiley and Wagner, 1972); and an excess of 20 to 50 fold of genome over antigenome has also been shown for Rabies virus (RV). Further analysis on SeV showed the important role played by the gene start gs1 in decreasing the strength of the replication activity of the G/Pr (Le et al., 2003). Indeed, when gs1 was introduced in the AG/Pr sequence, the balance between genome and antigenome RNAs was equalized by weakening AG/Pr replication. Finally, the presence of the leader (containing the start site), instead of the trailer in the G/Pr also favours the hypothesis that the AG/Pr is stronger than the G/Pr.
H. Sendai Virus strains

There are two known lineages of SeV: Z/H/Fushimi and Ohita M/Hamamatsu (Fujii et al., 2002; Itoh et al., 1997). The nucleotide sequences within each lineage are 99% identical and they are 89% identical between lineages.

Z/H/Fushimi come from viruses isolated in Japan in 1956 after an epidemic of newborn infants and adapted to grow in embryonated chicken’s eggs (Ishida and Homma, 1978; Skiadopoulos et al., 2002). These adapted viruses, which were continuously passaged in eggs over a period of several decades are moderately virulent for mice (50% lethal dose [LD₅₀] = 10³ to 10⁴ PFU) (Sakaguchi et al., 1994a).

Ohita M (SeV¹) and Hamamatsu, in contrast, are highly virulent (LD₅₀, <10²). They were both isolated from two completely separate, very severe epidemics of animal houses in Japan and were low-egg-“passaged”. This virus is presumably closer to the virus in its natural host, and it is known that SeV passage in eggs attenuates its virulence in mice. For instance, in an infectious model, in which Kiyotani et al used three-year old mice, the Hamamatsu strain was very virulent; but when serially “passaged” 30 times in eggs, it strain became attenuated. (Kiyotani et al., 2001).
I. The Defective Interfering (DI) genomes

General aspects:

The generation of uncompleted forms of the viral genome during the viral replication process called Defective Interfering (DI) genomes has been observed in almost all the RNA and DNA viruses including SeV (Huang and Baltimore, 1970). A table is represented summarizing the occurrence of DI in negative stranded RNA viruses (Table 2). The ease with which DIs are produced varies widely within different virus groups and depends on many different factors. These include growth conditions, multiplicities of infection, the host cell, relative rates of standard virus replication and DI enrichment, virus strain differences, and intrinsic rates of DI generation. The appearance of DI genomes arises during passage at high multiplicities of infection because of the need for complementation by the helper virus. It has also been reported that single clones from certain viruses (SeV, VSV and Influenza I) have a genetic capacity to regularly generate the same DI species (Holland et al., 1980; Kolakofsky, 1979).

A good way to get rid of most or all DI genomes in a given virus stock is to “plaque” the virus stock several times in a row (Baltimore and Huang, 1975). Since DI RNAs are not antigenetically distinct from their parent virus, their biological properties are attributable to the genome deletions that they contain. Most of the time, the DI genome can be separated by velocity gradient, because they are smaller than the standard helper virus genome.

Table 2:

<table>
<thead>
<tr>
<th>Virus Group</th>
<th>Member name</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Negative Strand</strong></td>
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<tr>
<td>Rhabdo</td>
<td>Vesicular stomatisis, rabies, others</td>
<td>Reichmann and Schnitzlein (1979)</td>
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<td>Paramyxo</td>
<td>Sendai</td>
<td>Kolakofsky (1979)</td>
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<td></td>
<td>Newcastle disease</td>
<td>Roman and Simon (1976)</td>
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<td></td>
<td>Measles</td>
<td>Rima et al. (1977)</td>
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<td></td>
<td>Mumps</td>
<td>Norval (1979)</td>
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<tr>
<td>Orthomyxo</td>
<td>Influenza, fowl plague</td>
<td>Nayak (1980)</td>
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<tr>
<td>Arena</td>
<td>Lymphocytic choriomenengitis, others</td>
<td>Pedersen (1979)</td>
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<td></td>
<td>Tacaribe</td>
<td>Gimenez and Comans (1980)</td>
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<td>Bunya</td>
<td>Bunyavera</td>
<td>Kacsak and Lyon (1978)</td>
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<td></td>
<td>Lacrosse</td>
<td>Bishop and Shope (1980)</td>
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<td><strong>Positive strand</strong></td>
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<tr>
<td>Picorna</td>
<td>Poliovirus</td>
<td>Lundquist et al. (1979)</td>
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<tr>
<td>Toga</td>
<td>Sindbis, Semliki Forest, West Nile</td>
<td>Stollar (1979)</td>
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<tr>
<td>Corona</td>
<td>Mouse hepatitis</td>
<td>Robb and Bond (1979)</td>
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The Origin of DI RNAs:

DI genomes (DIs) stocks are defective viruses that contain only a fraction of genetic information of the infectious non-defective (ND) virus genome and that require homologous parental virus as helper for replication. They also contain virus structural proteins and exhibit the capacity to replicate preferentially at the expense of the infectious helper virus in cells infected by both. DI RNAs originate from low frequency events during the replication or transcription of standard virus RNAs. Many different DIs can be generated but only a few are selected, depending on their capacity to interfere with the ND genome. By “interference”, we mean that DI particles can replicate and amplify their genome preferentially at the expense of the replication of the helper virus. Indeed, the latter encodes replication and encapsidation proteins and must compete with the DIs for these gene products. Small DIs are likely to be the majority that are selected, but defective full-size genomes can also be found to exert interference (Roux et al., 1991). In general, most of the DI particles of RNA viruses are only replicative entities that are not capable of transcription or translation. They can undergo extensive mutational changes but must conserve the segments that are necessary for efficient replication and encapsidation. It is important to underline the fact that there must be a balance between the generation of DIs and the rate of viral production. If the DI interference is too strong, there will be insufficient virus helper to support significant DI particle replication, leading to an increased yield of infectious virus and to a reduced yield of DI.

The formation of DI particles is expected to be a consequence of the viral polymerase. It has been suggested that DIs arise by virus rearrangement or recombination as a result of viral replicase “leaping” or skipping from one virus RNA template to another or from one segment of a template to another (Huang, 1977). During this “leaping” the RNA replicase carries uncompleted nascent strand to a new template, then uses this nascent strand as a primer for continuation of chain elongation at the new template elongation site. Depending on the leaving sites or continuation sites, different DIs can be observed. There are mainly two different forms of DIs that can be generated: the Internal deletion DI and the Copyback DI genomes (Fig 8). In the case of the internal deletion DI the viral polymerase starts synthesising the genome and at a certain point jumps forwards on its template. The internal deletion DI genome still conserve the 3’ and the 5’ ends of the ND genome and lacks various portions of the internal transcription units. It carries both the minus- and plus-strand polarity RNAs containing both promoters enabling the DI to transcribe from the minus-strand and replicate from both the minus- and plus-strand. In the case of the copycack DIs, the viral
polymerase detaches itself from the template, and instead of going forwards, it initiates copying in reverse direction using the nascent strand as template. Consequently, the copyback DI genome lacks most of the genomic 3’ end sequence from the ND genome and in addition, it contains complementary extremities to the genomic 5’end, as inverted repeats (Kolakofsky, 1976; Leppert et al., 1977) (Fig 8). The origin of terminal sequence complementarity in SeV and VSV DIs is generally thought to occur by some sort of strand-switching event during polymerisation, where the polymerase would drop off the template and start the synthesis of the daughter or another template. In this way the features of terminal sequence complementarity in DI genomes provide strong argument for the involvement of the viral polymerase in the origin of DIs.

The presence of inverted complementarity sequences (not found in the standard genome) was defined as the hallmark of the most abundant class of VSV DI (DI_011) RNAs also called the “snapback” DI. The first evidence for this structural feature came from electronic microscopic observations of circular DI RNAs with characteristic small panhandle or stems in SeV (e.g. the natural copyback DI-H4), (Kolakofsky, 1976) and in VSV (Perrault and Leavitt, 1978). The shortest inverted repeat reported for Paramyxoviruses amounts to 94 nucleotides, with a range between 94 and 168 nucleotides (110 nucleotides for SeV DI-H4) (Calain et al., 1992; Calain and Roux, 1993). In this case, the copy-back DI carries both the minus- and plus-strand polarity RNAs but contains the antigenomic (AG) promoter at both the genomes and antigenomes ends. Furthermore, copyback DIs in SeV seem to interfere more with the ND genome than other conventional DIs, presumably because of the strength of the two strong AG promoters they contain. As mentioned before, the replication efficiency of the copyback DI (e.g. DI-H4) is 20 fold higher than the one of the Internal Deletion DI-E307 (Calain and Roux, 1995). For VSV, the presence of the specific sequences at the 3’-terminus of both the genomic and antigenomic DI RNAs may also explain in part the replicative dominance of DI genome over the full-length genome, which contains these sequences only at the 3’-terminus of the antigenome (Pattnaik et al., 1995). Finally, it has been observed that some DI copyback particles could modulate the course of infection by interfering indirectly with the helper virus by inducing IFN activation. This aspect is likely dependent on the kind of DI genomes involved in the infection. The VSV “snapback” DI has been shown to strongly induce the IFN and interestingly, it was the minimal multiplicity of DI infection that was required to promote maximal effects on IFN induction (Marcus and Gaccione, 1989).

Another important aspect of DI genomes is that they are required for the establishment and/or maintenance of persistent infections in cell culture (Holland et al., 1979). This characteristic
can be observed in several different virus systems, such as RV (Kawai and Matsumoto 1977), VSV (Horodyski and Holland, 1980) and NDV. For SeV infection, this property can be explained (in part) by the fact that copyback DIs contain two trailer sequences that can bind to TIAR and thus prevent apoptosis leading to the establishment of a persistent infection (Iseini et al., 2002b). Even though the ability of DI genomes to modulate the intensity and the course of viral infections has been studied widely in in vitro systems, their possible roles in natural infections remain largely unexplored and unconfirmed (Barrett and Dimmock, 1986).

**Figure 8**: A schematic representation of DI genomes synthesis.

During the replication process, the viral polymerase synthesizes new SeV genomes from the antigenomic promoter (AG/Pr) and occasionally generates uncompleted forms of the viral genome called the Defective Interfering (DI) genomes. The dotted lines below the SeV antigenome indicate the path of the viral polymerase when it generates internal deletion DI genome (e.g. E307) or copyback DI genomes (e.g. DI-H4).
*J. The reverse genetics system*

In contrast to the positive RNA virus, which are potentially infectious by forming directly viral proteins, neither the genome nor the antigenome RNA of NNV are infectious. Indeed, they need to be transcribed before they can produce active proteins, such as the polymerase or the nucleoprotein, that are required for the synthesis of the viral proteins and the RNA encapsidation, respectively. For this reason, the recovery of infectious virus from cDNA (reverse genetics), which requires the co-expression of the N, P and L proteins in trans, has been delayed compared to the one of positive stranded viruses (Racaniello and Baltimore, 1981). Influenza virus was the first NNV to be recovered from cDNA. Luytjes et al. have described a system in which they use purified N proteins and synthetic RNA to assemble a synthetic gene-like RNA into a biologically active ribonucleoprotein (Luytjes et al., 1989). In contrast to influenza virus the paramyxo-ribonucleoproteins have resisted for a long time to be assembled in a functional form *in vitro*, probably because they are much more tightly structured (Baudin et al., 1994; Iseni et al., 1998). Indeed even after banding in CsCl density gradients, they remain highly active and the RNA within this structure remains resistant to RNase treatment at any salt concentration (Enami and Palese, 1991; Lynch and Kolakofsky, 1978). In 1991, paramyxoviridae derived RNA corresponding to a SeV minigenome was rescued for the first time (Krystal et al., 1986; Park et al., 1991). This artificial SeV RNA, containing the antisens coding region of the CAT gene flanked by the entire 5’ and 3’ noncoding termini of SeV, was generated *in vitro* by the T7 polymerase from a linearized plasmid. When introduced into cells that was infected with SeV, this RNA construct was transcribed, replicated, and packaged into infectious virions and significant CAT activity was observed. This further confirmed the fact that the cis-acting sequences necessary for the encapsidation, replication and transcription of SeV are located in the terminal sequences and that the helper virus provides the necessary viral proteins for the RNA recognition.

The concept of replicating mini-genomes using support plasmids providing N, P, and L proteins in trans was finally the key to the development of the technology. Indeed, the use of mini-genome RNA transcripts carrying only a reporter gene facilitated *in vivo* experiments, in which RNA encapsidation was strongly required. Moreover, a system named the Vac-T7 system, in which a recombinant Vaccinia virus encoding the bacteriophage T7 RNA polymerase was established (Fuerst et al., 1986). Cell were infected with the recombinant Vaccinia virus and transfected with plasmids encoding all the required viral genes and the viral RNA genome, which were all expressed under the control of the T7 polymerase
promoter. This approach conferred several advantages: (1) it provides the T7 polymerase, whose function is absolutely necessary for producing RNA genomes and for increasing the expression level of the N, P and L mRNA in the cytoplasm; (2) it allows further analysis regarding the individual function of each viral gene involved or not in the RNA amplification. The vac T7 system required the generation of correct viral RNA termini. Indeed, while the exact 5’ends were determined by the position of the T7 promoter transcription start signal, generation of the correct 3’ends required a ribozyme activity sequence derived from the antigenomic strand of the hepatitis delta virus RNA (Gershon et al., 1991; Perrotta and Been, 1990; Shih and Been, 2002).

More refinements to the original technique have been made using stably transfected cell lines BHK, which express the bacteriophage T7 RNA polymerase instead of vac-T7 infection. (Buchholz et al., 1999). This approach based on the critical thought that the assembly of free genome (or antigenome) RNA into a functional NC could be accomplished in a living cell, has been successful for rescuing VSV and SeV DIAs (Calain et al., 1992; Calain and Roux, 1993; Curran and Kolakofsky, 1991; Pattnaik and Wertz, 1990).

With time, this approach has been successful for many negative-stranded RNA viruses: RV (Conzelmann and Schnell, 1994), VSV (Pattnaik and Wertz, 1990), SeV (Garcin et al., 1995), RSV (Collins et al., 1991), MeV (Radecke et al., 1995), SV5 (Murphy et al., 1998), Rinderpest virus (Baron and Barrett, 1997), Canine distemper virus (Gassen et al., 2000) and Nipah virus (Yoneda et al., 2006).
**K. The Interferon System**

During evolution cells were forced to develop sophisticated defences for counteracting virus infection. Secretion of cytokines and chemokines is one of the first responses of an organism to pathogen infection. Among the cytokines are the interferons (IFNs). The IFNs are an heterogeneous family of cytokines, originally identified on the basis of their ability to strongly induce cellular resistance to viral infections (Pestka et al., 1987; Vilcek, 1996). The IFNs’ action on virus-infected cells and surrounding cells and tissues elicits an antiviral state. The IFNs exert various biological effects such as interference with virus replication, modulation of the immune response, antiproliferative activities and apoptosis. A potent inducer of IFNs is intracellular double stranded RNA (dsRNA) which is generated during replication and transcription of viral genome. There are three types of IFNs: 1) Type I IFNs includes IFNα and IFNβ, plus additional IFNs recently defined: IFNε,κ and ω; 2) Type II IFNs, containing IFNγ, also called “immune interferon”; 3) Type III IFNs or IFNλ, a novel interleukin 10-related cytokines (Fig. 9). IFNα is produced by peripheral blood leucocytes and many other cell types in response to viral infection or treatment with dsRNA. IFNβ is mainly produced by fibroblasts or epithelial cells. IFNγ is produced exclusively by immune cells. Although both classes of IFNs exhibit potent antiviral activity, Type I IFNs play a more important role in the innate immune response to viral infection, whereas IFNγ plays a much more significant role in the subsequent acquired immune response (Schindler, 1999).

A common property of both IFN types is to induce immediate transcriptional responses through a JAK-Stat signal transduction pathway (Schindler and Darnell, Jr., 1995). All type I IFNs bind to a class II cytokine receptor composed of IFNα receptor 1 (IFNAR1) and IFNAR2 chains (Novick et al., 1994), which are associated with the Janus kinase (JAKs) TYK2 and JAK1 respectively (Darnell, Jr. et al., 1994). Ligand-bound, tyrosine-phosphorylated receptor complexes bind the SH2 domains of signal transducers and activators of transcription (Stats) 1 and 2, causing phosphorylation of the proteins on tyrosines 701 and 692, respectively. Interaction of Stats through reciprocal SH2 domain-phosphotyrosine binding results in the formation of two distinct transcription factor complexes: 1) ISGF3, a heteromeric complex consisting of Stat1 and Stat2 in association with a third protein, p48 or IRF9, associates specifically with and transactivates genes containing the interferon-stimulated response elements (ISRE) in their promoter or enhancer regions. 2) A simpler complex, consisting solely of the Stat1 homodimer, is also active as a transcription factor and binds to different DNA sequences, termed IFNγ activated site (GAS) elements (Katze et al.,
The receptor for IFNγ is structurally related to that for IFN type I. It consists in IFNγ receptor 1 (IFNGR1) and IFNGR2 chains in association with Jak1 and Jak2 kinases. Once tyrosine-phosphorylated in the presence of ligand, it binds Stat1 and causes phosphorylation of tyrosine 701. Stat1 homodimers are formed, move to the nucleus, and regulates transcription of promoters containing GAS sequences (Decker et al., 2002). Finally, type III IFNs display activities and functions much like the type I IFNs and utilise a different receptor complex to signal (IL10R2 and IFNLR1 chains).

The regulation of IFNs synthesis is well characterized and requires the participation of several transcriptional factor complexes that already pre-exist in the cell and are activated upon virus infection. These factors bind to regulatory sequences called positive regulatory domains, which lie within 200 nucleotides upstream of the transcription start sites on both IFNα and IFNβ genes. Four different transcription factors are known to bind to the IFNβ enhancer: the ATF-2/ C-Jun heterodimer, the NF-kB heterodimer and two interferon-regulatory factors (IRFs), IRF-3 and IRF-7. Together with general transcription factors including TFIID and RNA polymerase II, this collection of DNA-bound proteins assembles to form an “enhanceosome” (Thanos and Maniatis, 1995). The IFNα enhancer also contains multiple positive regulatory domains and is activated by binding to similar transcription factors. The IRFs are extremely important and function in a number of processes such as pathogen...
response, cytokine signaling, cell growth regulation, and hematopoiesis. IRF-3 and IRF-7 are two of the nine members of the IRF family (Mamane et al., 1999), residing in the cytoplasm of uninfected cells, and undergoing nuclear translocation upon viral infection. IRF-3 and IRF-7 are crucial to maximally induce the expression of IFNα/β (Sato et al., 2000). IRF-3 is constitutively expressed in normally growing cells, whereas IRF7 is mainly dependent on IFNα/β signaling. Upon viral infection, IRF3 is activated by phosphorylation on its serine residues by IKKe and TANK-binding kinase-1 (TBK-1) (Fitzgerald et al., 2003; Sharma et al., 2003). Phosphorylated IRF-3 homodimerizes and translocates to the nucleus, where it recruits the transcriptional coactivators p300 and CREB-binding protein (CBP) to initiate IFNα/β mRNA synthesis by binding to the IFNα/β enhancer regions (Hiscott et al., 1999). IFNα/β are secreted and feed back onto cells in a paracrine manner to prime neighbouring cells for possible infection and in an autocrine manner to induce multiple IFN stimulated genes (ISGs) including ISGF3 and IRF-7. As a result the “first wave” of IFN leading to the cellular antiviral state is in place (Sato et al., 1998) (Fig. 10).

**Figure 10**: Type I IFN induction, signaling and action. Left panel: RNA, a characteristic by-product of virus replication, leads to activation of the transcription factors NF-κB, IRF-3 and AP-1. The cooperative action of these factors is required for full activation of the IFNβ promoter. IRF-3 is phosphorylated by the kinases IKK and TBK-1 which in turn are activated by the RNA-sensing complex of RIG-I, Mda5 and IPS-1/MAVS. Right panel: Newly synthesized IFNβ binds to the type I IFN receptor (IFNAR) and activates the expression of numerous ISGs via the JAK/Stat pathway. IRF-7 amplifies the IFN response by inducing the expression of several IFNβ subtypes. Mx, ISG20, OAS and PKR are examples of proteins with antiviral activity (Haller et al., 2006).
Interferons confer the first line of defence against pathogen invasion by activating the ISGs. The ISGs carry antiviral functions, targeting the stability or translation of viral RNA. The three main IFN-induced systems are the following: the Mx proteins, 2′-5′oligo (A) synthetase (OAS) and ribonuclease L, and the dsRNA-dependent protein kinase PKR. Mx (for “myxovirus”) proteins are inducible-IFN proteins with the ability to hydrolyse GTP (Haller et al., 2007). Expression of Mx affects virus replication by interfering with the transcription of influenza virus and other negative-single stranded viruses. Mx proteins inhibit the activity of the vRNAP, thus blocking viral mRNA production. They also interfere with the transport of influenza virus RNP complexes from the cytoplasm to the nucleus. The exact function/action of Mx is not yet understood and the role of the GTPase activity also remains unclear. The 2′-5′oligo (A) synthetase is produced in IFN-treated cells, and is activated by binding to dsRNA in virus infected cells. This enzyme has the sole ability to produce oligomers of ATP via a 2′, 5′ linkage in contrast to the normal 3′, 5′ linkage found in the natural RNAs. These oligomers bind to and activate ribonuclease L, which degrades viral and cellular RNAs (Player and Torrence, 1998). PKR is a well characterized effector of anti-viral responses in mammals. PKR is a serine-threonine protein kinase that is present in normal cells at low levels. Like 2′-5′oligo (A) synthetase, PKR is activated by dsRNA or stem loop RNA structures. Activated PKR phosphorylates a variety of cellular proteins, such as the α-subunit of translation initiation factor eIF-2, thus inhibiting the translation. When PKR is overexpressed, programmed cell death is activated (Takizawa et al., 1996). Additionally PKR is believed to play an important role in regulation of cellular protein synthesis in absence of virus infection (Williams, 1999), as well as in signal transduction and cell growth (Koromilas et al., 1992).
L. Sensors of RNA virus infection

Many signaling pathways leading to the IFNα/β induction upon virus infection, have been recently discovered. These involve specific cellular receptors that can detect the presence of virus by recognising viral molecular signatures. These viral molecular signatures are part of the PAMPs (Pathogen-Associated Molecular Patterns) that contain many other potential patterns from other pathogen, including bacteria and fungi. The PAMPs are recognised by a wide range of receptors, called PRRs (Pattern Recognition Receptors) that comprise the Pathogen-Resistance Protein (R-protein) in plants, the Toll-Like Receptors (TLRs), the Nod-Like Receptors (NLRs) and the recently discovered Rig-like receptors (RLRs) in animals (Fig.11).

The R proteins were the first PRR to be discovered. These are crucial for immune defence against all the possible pathogen-derived molecules invading plants. Considering the fact that these R genes are of real importance for the survival of plants, it is not surprising to find that kind of pathogen-resistance protein in other organisms (Nimchuk et al., 2003). The next PRR to be discovered was the Toll receptor that was identified in the fruit fly, Drosophila melanogaster, as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos. In the mid-90s, toll-mutant flies were shown to be highly susceptible to fungal infection (Lemaitre et al., 1996; Yamamoto and Akirz, 2005). The receptor Toll was identified as a key mediator of innate immune defences in Drosophila melanogaster. A year later, the identification of a Toll-like receptor (TLR) in the human genome was reported and it was later called TLR4 (Medzhitov et al., 1997). Sequencing of human and murine genomes further allowed the identification of 11 TLRs in mice and 10 TLRs in human. The ability of the TLRs to recognise microbes and directly initiate specific signal transduction cascades that alert the host defences, can also be observed in the two additional families of innate receptors: the NLRs and the RLRs. Unlike the TLRs that are essentially found at the plasma membrane, these families consist of soluble proteins that survey the cytoplasm for signs that broadcast the presence of intracellular invaders. Until now, the NLRs have been shown to detect bacteria and many other pathogens, whereas the RLRs only recognise viruses (Creagh and O'Neill, 2006). NLRs share high structural and functional homology with plant R genes and search of the human genome database revealed 22 R-gene homologous classified in two main subclasses: the NODs (Nucleotides Oligomerization Domain leucine-rich repeat protein) counting 5 members and the NALPs (NACHT Leucine rich domain and Pyrin-containing
protein) containing 14 members. Some of the NLRs activation is proposed to occur via a mechanism similar to the mechanism described for the apoptosome (Reith and Mach, 2001).

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<tr>
<td><strong>NACHT</strong></td>
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<td><strong>RLRs</strong></td>
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</table>

**Figure 11**: Domain organization of the PRRs. All of the proteins have the NACHT-LRR configuration, with the exception of the RLRs, which contain a helicase domain. The A and B represent the Walker A and Walker B motifs. Not all of the possible N-terminal domains are represented; the caspase-recruitment domains (CARD) are specific to the RLRs and are also present in the NLRs when there are no BIR (baculoviral inhibitory repeat) or acidic domains. The Toll-IL-1 receptor or coiled-coil domains (TIR/CC) are N-terminal domains specific to the R protein or the TLRs (Adapted from Ting and Davis, 2005).

It is likely that the initiation of innate immunity involves an important cooperation between the TLRs, the NLRs and the RLRs depending on the pathogen, providing a tightly controlled combinatorial repertoire for triggering host defences. TLRs seem to be specifically active in immune cells such as dendritic cells whereas NLRs and especially RLRs are expressed in more various cell lines. The RLR family comprises three DExD/H-box-containing RNA helicases: retinoic-acid-inducible gene 1 (RIG-1), melanoma differentiation-associated gene 5 (Mda-5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and Mda-5 are both ubiquitously expressed in most tissues and are part of the ISGs, which allows autocrine and paracrine amplification of the sensing system. RIG-I and Mda-5 encode two caspase recruitment domains (CARD) at the N-terminus, followed by an RNA helicase domain. The helicase domain recognises viral RNA and regulates signal transduction in an ATPase-dependent manner, whereas the CARD domain is involved in the signal transduction downstream (Kang et al., 2004). LGP2 lacks completely the CARD domains and functions as a dominant-negative regulator of RIG-I/Mda-5 mediated signaling (Rothenfusser et al., 2005; Yoneyama et al., 2005). The recognition of the molecular viral signature via the helicase domain of RIG-I and Mda-5 likely induces a conformational change enabling their CARD
domains to interact with the CARD-like domain of a protein anchored to the outer mitochondrial membrane called Cardif (MAVS/IPS-1/VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). This interaction leads indirectly to the activation of several different kinases such as TBK-1/IKKe and IKKγ and activate IRF-3 and NF-κB respectively, inducing type I IFNs and the antiviral state of the cell (Fig. 12) (Hornung et al., 2006; Pichlmair et al., 2006).

Figure 12: RIG-I and Mda-5 are cellular RNA helicases that, upon activation, stimulate IFN gene expression. RIG-I recognises RNAs with 5′triphosphates, whereas Mda-5 recognises dsRNA (From Basler and Garcia-Sastre, 2007).

Studies of RIG-I and Mda-5-deficient mice have revealed that RIG-I is essential for the recognition of a set of specific ssRNA viruses, including Paramyxoviruses, Flaviviruses, Orthomyxoviruses and Rhabdoviruses, whereas Mda-5 is important for the recognition of a different set of RNA virus that includes Picornaviruses and alphaviruses (Basler and Garcia-Sastre, 2007; Kato et al., 2006). In vitro studies have also shown that both RIG-I and Mda-5 can bind to polyI/C and respond to polyI/C and RNA viruses (Yoneyama et al., 2005). In addition to this, it has been shown that RIG-I is more likely to recognise RNAs with 5′triphosphates than dsRNA, whereas Mda-5 specifically recognises dsRNA (Basler and Garcia-Sastre, 2007). The role of these helicases is to distinguish between self RNA and non-self RNA coming from the viruses. At the end of the year 2006, Veit Hornung et al. provided evidence that uncapped unmodified 5′-triphosphate RNA present in viruses known to be recognised by RIG-I, but absent in viruses known to be detected by Mda-5, serves as PAMP for the detection of viral infection by RIG-I in the cytosol of eukaryotic cells and that this
property is not confined to immune cells. They further observed that RIG-I does not activate the IFNβ activation with RNA containing 5’di- or 5’-monophosphate (Hornung et al., 2006). Many of the RNA species in the cytosol are known to lack free 5’-triphosphate group although all RNA transcripts generated in the nucleus of a eukaryotic cell initially contain a 5’tri-phosphate. Indeed cellular self RNA escapes detection by RIG-I because they are known to undergo several modifications before being transported to the cytoplasm: messenger RNA acquires a 7-methyl-guanosine cap structure at its 5’-end; transfer RNA undergoes 5’ cleavage and a series of nucleotide modifications; and ribosomal RNA undergoes several cleavages and finally associates with ribosomal proteins (Bowie and Fitzgerald, 2007) (Fig. 13).

**Figure 13:** Discrimination of self and non-self RNA by RIG-I. Viral infection leads to the accumulation of non-self RNAs in the cytoplasm, such as dsRNA and 5-triphosphate RNA. Cellular RNA synthesis takes place in the nucleus by 3 different RNA polymerases. The 5’ ppp of these RNA are eventually removed or masked, thus self RNA species do not activate RIG-I (From (Yoneyama and Fujita, 2007).

### M. Viral antagonists of the interferon system

Many viruses including SeV have developed strategies for counteracting the host type interferon I response and this at different levels. For that purpose viruses use most of the time non-structural proteins which are non-essential for virus growth. These antagonists are often multifunctional proteins that interact with multiple viral or host cell components and are involved in regulating many different functions in infected cells. Hence the IFN system can be triggered at different levels: 1) the IFN induction pathway comprising the IRF-3 activation, 2)
the basic transcription level, such as the inhibition of mRNA exportation or inhibition of polIII from Flu, 3) IFN signaling pathway including the JAK-Stat activation and 4) the IFN effectors level. Some viruses can contain more than one antagonist inhibiting one or several different components of the IFN induction and signaling pathway. Moreover, the virus-induced IFN response is generated in a cascade-like manner. Consequently, viral proteins blocking one cellular component in this circuit also affect distant signaling molecules or effectors, which amplify their inhibitory effect. Figure 14, from O. Haller et al. illustrates the range of activities mediated by IFN antagonists of various viruses (Fig. 14). As mentioned before, SeV mainly uses the non-structural C and V proteins to act at the level of the IFN induction pathway by blocking RIG-I activation and also at the level of the IFN feedback loop where C blocks Stat-1 signaling pathway. In many NNV, the phosphoprotein is the main antagonist. The P protein of RV prevents IRF-3 phosphorylation by TBK-I (Brzozka et al., 2005). In the same way, the P protein of Ebola Virus interferes with IRF-3 activation (Basler et al., 2003). The P protein of Borna disease virus directly binds to TBK-I and reduces its activity (Unterstab et al., 2005).

![Figure 14: Viral inhibitors of the virus-induced IFNα/β response loop: Viral gene products interfere with the type I IFN system at all levels. For example, V of paramyxoviruses were found to be IFN antagonists at the level of the IFN induction pathway. Many others viral proteins are involved in blocking the IFN activation at different levels. For more details see review (From (Haller et al., 2006).](image-url)
Vaccinia Virus from the Poxvirus family, as well as Influenza I virus, also possess genes that can interfere with the cellular activation pathway of IFN signaling. E3L and NS1 are viral regulatory protein found in Vaccinia Virus and Influenza virus respectively. E3L can bind to dsRNA and inhibit the dsRNA-stimulated enzymes, PKR and RNA-specific adenosine deaminase. A vaccinia deficient in E3L is highly sensitive to the activity to type I IFN and restricted growth in some cell lines such as Hela but not in other as chicken fibroblasts (Fischer et al., 2006). NS1 from Influenza A virus can also act against the IFN type I response. It has been suggested that NS1 can prevent IFNβ induction in several ways: by sequestering dsRNA through its amino terminus (Donelan et al., 2003; Hatada and Fukuda, 1992), by forming a complex with RIG-I to abrogate RIG-I signaling (Pichlmair et al., 2006), by binding to PKR (Li et al., 2006), and by inhibiting posttranscriptional processing of the 3’end of cellular specificity factor and poly(A)-binding protein II (Chen et al., 1999; Fortes et al., 1994; Li et al., 2006).
"SENDAI VIRUS TARGETS INFLAMMATORY RESPONSES, AS WELL AS THE INTERFERON-INDUCED ANTIVIRAL STATE, IN A MULTIFACETED MANNER"

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INTRODUCTION TO PAPER ONE:

Upon viral infection, cells respond by protecting themselves by inducing specific antiviral responses, such as IFNs. IFNs induce an antiviral state via the JAK/Stat pathway that leads to the activation of the ISGs expression. On the other hand, SeV infection counteracts IFN action by producing several specific proteins. SeV is known to use its C protein 1) to evade the host IFN response by binding to Stat1, preventing its activation in response to IFN, and 2) to target Stat1 for degradation. The leader and trailer products are short promoter-proximal products generated during abortive antigenome and genome synthesis. Moreover, the trailer is known to play a role in the regulation of programmed cell death, which is part of the antiviral program.

We have based our study on cDNA microarrays done in collaboration with Ian Kerr’s laboratory in London (ICRF) (Schlaak et al., 2002) to study the cellular response to IFNα/βs. These genes comprise known ISGs and genes of intrinsic interest that might or might not be induced by IFNs in different cell systems. They also include genes involved in cell proliferation, immune response, and response to a variety of cytokines. We have used these arrays to compare the activation of various cellular genes in response to infection by different strains and mutants of SeV in 2FTGH cells (human fibroblasts). Knowing the importance of the C gene and the promoter region in disturbing the antiviral system of the cell, two different SeV strains (SeV\textsubscript{M} or SeV\textsubscript{Z}) containing specific mutations in their C proteins (SeV C\textsubscript{F170S} and SeV C\textsubscript{Δ1015}) or in the promoter regions (SeV GP31-42 and SeV GP42) were used in our study.

The mutant SeV\textsubscript{C\textsuperscript{F170S}}, represents a SeV\textsubscript{M} strain that has been isolated after 5 passages in LLC-MK2 cells. It has three mutations and one, F170S is located in the carboxyl-terminal of all four C proteins (C', C Y1 and Y2) (Itoh et al., 1997). This base change is silent in the Open reading frame of P, V and W but this phenylalanine at position 170 of C is critical for blocking STAT1 activation, thus inhibiting the IFN signaling (Garcin et al., 2002; Gotho et al. 2001). This strain appears to be strongly attenuated in mice (LD\textsubscript{50} of > 8.10\textsuperscript{5} PFU) (Wang et al., 1994). The mutant SeV\textsubscript{C\textsuperscript{Δ1015}} is a recombinant of SeV\textsubscript{Z}. The codons 13 to 18 of P (W and V) and the codons 10 to 15 of C have been deleted. These deletions affect the N-terminal part of the C protein, a region essential for Stat1 degradation. It is an attenuated strain containing mutated C and C’ and WT Y1 and Y2 proteins. This deletion might alter the function of P, W and V, even though the P protein seems to be fully functional. Mutants SeV GP31-42 and SeV GP42 are SeV Z stocks carrying specific mutations in the leader region within the genomic
promoter. Compared with the WT SeV, SeV GP42 has its first 42 nucleotides from the leader promoter replaced into those of the trailer promoter. SeV GP31-42 has nucleotides from the leader promoter between the 31st and 42nd changed into those of the trailer promoter (Fig.15). Nucleotides 31-41 of the trailer promoter are important for binding to TIAR, a host RNA binding protein important for virus-induced apoptosis. Consequently, infections with mutants SeV GP42 and SeV GP31-42 lead to persistent infection. Finally, it is important to take into account that this gain of function can be due to either the over-expression of the trailer or the absence of the leader.

![Diagram](image.png)

**Figure 15**: SeV mutants carrying mutations in the leader region, which is part of the genomic promoter. SeV GP42 has its first 42 nucleotides replaced by the 42 first nucleotides of the trailer region. SeV GP31-42 contains nucleotides 31 to 42 from the trailer region.

Upon infection with SeV mutant viruses (SeV C\(^{F170S}\), SeV C\(^{Δ10-15}\), SeV GP31-42 and SeV GP42) 15 mRNA levels were found to increase significantly and three distinct patterns of gene activation could be observed. But, the levels of these mRNAs remain unchanged upon SeV WT infection (SeV\(^M\) and SeV\(^Z\)), presumably because these SeV stocks carry functions to counteract the cellular host defence. (Fig. 16):

A) The first group includes a series of genes that have their mRNA level increased by SeV C\(^{F170S}\) alone. SeV C\(^{Δ10-15}\) and others SeV mutant infections have no effect on these mRNA levels. The C\(^{F170S}\) substitution inactivates the ability of all four C proteins (C', C Y1 and Y2) to bind STAT1 in a stable manner and to counteract IFN signaling. It is likely that any of the four C proteins can block the increase of mRNA levels upon SeV infection.

B) The second group is only represented by the IL-6 gene. It is activated by SeV C\(^{F170S}\) as well as SeV C\(^{Δ10-15}\), but not by the WT viruses or other promoter mutants. Consistant
with this, the N-terminal region in the longer viral C proteins has a role in preventing the increase of IL-6 messengers upon SeV infection.

C) The third group includes genes whose mRNA levels increase upon SeV C\textsuperscript{F170S}, SeV C\textsubscript{\Delta10-15} and SeV GP42 infection, but not upon WT viruses or the SeV GP31-42 mutant. A function provided by either the C or the first 30 nucleotides of the genomic promoter (le) is required to prevent activation of these genes.

We then decided to confirm the above results by a more quantitative method, Real-time PCR (Taqman). The following host genes activated by our mutant SeV infections were selected, each one representative of one group: 6-16 (ISG) for group 1, IL-6 for group 2 and IL-8 for group 3. Since 6-16 is an ISG, we used it as a positive control. The chemokines, IL-6 and IL-8 were chosen because they are not part of the ISGs (as they are not activated upon simple IFN treatment) and are presumably involved in other different signaling pathways induced by virus infection. We also decided to examine the IFN\textbeta gene, because of its role in the early host response upon viral infection and the cellular gene GAPDH as an internal control to normalize the level of mRNAs.

Figure 16: Schematic representation of the viral mutations and their effects on the host gene activation. The C proteins are shown as two telescoping boxes representing the longer (C’ and C) and the shorter (Y1 and Y2) C proteins. The requirement for the various WT genetic elements to prevent host gene activation is shown (Instab: instability; sig: signaling) (From Strahle et al., 2003).
We examined the levels of each gene (as well as protein secretion in the case of IL-8) in different conditions: 1) upon WT and mutated SeV infections to confirm the microarrays; 2) upon dsRNA treatment or IRF-3 overexpression followed by WT and mutated SeV infections, to observe whether the antiviral response was enhanced by these pre-treatments; 3) by changing cell types: we used U5A and U3A cells that are 2fTGH cells, which have been generated by X irradiation. U5A are defective in the IFNα/β receptor 2 chain (IFNAR2) and U3A are defective in the Stat 1 gene. The use of these cells allowed us to determine whether induction of our genes upon WT and mutated SeV infections were dependent on IFN signaling and required Stat1; 4) Because SeV appears to use the V and C proteins to counteract the IFNβ expression and because the influence of the presence of the leader and the trailer RNAs is still unclear, we examined our SeV mutants as well as other recombinants SeV for their relative activation of IFNβ: I) SeV AGP55 which transcribes two leader RNAs from both promoters; II) SeV GP48 which transcribes two trailer RNAs and carries the same phenotype than SeV GP1-42; III) and SeV V minus/W++ that do not expressed any V proteins. The results of our study revealed that infection of SeV containing specific mutations in their C proteins and leader region activates specific cellular genes. The absence of the leader (1-42), C Δ1015, C F170S, and V-/-W++ mutations appear to disrupt four distinct elements in the SeV program to counteract the cellular antiviral response. This means that the products of the C and V proteins together with the presence of the leader RNA transcripts prevent the expression of these cellular genes involved in the IFN-induced antiviral state and in the inflammatory responses (Fig. 17).

![Figure 17](image)

**Figure 17:** Upon SeV infection, the V and C accessory proteins with the leader have a role in preventing the expression of specific cellular genes central to the host antiviral response as well as inflammatory response.
Senda Virus Targets Inflammatory Responses, as Well as the Interferon-Induced Antiviral State, in a Multifaceted Manner

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We have used cDNA arrays to compare the activation of various cellular genes in response to infection with Sendai viruses (SeV) that contain specific mutations. Three groups of cellular genes activated by mutant SeV infection, but not by wild-type SeV, were identified in this way. While some of these genes are well known interferon (IFN)-stimulated genes, others, such as those for interleukin-6 (IL-6) and IL-8, are not directly induced by IFN. The gene for beta IFN (IFN-β), which is critical for initiating an antiviral response, was also specifically activated in mutant SeV infections. The SeV-induced activation of IFN-β was found to depend on IFN regulatory factor 3, and the activation of all three cellular genes was independent of IFN signaling. Mutations that disrupt four distinct elements in the SeV genome (the leader RNA, two regions of the C protein, and the V protein) all lead to enhanced levels of IFN-β mRNA, and at least three of these viral genes also appear to be involved in preventing activation of IL-8. Our results suggest that SeV targets the inflammatory and adaptive immune responses as well as the IFN-induced intracellular antiviral state by using a multifaceted approach.

Alpha/beta interferons (IFN-α/β) are cytokines that act in a pleiotropic manner to limit viral replication and spread (2, 57). In fibroblasts (e.g., the bronchial epithelial target of many paramyxoviruses), the product of the single IFN-β gene is directly induced by viral infection, and IFN-β feeds back onto cells in an autocrine manner to induce multiple IFN-α genes and in a paracrine manner to prime neighboring cells for their possible infection (60). Since most viruses induce IFN-β to some extent, intracellular double-stranded RNA (dsRNA) generated from the viral genome is traditionally assumed to be the common signature of virus replication that sets the IFN system in motion (22, 32). dsRNA is thought to induce the formation of an enhancosome at the IFN-β promoter that includes IFN regulatory factor 3 (IRF-3) and NF-κB (among other transcription factors) (65). IFNs induce a cellular state that is non conducive for viral replication by signaling through their cell surface receptor, leading to the phosphorylation of cytoplasmic STAT proteins and their nuclear translocation. IFN-α/β responses are regulated primarily via IFN-stimulated gene (ISG) factor 3, a heterotrimeric transcription factor composed of STAT1, STAT2, and IRF-9 (p48). ISG factor 3 binds to a DNA element (IFN-stimulated response element) in the promoters of ISGs and activates their expression (7).

The extravasation of neutrophils, eosinophils, basophils, and mononuclear cells is the salient feature of the innate response to microorganisms in the lung. Localized and systemic pro- and anti-inflammatory cytokines thus also play an important role in the outcome of viral infection and pathogenicity of this organ (58). The CC chemokine interleukin-8 (IL-8) is secreted from epithelial surfaces in a polar fashion during infection with pathogenic bacteria such as Salmonella enterica serovar Typhimurium and sets up a subepithelial chemotactic gradient directing neutrophils and other immune cells to the site of infection (27). In polarized epithelial monolayers, S. enterica serovar Typhimurium-induced IL-8 expression is controlled via the activation of the mitogen-activated protein kinase cascade and IκB kinase, followed by NF-κB translocation to the nucleus and production of IL-8 mRNA. IL-8 secretion by primary human monocytes in response to dengue virus infection is also tightly linked to NF-κB activation (3). Sendai virus (SeV) infection of human embryonic kidney 293 cells induces the expression of the CXC chemokine RANTES in an IRF-3- and NF-κB-dependent manner (23, 41). NF-κB, like IRF-3, is found in the cytoplasm of unstimulated cells, retained in a complex with the inhibitory IκB proteins. Upon stimulation with many inducers, including dsRNA and virus infection, IκB is rapidly phosphorylated and degraded, resulting in NF-κB release and translocation to the nucleus (30, 33).

Given the importance of the host innate immune response to virus infection, viruses have, during their coevolution with cells, developed strategies to regulate cytokine synthesis and action. SeV, a model paramyxovirus and respiratory pathogen of mice, is known to use its C protein to evade the host interferon response by at least two mechanisms. (i) C binds STAT1, preventing its activation in response to IFN, and the carboxyl part of the C protein (i.e., residues 24 to 204, or the Y proteins) is sufficient for this activity. A phenylalanine at position 170 of C is sufficient for this activity. A phenylalanine at position 170 of C is also critical for blocking STAT1 activation (18, 59). (ii) C also targets STAT1 for degradation, and the amino-terminal residues of the C proteins (resides 1 to 23, which are absent...
in the Y proteins) are essential for this activity (reference 17 and references therein).

This paper reports that SeVs carrying specific mutations in the C gene, in contrast to wild-type SeV (SeV-wt), activate IL-8 and IFN-β expression as well as that of several ISGs. Our results suggest that the products of virtually all of the viral accessory genes (C and Y proteins and leader RNA) act to prevent the expression of these cellular genes that are central to the overall host antiviral response.

**MATERIALS AND METHODS**

**Cells and viruses.** 2C4 cells (39), 2TGH cells (48), and their derived cell lines U3A (45) and USA and USA-IFNAR (43) were obtained from IM Kerr (Imperial Cancer Research Fund, London, United Kingdom) and grown in Dubllecceo's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of the relevant maintenance drug (hygromycin at 250 μg/ml or G418 at 400 μg/ml). The generation of recombinant SeV (rSeV) expressing alternate C and V (and P) proteins is described elsewhere (8, 19, 37, 38). All SeV stocks were grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Virus titers were determined by plaquing on LLC-MK2 cells.

**Virus infections.** Cells were infected at a multiplicity of infection of 20 in Dulbecceo's modified Eagle's medium containing 10% fetal bovine serum. After an absorption period of 1 to 2 h, the inoculum was removed and replaced with fresh medium.

**Plasmids, transient transfections, and luciferase assay.** IRF-3 (54), IRF-3AN (41), and IRF-3-ribozyme (67) were obtained from John Hiscott and Paula Pitha. pDsRed2, expressing red fluorescent protein (RFP), was from Clontech. The reporter plasmid with the firefly luciferase gene under the control of the human IFN-β promoter was described by King and Goodbourn (35) and is referred to here as pIFN-β-luc. pTK-rl-luciferase, used as a transfection standard, contains the herpes simplex virus thymidine kinase promoter region upstream of the Renilla luciferase gene (Promega). For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pIFN-β-luc, 0.3 μg of pTK-rl-luciferase, 1 μg of IRF-3-expressing plasmid, and 6.9 μl of Fugene (Roche) according to the manufacturer's instructions. At 24 h posttransfection, the cells were cultured with (or were not) infected with SeV recombinants or treated with 50 μg of poly(I)-poly(C) (Sigma, St. Louis, Mo.) per ml. Twenty hours later, cells were harvested and assayed for firefly and Renilla luciferase activities (dual-luciferase reporter assay system; Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of Renilla luciferase.

**RNA extraction and quantification.** Total RNA was extracted from Trizol reagent (Invitrogen), and two dilutions were electrophoresed on agarose-HCHO gels. The gels were stained with ethidium bromide, and the intensities of the 18S and 28S rRNA bands were quantified by using the ChemiDoc System (Bio-Rad) and One-D-scan software. All samples were diluted to a final concentration of 1 μg/ml so that their subsequent transcription into DNA, if not quantitative, would be little influenced by this parameter.

**RT and real-time PCR via TaqMan.** Ten microliters of total RNA was mixed with 0.5 μg of random hexamer primer (Promega) and subjected to a reverse transcription (RT) reaction with Superscript enzyme (Gibco), as described by the manufacturer, in a total volume of 50 μl. Two microliters of each cDNA was then combined with 1 μl of internal control (either 20× ribosomal 18S or human glyceraldehyde-3-phosphate dehydrogenase [GAPDH] [Applied Biosystems]), 11 μl of 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: for the IFN-β gene, 5′-CAGCAATTTCATCAGTGAACG-3′ (forward), 5′-TCTCTTGCTGCTTGAGCCGTG-3′ (reverse), and 5′-CTTTGCGCAATTTGAGGAGCTTG-3′ (probe); for the IL-8 gene, 5′-CGCGCGTCTCCAGCTTGAACGCA-3′ (forward), 5′-TTAAGTCTTAGTGCTGGCTG-3′ (reverse), and 5′-GTGAAATTTCAATCAGTGATC-3′ (probe); for the IL-6 gene, 5′-CTTTGCTTGCTGCTTGAGCCGTG-3′ (forward), and 5′-CAGCGCGTCTCCAGCTTGAACGCA-3′ (reverse), and 5′-GGC-3′ (probe); for the IL-6 gene, 5′-CGCACTACGATGGCGATGCTG-3′ (forward), 5′-GATCTGAGGCCCAGCTTGAACGCA-3′ (reverse), and 5′-CGAAGTACATGCTGCTGCTG-3′ (probe) and for the N gene of SeV, 5′-GAATCCATGCTGCTGCTGCTG-3′ (forward), 5′-TGGCGCGTCTCCAGCTTGAACGCA-3′ (reverse), and 5′-CGAAGTACATGCTGCTGCTGCTG-3′ (probe).

**SeV, 5′-CGAAAGGTATGCAGCAGCGC-3′ (forward), 5′-GGGTCTGATGCTGCTGCTGCTG-3′ (reverse), and 5′-CAACCGTGGAGGTGAGGAGAAGT-3′ (probe). Real-time PCR was carried out in a 7700 sequence detector (Applied Biosystems, Foster City, Calif.). Generation of customized cDNA arrays. Macroarrays were prepared as described previously (55). 5′ IMAGE clones cloned 0.5 to 0.8 kb in length were chosen and obtained from the Human Genome Mapping Project (Hinxton, United Kingdom), plated onto L agar plates and grown overnight at 37°C. Single colonies were picked and propagated overnight in Luria-Bertani medium containing 50 μg of ampicillin per ml. Bacterial lysates were generated by heat lysis in distilled water. From these lysates, recombinant SeV were amplified by PCR as described above. After purification (QiAquick PCR purification kit; Qiagen, Crawley, United Kingdom), PCR products were sequenced (ABI Prism; Applied Biosystems). PCR-amplified cDNAs were transferred into 96-well plates and spotted manually onto dry nylon membranes (Hybond N+; Amersham Pharmacia, Little Chalfont, United Kingdom) in triplicates by using 96-pin replicators (Nalge Nunc, Naperville, Ill.; V&P Scientific, San Diego, Calif.). Membranes were air dried, denatured by alkaline treatment, and then neutralized. The membranes were again air dried and UV cross-linked prior to the experiment.

**Generation of labeled cDNA, hybridization, washing of membranes, and analysis.** Radiolabeled cDNA was generated from 10 μg of total RNA by RT with 400 U of reverse transcriptase (Superscript II; Gibco) in the presence of 30 μCi of [α-32P]dCTP. After RT, residual RNA was hydrolyzed by alkaline treatment at 70°C for 20 min. For removal of unincorporated nucleotides, the cDNA was purified by using G-50 columns (Amersham Pharmacia) according to the instructions of the manufacturer. Before hybridization to the arrays, the labeled cDNA was mixed with 50 μg of Cot-DNA (Gibco) and 10 μg of poly(A) DNA (Sigma), denatured at 95°C for 5 min, and hybridized for 1 h to minimize nonspecific binding. The cDNA was then added directly to the membranes, which had been prehybridized in 20 ml of hybridization buffer for at least 30 min. The membranes were hybridized for 16 h at 65°C in hybridization bottles (Amersham Pharmacia) in a rotary hybridization oven. After hybridization, the hybridization buffer was discarded and replaced by 150 ml of washing buffer. The membranes were washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS, twice in 0.2× SSC-0.1% SDS, and once in 0.1× SSC-0.1% SDS for 20 min each at 65°C. The membranes were then exposed to phosphorimage screens for 48 h and scanned with a phosphorimager (Storm; Molecular Dynamics, Little Chalfont, United Kingdom). For analysis, images were analyzed with ImageQuant (Molecular Dynamics). Further data analysis was performed with Excel (Microsoft).

**IL-8 assay.** Levels of IL-8 in supernatants and in cell lysates were determined by a sandwich enzyme-linked immunosorbent assay with paired monoclonal antibodies (Pierce) as described by the manufacturer. The IL-8 concentrations were normalized by using total protein levels in the lysate.

**RESULTS**

In our experiments we have used two strains (or lineages) of SeV whose history is relevant to studies of virulence. The natural host of SeV has not been clearly identified, and this virus is sometimes referred to as murine parainfluenza virus type I because it efficiently infects mice, causes disease, and spreads readily to uninected animals. However, there is no virologic or serologic evidence of SeV in wild mouse populations (29). There are two known lineages of SeV, Z/H/Fushimi and Ohita M/Hamanatsu (12, 31, 53, 62). The nucleotide sequences within each lineage are 99% identical, and they are 89% identical between lineages. Z/H/Fushimi comes from viruses isolated in the early 1950s after an epidemic of pneumonia of newborn infants in Sendai, Japan (29, 56). These SeVs have been passaged extensively in eggs in various labs since the 1950s, and they are moderately virulent for mice (50% lethal dose [LD50] = 102 to 104 PFU). All of the SeV2 strains used in this study (including the wt) are recombinants.

Ohita M (SeVM) and Hamanatsu, in contrast, are highly virulent (LD50 <102), low-egg-passage (nonrecombinant) viruses isolated from two completely separate, very severe epizootics of animal houses in Japan. This lineage is presumably
closer to the virus in its natural (unknown) host, and it is known that SeV passage in eggs attenuates its virulence in mice. SeV was avirulent (LD50 > 10^5) and contained only two amino acid substitutions, C\textsuperscript{F170S} and L\textsubscript{E2050A} (31). When placed in the rSeV background, the C\textsuperscript{F170S} mutation was found to account for most or all of the loss of virulence (15). This virus is referred to here as SeV\textsuperscript{M-C\textsuperscript{F170S}}. SeV\textsuperscript{M-C\textsuperscript{F170S}} appeared to initiate the infection of mice normally, but the infection was limited to the first day. This was the first evidence that the C gene, like the SeV V gene (34), was involved in countering host innate defenses. Although SeV\textsuperscript{Z-wt} is attenuated relative to SeV\textsuperscript{M-wt} in laboratory mice, rSeV\textsuperscript{Z-wt} still replicates as efficiently in the monkey and chimpanzee models of human respiratory disease as human parainfluenza virus type 1 (56), the virus which is most closely related to SeV and which is endemic in children.

We have used a cDNA array designed to study the human cell response to IFN-α/β (55) to monitor the effects of various SeV infections on host mRNA levels. Around 150 genes of interest were selected from the UniGene database. These genes comprise known ISGs and genes of intrinsic interest which might or might not be induced by IFNs in different cell systems. They include genes involved in cell proliferation, immune responses, and the responses to a variety of cytokines (see Table 1 of reference 55). We compared matched sets of SeV carrying two different mutations in the C gene (SeV\textsuperscript{M-C\textsuperscript{F170S}} and SeV\textsuperscript{Z-C\textsuperscript{A10-15}}), whose products interact with STAT1 in different ways (to interfere with IFN signaling and to induce STAT1 instability, respectively) (Fig. 1D). We also used matched sets of SeV carrying mutations in the viral replication promoters (SeV\textsuperscript{Z-GP1-42 and -GP31-42}) that prevent apoptosis and lead to persistent infections (the numbers refer to the nucleotides of the genomic promoter that have been replaced with the equivalent sequences of the antigenomic promoter) (16, 20). Promoter mutations are thought to act via mutant leader RNAs that are abundantly transcribed from the genomic replication promoter and which bind to cellular RNA-binding proteins that regulate mRNA fate (28).

Upon infection with SeV\textsuperscript{M-wt} or SeV\textsuperscript{Z-wt}, many of these mRNA levels remain unchanged (Fig. 1A to C). This lack of response is presumably due in part to active SeV countermeasures that neutralize the cell’s antiviral response (24). Upon infection with the mutant viruses, the mRNA levels of 15 of the 150 genes examined were elevated, and three patterns of gene activation were seen (all values are triplicates, and a twofold difference is very significant [55]). One series of genes (group 1, nine genes) (Fig. 1A) is activated by SeV\textsuperscript{M} infection alone; these mRNA levels are unchanged in SeV\textsuperscript{C\textsuperscript{A10-15}} and SeV\textsuperscript{GP1-31-42} infections. The C\textsuperscript{F170S} substitution inactivates the ability of all four C proteins (C', C, Y1, and Y2) to stably bind STAT1 and to Interdict IFN signaling (18, 59). According to this view, any of the four C proteins may function to prevent these mRNA levels from increasing during SeV infection (Fig. 1D) (14). The IL-6 gene is the sole representative of group 2; it is activated by SeV\textsuperscript{C\textsuperscript{A10-15}} as well as SeV\textsuperscript{C\textsuperscript{F170S}}, but not by the promoter mutants or the wt viruses (Fig. 1B). According to this view, a second function of the C gene, specific to the NH\textsubscript{2}-terminal 23 amino acids present only in the longer C proteins, is also required for SeV to prevent IL-6 activation. The third group, consisting of five genes, is activated by SeV\textsuperscript{C\textsuperscript{A10-15}} and SeV\textsuperscript{GP1-42} infections as well as SeV\textsuperscript{C\textsuperscript{F170S}} but not by SeV\textsuperscript{GP1-31-42} or SeV\textsuperscript{Z-wt} infections (Fig. 1C). Apparently, a third function provided specifically by the first 30 nucleotides (nt) of the genomic promoter (or leader RNA) is also required to prevent activation of genes such as that of IL-8 (Fig. 1C). This third function is not the ability of mutant leader RNA to bind TIAR, a host RNA-binding protein important for virus-induced apoptosis, as this occurs with SeV\textsuperscript{GP1-31-42} as well (28). In summary, comparative analysis of host gene activation with SeV with specific mutations has identified three groups of cellular genes that respond differently to SeV infection.

Real-time RT-PCR estimations of mRNA levels. IFN signaling through the JAK/STAT pathway activates many ISGs (such as 6-16, PKR, etc.) that collectively contribute to the cellular antiviral response. SeVs that interdict IFN signaling would therefore also prevent the activation of these ISGs. While many of the genes activated by the mutant SeVs are well known ISGs, IL-6 and IL-8 are known to be non-ISGs; treatment of 2TGH cells with 1,000 IU of IFN-α does not increase IL-6 or IL-8 mRNA levels over those of the untreated control (55). We therefore continued our study of selected host genes activated by SeV by real-time RT-PCR (TagMan), a method that is more quantitative than DNA arrays. We first examined IL-8 (a chemokine) and IFN-β, an early host response protein whose gene was absent in the DNA array. 2C4 cells (a 2TGH-derived cell line) were infected with 20 PFU of the various SeVs per cell, and the levels of various host mRNAs were determined, including that of GADPH as an internal control (see Materials and Methods). All four SeVs (SeV\textsuperscript{M-wt}, SeV\textsuperscript{Z-wt}, SeV\textsuperscript{Z-wt}, and C\textsuperscript{A10-15}) grow relatively well in 2C4 cells, as indicated by their accumulated N mRNAs (Fig. 2 and 3) or N proteins (data not shown); however, the SeV\textsuperscript{M-wt} infections accumulated ca. 2-fold less N mRNA than the three other SeV infections (Fig. 2C). The relative levels of virus replication are presumably important in stimulating the host response, and SeV\textsuperscript{M-wt} infections might therefore be expected to activate IL-6 and IFN-β less strongly than the other SeVs for this reason alone. We found that IL-8 and IFN-β mRNA levels were elevated >20-fold in SeV\textsuperscript{M-C\textsuperscript{F170S}} versus SeV\textsuperscript{M-C-wt} infections. A strong difference was also found between SeV\textsuperscript{Z-C\textsuperscript{A10-15}} and SeV\textsuperscript{Z-C-wt} infections, where N mRNAs had accumulated identically (Fig. 2). Moreover, IL-8 and IFN-β mRNA levels were also elevated in other SeV\textsuperscript{M-C\textsuperscript{F170S}} versus SeV\textsuperscript{M-C-wt} infections of 2C4 cells, where N mRNAs had accumulated identically (Fig. 3). Thus, specific mutations in two different regions of the SeV C proteins lead to increased activation of IL-8 and IFN-β. In all cases, IL-6 and IFN-β mRNA levels were more strongly increased by SeV\textsuperscript{M-C\textsuperscript{F170S}} than by SeV\textsuperscript{Z-C\textsuperscript{A10-15}}.

We also examined the effects of various SeV infections of cells transfected with plasmids expressing IRF-3 (or RFP as a neutral control), and in some cases the cells were also treated with 50 μg of poly(I)-poly(C) (dsRNA) per ml. Elevated IRF-3 levels should enhance the antiviral response of the cells to SeV infection, and the combined treatment is thought to approximate a virus infection in itself and should potentiate the antiviral response. This appears to be so, as the levels of IL-6, IL-8,
FIG. 1. Comparison of host mRNA levels in 2fTGH cells infected with various SeVs. (A to C) Parallel cultures of 2fTGH cells were infected with 20 PFU of the various SeVs per cell. Total cytoplasmic RNA was prepared with Trizol at 24 hpi, and equal amounts (10 µg) were used as a template for oligo(dT)-primed [³²P]cDNA synthesis. The [³²P]cDNA was annealed to triplicate DNAs arrayed on nylon membranes, and the [³²P]cDNA bound was quantitated in a PhosphorImager. The graphs show the fold increase in each mRNA relative to the mock control. (D) Schematic representation of the viral mutations and their effects on host gene activation. The C proteins are shown as two telescoping boxes representing the longer (C' and C) and shorter (Y1 and Y2) C proteins, whose activities during infection, and the mutations investigated, are indicated. The promoter mutation GP42 is thought to exert its effect via mutant leader (Le) RNA. The presumed requirement for the various wt genetic elements to prevent host gene activation is shown. The names of the mutant SeVs used are also indicated. Instab., instability; sig., signaling.
and IFN-β mRNAs induced by SeV infection in general increased with increasing proresponse treatment (i.e., RFP, IRF-3, and IRF-3 plus dsRNA) (Fig. 3). IRF-3 overexpression (Fig. 3A to C, IRF-3 versus RFP) enhanced IFN-β and IL-6 activation by SeV but did not enhance that of IL-8. The additional dsRNA treatment had little effect on further enhancing IFN-β and IL-6 mRNA levels but strongly enhanced IL-8 mRNA levels. These differences in the enhancement of IL-6 and -8 and IFN-β activation upon treatment with IRF-3 with or without dsRNA presumably reflect different activation pathways in response to SeV infection. It is possible that IL-8 activation requires both IRF-3 and dsRNA, whereas IRF-3 is sufficient for IL-6 and IFN-β activation.

SeV activation of IL-6, IL-8, and IFN-β is independent of IFN signaling. 2fTGH human fibrosarcoma cells were chosen for these experiments because sublines defective in specific components of the IFN signaling system have been generated from these cells by X irradiation (48). U5A cells, for example, are defective in the IFN-α/β receptor 2 chain, which is essential for IFN-α/β signaling, and these cells have been restored to IFN sensitivity by complementation with the IFNAR2 gene (U5A+IFNAR2 cells) (43). Even though IL-6 and -8 are not activated upon simple IFN treatment of uninfected cells, IFN secreted during SeV infection may act differently, as additional signaling pathways are being induced by the virus infection. Moreover, C<sup>F<sub>170S</sub></sup> (in contrast to C<sub>Δ10-15</sub>) does not prevent IFN signaling, and it is important to know whether this phenotype is responsible for the activation of IL-6 and -8. We therefore examined the various SeV infections of U5A as well U5A+IFNAR2 cells to determine whether activation of IL-6 and IFN-β by SeV-C<sup>F<sub>170S</sub></sup> and SeV-C<sub>Δ10-15</sub> required IFN signaling. We also examined the activation of the 6-16 gene, a known ISG, as a positive control (10). As shown in Fig. 4, both SeV-C<sup>F<sub>170S</sub></sup> and SeV-C<sub>Δ10-15</sub> activated IL-6, IL-8, and IFN-β in U5A cells relative to SeV-wt infection. The IFNAR2-complemented cell line yielded similar results, except that the activation of these genes was paradoxically reduced in U5A+IFNAR2 cells relative to U5A cells. In contrast to the case for IL-6, IL-8, and IFN-β, little or no activation of 6-16 occurred in SeV-C mutant-infected U5A cells, whereas a modest activation was evident in U5A+IFNAR2 cells. Moreover, 6-16 was the only mRNA whose levels in U5A+IFNAR2 cells exceeded those in U5A cells. The activation of IL-6, IL-8, and IFN-β during SeV infection, in contrast to that of 6-16, is thus largely independent of IFN signaling.

STAT1-defective U3A cells. The SeV C proteins interact with STAT1 in two ways (Fig. 1D). C and STAT1 form a stable complex in vitro and during SeV infection, and this complex is associated with a loss of IFN signaling. These events are blocked by the C<sup>F<sub>170S</sub></sup> mutation but not by C<sub>Δ10-15</sub>. The shorter Y proteins are also active in this respect. The longer C proteins alone also induce STAT1 instability, and in contrast to their effects on IFN signaling, this effect does not require F170 (Fig. 1D). To examine whether SeV-C mutant-induced activation of IL-6, IL-8, and IFN-β requires STAT1, U3A cells, which are known to be defective for STAT1, were examined (45). However, we were unable to examine the companion U3A+STAT1 cells, as these cells were found to have lost STAT1 expression. Moreover, attempts to reconstitute U3A cells with STAT1 failed (data not shown).

The results of the U3A cell infections are shown in Fig. 5. As before, IL-6 and -8 and IFN-β mRNA levels were all clearly increased in SeV-C<sup>F<sub>170S</sub></sup> infections relative to SeV-wt infections. However, in contrast to the case for U5A and 2fTGH cells, SeV-CΔ10-15 infections did not contain enhanced mRNA levels relative to SeV-wt infections. Given that STAT1 is probably not the only gene that has been lost in U3A cells that have been X irradiated (which randomly destroys chromosomal DNA), we can conclude only that enhanced mRNA levels due to SeV-CΔ10-15 infection require STAT1 and/or
another, unknown gene, whereas activation due to SeV-C<sup>F170S</sup>
requires neither STAT1 nor any other gene destroyed in U3A cells.

**IL-8 gene activation and IL-8 secretion.** IL-8 is not known to act intracellularly. We therefore examined whether the SeV-induced IL-8 gene activation in U5A/U5A<sub>H11001</sub> IFNAR2 cells also led to increased IL-8 protein synthesis and secretion. When IL-8 protein levels in cytoplasmic extracts of the various SeV-infected cells were examined, they were found to roughly mirror the mRNA levels (Fig. 4E). However, when the culture supernatants were examined, a somewhat different result was found (Fig. 4F). Whereas the increased IL-8 mRNA level of SeV-C<sub>Δ10-15</sub>-infected U5A cells was accompanied by strongly increased IL-8 secretion, that of U5A+IFNAR2 cells led to only a modest increase in IL-8 secretion. Moreover, the in-

![Graph A. IFNβ levels with different SeV treatments](image1)

![Graph B. IL-8 mRNA levels with different SeV treatments](image2)

![Graph C. SeV-N mRNA levels with different SeV treatments](image3)

![Graph D. SeV-N mRNA levels with different SeV treatments](image4)

**FIG. 2.** Effects of SeV C gene mutations on IFN-β and IL-8 mRNA levels during infection. Parallel cultures of 2C4 cells were infected (or not) in triplicate for 24 h with 20 PFU of the various SeVs per cell. Total cytoplasmic RNA was prepared from each culture, and the same amount of RNA (ca. 1 µg) was transcribed into cDNA with random hexadeoxynucleotides and murine leukemia virus reverse transcriptase. The relative amounts of IFN-β and IL-8 gene sequences, relative to that of GADPH as an internal control, were determined by real-time PCR (see Materials and Methods). The average levels of the mRNAs and their deviations in the triplicate infections are shown. The relative SeV N mRNA levels of the SeV<sup>Z</sup>-C-wt and -C<sub>Δ10-15</sub> infections were also determined (SeV<sup>Z</sup> and SeV<sup>M</sup> are 10% different in sequence, and their detection requires different primers and probes).

**FIG. 3.** Effects of IRF-3 and dsRNA treatment on SeV-induced host gene activation. Parallel cultures of 2C4 cells were transfected with pRFP or pIRF-3. The cultures were then infected (or not) with 20 PFU of the various SeVs per cell at 20 h posttransfection, and some of the cultures were also treated with 50 µg of poly(I)-poly(C) per ml. The cells were harvested at 24 hpi. The relative amounts of IFN-β, IL-6, and IL-8 gene sequences present were determined as described for Fig. 2.
creased IL-8 mRNA levels in either SeV-C$^{170S}$-infected cell line did not lead to clearly increased IL-8 secretion.

IL-8 expression can be controlled at both the transcriptional and posttranscriptional levels. In polarized epithelial monolayers, S. enterica serovar Typhimurium-induced IL-8 secretion requires not only the activation of NF-κB and production of IL-8 mRNA but also the activation of the small, Rho family GTPases Cdc42 and Rac1, which regulate endocytic protein traffic from the Golgi network to the basolateral surface of the cell. In the absence of Cdc42 or Rac1 function, IL-8 mRNA levels increase in response to bacterial infection but IL-8 is not secreted (3), similar to the case for our SeV-C$^{170S}$-infected cell lines.

FIG. 4. SeV infection of cells defective for the IFN receptor and their complemented pseudo-wt derivatives. (A to D) Parallel cultures of U5A or U5A+IFNAR2 cells were infected (or not) in duplicate with 20 PFU of the various SeVs per cell for 24 h. The relative amounts of IFN-β, IL-6, IL-8, and 6-16 mRNAs present were determined as described for Fig. 2. (E and F) Equal samples of the culture supernatants (F) or cytoplasmic extracts of the cultures (E) were analyzed for IL-8 protein levels by enzyme-linked immunosorbent assay.
U5A cells. The requirement for Cdc42 and Rac1 activation, moreover, is cell type dependent (9). The C°F170S and CΔ10-15 mutations thus appear to affect IL-8 secretion differently.

SeV prevents IFN-β gene activation in several ways. The manner in which IFN-β transcription is induced by virus infection is well studied, and the activation of IRF-3 is central to this process. IRF-3 is expressed constitutively and is found in the cytoplasm in an inactive, unphosphorylated state. Upon virus infection or dsRNA treatment of cells, IRF-3 is phosphorylated by an unknown kinase and translocates to the nucleus, where, together with other transcription factors such as NF-κB (which is itself also directly activated by virus infection or dsRNA), it activates IFN-β transcription (41, 65, 68). Phosphorylation of IRF-3 after viral infection is the first step in the activation of a gene program that includes a positive feedback loop of IFN-α/β and IRF family members (60). The results described above suggest that the SeV C gene encodes functions that prevent virus-induced IFN-β transcription (directly or indirectly). During the course of this work, it was reported that the SeV V protein, as well as the V proteins of the rubulaviruses SV5 and hPIV2, also prevented IFN-β transcription (26, 50, 64).

Given that SeV appears to use two viral genes (C and V) to neutralize IFN-β expression, we have examined a broader panel of mutant rSeV infections for their relative activation of the IFN-β promoter compared to that of dsRNA treatment. Besides SeV-C°F170S and CΔ10-15, we examined two promoter mutants, SeV-AGP55, in which the first 55 nt of the antigenomic promoter is replaced with the equivalent leader sequences of the genomic promoter (38). SeV-AGP55 transcribes leader RNA from both promoters (and no trailer RNA). The converse SeV-GP48 has the first 48 nt of the genomic promoter replaced with the equivalent trailer sequences, and SeV-GP48 transcribes basically trailer RNA from both promoters (and no leader RNA; GP48 and GP1-42 are identical in this respect [data not shown]) (19, 20). Finally, we examined SeV-V°/ W°, which contains a stop codon at the beginning of the V open reading frame (ORF), such that edited V mRNAs are translated into W-like proteins, and specifically no V protein is expressed (8).

2fTGH cells were transfected with a reporter plasmid in which luciferase is controlled by the IFN-β promoter (pIFNβ-luciferase). To determine whether virus-induced IFN-β transcription required IRF-3 activation, the cells were cotransfected with either a dominant-negative mutant of IRF-3 (IRF-3°N), an anti-IRF-3 ribozyme, or an empty control plasmid (see Materials and Methods). The transfected cultures were then infected (or not) with the various SeVs (or treated with dsRNA) 24 h later and were harvested at 20 h postinfection (hpi). As shown in Fig. 6, with the notable exception of SeV-AGP55, all of the mutant SeV infections induced the reporter more strongly than SeV-wt and as well as dsRNA treatment. In all cases, the coexpression of IRF-3°N or an anti-IRF-3 ribozyme prevented the SeV-induced expression of the reporter. Thus, mutations in two regions of the C protein that carry out different functions, as well as the loss of leader RNA expression or the expression of the V protein, all lead to IFN-β promoter activation in an IFN-3-dependent manner. Overexpression of the W protein cannot compensate for the lack of V protein, so the highly conserved Cys-rich carboxyl domain of V is specifically required. Only the loss of trailer RNA expression (SeV-AGP55) did not result in IFN-β activation.

Leader and trailer RNAs, the promoter-proximal products of viral RNA synthesis, are AU rich and are thought to bind to cellular RNA-binding proteins that bind AU-rich elements (28). In contrast to leader RNA, trailer RNA is expressed relatively late in infection (upon antigenome accumulation) and would not be expected to counteract immediate-early response genes. However, many of the other viral products that do not have a dedicated essential role in the replication ma-
The CF170S mutation is the only one of the four mutant SeVs not to have lost virulence to SeV-wt, and consistent with this failure, SeV-GP31-42 was the mutant SeV examined by DNA arrays, only the leader(31-42) groups of cellular genes were identified in this way (Fig. 1). Of the four mutant SeVs not to have lost virulence in mice (M. Itoh, unpublished data), the CF170S mutation appears to be the most important in cell culture infections as well as in mice (15). This mutation activates IL-6, IL-8, and IFN-β more strongly than CΔ10-15, and only this mutation activates all three groups of cellular genes that respond to SeV infection.

The IFN-β gene is both a primary response gene and an ISG, and it plays a central role in initiating the IFN-induced antiviral response. This is the first report that the SeV C proteins and leader RNA counteract the expression of this key primary response gene, and it confirms that the report of Poole et al. (50) that the product of our hemagglutinin-tagged V gene is active in this respect. As expected, activation of IFN-β required activation of IRF-3 (Fig. 6) and was independent of IFN signaling (Fig. 4), but the details of how this occurs remain to be elucidated. We have also provided evidence that the SeV C proteins and leader RNA counteract the expression of the chemokine IL-8. Infections by other viruses, e.g., respiratory syncytial virus (RSV) (44), dengue virus (3), hepatitis C virus (49), and human immunodeficiency virus type 1 (46, 52), are known to induce IL-8 secretion, as do infections by bacteria and parasites, including Mycobacterium tuberculosis (66). In a related vein, cytomegalovirus, a large DNA virus, encodes a chemokine receptor that may facilitate virus replication (13), and human herpesvirus 8/Kaposi’s sarcoma virus carries four ORFs whose products are related to chemokines (42). Viral modulation of chemokine expression presumably represents one aspect of the continuous battle between viral parasites and antiviral, inflammatory, and immune responses of the host. SeV infection has been reported to induce the CXC chemokine RANTES via the activation of IRF-3 and NF-κB (23, 40). IL-8 may not have been noticed in these earlier studies, because SeV-wt induces very little IL-8 (Fig. 2). Our results suggest that SeV targets the inflammatory and adaptive immune responses (IL-6 and IL-8) as well as the IFN-induced intracellular antiviral state (IFN-β and STAT1). As IFN-β and IL-8 transcription both depend on NF-κB activation, SeV may target this key transcription factor as well.

The leader(1-42), CΔ10-15, CΔ170S, and VΔW++ mutations appear to disrupt four distinct elements in the SeV program to counteract the cellular antiviral response. The facts that they all lead to enhanced levels of IFN-β mRNA and that at least three of them increase IL-8 mRNA levels suggest that SeV employs a multifaceted approach to inhibit viral clearance by inflammatory cells as well as to prevent the IFN-induced antiviral state, sometimes using the same viral macromolecules due to its limited coding capacity. The best-studied example of paramyxovirus-induced activation of IL-8 is that of RSV (21, 69). The IL-8 promoter in A549 cells can be induced by RSV infection in at least three distinct pathways: via tumor necrosis factor alpha (which requires only an intact NF-κB binding site), directly by intracellular RSV replication (which also requires other transcription factor binding sites) (5), and via the interaction of the viral F protein with Toll-like receptor 4 (in which IRF-3 plays an important role) (36). Measles virus H protein interaction with Toll-like receptor 2 also activates IL-8 (1). If all three parallel cellular pathways for IL-8 expression operate during SeV infection of 2TGH cells, several different SeV products will be required to effectively prevent IL-8 activation (Fig. 1D).

Rubulaviruses do not express C proteins, but their V proteins have recently been found to prevent IFN-β expression by preventing the activation of IRF-3 and NF-κB, as well as in-
roducing the degradation of STAT1 or STAT2 (26, 50). The Rubulavirus V proteins thus also counteract more than one arm of the innate antiviral response. The versatility of these viral gene products continues to surprise us. The SeV C proteins have been more intensively studied than SeV V protein or leader RNA. Like the influenza A virus NS1 protein (6, 63) and hepatitis C virus NS5A protein (49), the SeV C proteins are pleiotropic polypeptides that have multiple activities during infection, presumably due to their interaction with various viral and cellular proteins. Their multiple functions, deciphered in large part via C gene mutations, include (i) stimulation of viral RNA synthesis early in infection (SeV-C/\(^{\text{C}}\)-infections exhibit a 10-h delay in the accumulation of viral products) (37); (ii) inhibition of viral RNA synthesis in a promoter-specific manner late in infection, by interacting with the \(P_r\_L\) vRdRP (this selective inhibition may promote the switch from mRNA synthesis to genome replication and increase the fidelity of vRdRP promoter recognition) (4, 61); (iii) a role in virion assembly, possibly by interaction with the matrix (M) protein (SeV-4C\(^{-}\) particles are poorly infectious and amorphic) (25); (iv) interaction with STAT1 in two separate ways, to inhibit IFN signaling and to induce STAT1 instability (17); and (v) inhibition of the IRF-3-dependent activation of IFN-\(\beta\) and the activation of IL-8 expression in an IFN signaling-independent manner (this work).

How C interacts specifically with all of its viral and cellular partners remains an enigma and is reminiscent of acidic activation domains of transcription factors that interact with multiple partners. Acidic activation domains are “natively disordered” (11, 51), and this property apparently allows them to bind different surfaces with high specificity (multiple induced fits) and limited stability. The NH\(_2\)-terminal portion of the measles virus P protein that contains the overlapping C protein ORF is, in fact, a recent example of such natively disordered proteins and for numerous cell lines, Paula Pitha (Baltimore, Md.) and Jerome Pugin lab (University of Geneva) for help with the IL-8 protein assays.

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ADDITIONAL PROOF

Fujii et al. (Y. Fujii, T. Sakaguchi, K. Kiyotani, C. Huang, N. Fukuhara, Y. Egi, and T. Yoshida, J. Virol. 76:8540–8547, 2002) have shown that mutations in the leader region specifically attenuate virus virulence in mice.

REFERENCES


III. PUBLICATION 2

"SENDAI VIRUS DEFECTIVE-INTERFERING GENOMES AND THE ACTIVATION OF THE INTERFERON-BETA"

Laura Strahle, Dominique Garcin, Daniel Kolakofsky

INTRODUCTION TO PAPER TWO:

dsRNA is known to be a common viral molecular signature that is generated during virus replication and acts a pathogen-associated molecular pattern (PAMP). These PAMPs are recognised in the cytoplasm by RIG-I and Mda-5, which activate TBK1 and IKKe leading to the phosphorylation and dimerization of IRF-3 and IRF-7. Activated IRF-3 and IRF-7 translocate into the nucleus where transcription of early specific genes are activated. IFNβ is first secreted and feeds back onto cells in an autocrine manner, to induce multiple ISGs resulting in an antiviral state and in a paracrine manner, to prime neighbouring cells for possible infection.

SeV stocks available commercially are known to induce IFNβ and are commonly used by many laboratories. Plus, these stocks are known for a long time to contain DI genomes. The paramyxoviruses DI genomes can be of two types: internal deletion or copyback DI genomes (cf. Introduction). DI genomes have the capacity to form dsRNA by at least two ways: 1) if the level of the N protein is not sufficient, DI genomes (and antigenomes) can self-anneal; and 2) some DI genomes contain termini that are perfectly complementary, thus are likely free to form dsRNA. Moreover, DI genomes (especially those from the copyback variety) interfere robustly with the ND helper genome by competing for replication and consequently reducing the production of viral proteins involved in counteracting the antiviral state. The effect of reduced level of SeV accessory proteins on the innate antiviral response is confirmed by the fact that mutated SeV stocks (containing deficient V and C proteins or leader) lead effectively to enhanced levels of IFNβ mRNA (cf. Paper one).

Because the induction of the IFNβ activation is known to be due to the presence of DI genomes, we investigate in this study whether the induction of IFNβ is truly due to the presence of DIs (since plaqued purified WT SeV does not activate IFNβ) and whether this property is specific to a certain type of DI genome. Moreover, the interference between both the DI and the ND genomes was also examined. Finally, because of the involvement of the viral C and V proteins in the counteraction of the innate immune response, their effect on the IFNβ activation were tested upon DI infection (Fig. 19).
Figure 19: Upon DI infection, a strong IFNβ activation is observed. Two questions are raised in this paper: (1) Is the IFNβ activation dependent on the presence of the DI genome? And what kind of DI is responsible? (2) Does overexpression of the viral C and V proteins inhibit IFNβ induction upon DI infection?

We first compared three SeV stocks containing different kind of DI genomes and their ability to activate IFNβ in non-IFN sensitive cells (293T). Levels of IFNβ were monitored depending on the different SeV stocks, but the copyback DI variety (DI-H4) was found to be the most potent IFNβ inducer. Moreover, because IFNβ activation requires modification of IRF-3, the dimerization and the phosphorylated forms of IRF-3 were analysed upon DI infection. The formation of dimers, as well as hyper-phosphorylated forms of IRF-3 were observed in DI infections, as opposed to SeV ND infection.

In order to estimate independently the DI and the ND genomes by RT/PCR, we generated a tagged version of the copyback DI-H4 genome, containing a sequence from the YFP gene. This enabled us to follow the evolution of this new stock, containing both the DI and ND genomes (generated by multiple passages in embryonated chicken eggs), in parallel with the activation of IFNβ. The correlation between the level of DI genomes and the level of IFNβ activation was also observed. We found that the activation of the induction of IFNβ upon DI-
H4 infection was proportional to DI genome replication and that the ratio of DI to ND genomes during infection.

Finally, we used IFN competent cells (2fTGH) to study the general aspect of the DI-H4 infection. Since the C and V proteins can block dsRNA signaling, we examined the effect of their overexpression upon DI-H4 infection, considering that DI infection produces less viral proteins. Indeed, overexpression of C and V proteins was effective in blocking the DI-H4-induced activation of IFNβ as well as the poly(I/C) activation. This result further confirms the idea that DI-H4 induces IFNβ via dsRNA.

This study provides evidence that the strong induction of IFNβ activation upon SeV infection (SeV stock containing DI genomes) is mainly due to the presence of copyback DI genomes. The level of IFNβ activation was found to be proportional to that of DI genome replication. This activation can be inhibited by the overexpression of the C and V proteins, whose concentrations are reduced in DI infected cells, because of the strong interference of the DI versus the ND genome.
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.

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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...
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 rubulavirus, respirivirus 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 (CEF}s) 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
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 (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.

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 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...
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 genome.
genes 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 embryonated 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
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 254 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.

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 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 IFN\(\beta\). 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 IFN\(\beta\) 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 IFN\(\beta\). 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 IFN\(\beta\) 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 IFN\(\beta\) 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 IFN\(\beta\) 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 IFN\(\beta\) (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-cytotoxic 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 (Izen 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 (10⁹ pfu/ml), 0.1 ml of a 10⁵ dilution (ca. 1000 pfu) was inoculated per egg. In the case of DI stocks, 0.1 ml of a 10² to 10⁴ 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 plaqueing 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 Imaiizumi 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-rfl-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-rfl-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-N1 (BD Biosciences Clontech), between the Axel and HindIII sites. For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pIFNβ-GFP and CaPO₄ (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/Cstop (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 specific oligonucleotides and Taqman probes and by Northern blotting using a biotinylated riboprobe generated in vitro by T7 RNA polymerase transcription (RT) reaction as described by the manufacturer (Eurogentec), 20 pmol (each) of forward and reverse primers Human GAPDH (Applied Biosystems), 11 μg were used: N gene: 5′-GCAATAACCCCAACGA-3′ (Fwd); 5′-GACGATACCGCAGCAGTAGC-3′ (Rev); 5′-CCGACAACCACTACCTGAGCTA-3′ (Fwd); 5′-GCAATAACCCCAACGA-3′ (Probe). YFP gene: 5′-TGCCTGAGCCGATCGG-3′ (Probe). Real-time PCR was carried out in the 7700 Sequence Detector (Applied Biosystems).

**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′-GCAATAACCGTGTGATCAGCAGC-3′ (Fwd); 5′-TGCCCTGAGCCGATCGGA-3′ (Rev); 5′-CAGCAGATGACGATCCAGCAGC-3′ (Probe). YFP gene: 5′-CCGACAACCACTACCTGAGCTA-3′ (Fwd); 5′-GAATCCAGCAGAACCATGTCGTA-3′ (Rev); 5′-AAAGACCCACACAGCAGCAGC-3′ (Probe). Real-time PCR was carried out in the 7700 Sequence Detector (Applied Biosystems). Calain, P., Curran, J., Kolakofsky, D., Roux, L., 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology 191, 62–71.


IV. PUBLICATION 3

"SENDAI VIRUS ACTIVATION OF THE IFNβ PROMOTER REQUIRES RIG-I AND IS INHIBITED BY THE VIRAL C PROTEINS"

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INTRODUCTION TO PAPER THREE:

All viruses have evolved to evade the cellular innate immune system including the IFN system by various mechanisms. Interestingly, for the Paramyxoviruses, the C and V accessory proteins are likely to be responsible for this counteraction of the innate immune response. Paper one showed that mutations within distinct regions of the V and C proteins or of the leader (absence of leader or over-expression of the trailer) all increased the level of IFNβ mRNAs. SeV V and C proteins as well as the region of the leader are thus important for antagonizing IFN signaling. Paper two showed that the SeV stocks containing specifically copyback DI genomes were responsible for the strong IFNβ activation. This phenomenon upon DI infection seemed to be due 1) to the ability of the DI genomes (and antigenomes) to self-anneal and form dsRNA, and 2) to the reduced production of the viral V and C proteins. Innate immunity upon viral infection has been extensively studied these past few years. As mentioned previously the two helicases RIG-I and Mda-5 were discovered to be important components responding to cytoplasmic RNA. Although these helicases transmit an identical signal leading to the activation of IRF-3, IRF-7 and NF-kB, they appear to target different viral products. In addition to this, RIG-I was found to detect other viral products than dsRNA. Indeed Sousa et al. showed that influenza A virus infection does not generate dsRNA and that RIG-I could be activated by single-stranded viral genomic RNA bearing 5’triphosphates (5’pppRNA).

In this study, the contribution of RIG-I (and Mda-5) in the detection of SeV infection is examined in mouse embryo fibroblasts (MEFs). Because 5’triphophorylated products have become new potential targets of RIG-I, we decided to test whether SeV infections induced IFNβ activation by producing pppRNAs as well as dsRNA. The involvement of both helicases was also analysed. For this, we used polyI/C, in vitro pppRNA (leader and trailer RNAs) and two different types of SeV infection: 1) SeV-DI-H4, containing copyback DI genomes and overexpressing 5’ppptrailer RNAs, as well as small amount of unencapsidated DI H4-genome RNA that can self anneal to form dsRNA panhandles with 5’ppp ends and 2) SeV-GFP(+-), a mixed stock of SeV capable of producing dsRNA with capped ends. For the mixed stock, we produced two different SeV stocks: one of them carries a GPF sequence, producing + mRNA that is translated in GFP expressing proteins, the other contains the inverse GFP sequence. The coinfection of these viruses produces dsRNA from both mRNA
GFP + and -, which enabled us to test more precisely the effect of dsRNA (annealed capped mRNAs) on the activation of IFNβ (Fig. 20).

**Figure 20**: SeV-GFP(+) expresses the protein GFP from a transgene between the M and the F genes. SeV-GFP(-) synthesises a mRNA containing the complement of the GFP ORF, but no GFP protein can be expressed. Upon coinfection (= SeV-GFP(+/-)), both messengers (GFP+ and GFP-) are generated and can form dsRNA with capped 5'ppp ends. These infections are tested for their induction of IFNβ activation.

The formation of dsRNA in the coinfection was tested by using the dsRNA-binding domain of E3L (Vacinia virus) proteins. We also used a dominant-negative form of RIG-I to examine whether the activation of the IFNβ upon RNA treatment or different SeV infections was dependent on RIG-I.

Finally, since the viral C and V proteins are responsible for counteracting the host innate immune response, we over-expressed the V or C proteins in infected (SeV-DI-H4 or SeV-
GFP(+/-))(Fig.21), or transfected (dsRNA or pppRNA) cells and also used SeV infections carrying mutation within the C or V genes (SeV-C\textsuperscript{minus} or SeV-V\textsuperscript{minus}). We also tested whether they could inhibit the RIG-I dependent-IFNβ activation. In order to see which region was responsible for the inhibition of RIG-I, two different domains of the C protein (C\textsuperscript{1-23} and C\textsuperscript{24-204}) carrying different function were also compared.

![Diagram](image)

**Figure 21**: Upon coinfection (SeV-GFP(+/-)) or upon SeV-DI-H4 infection, the cellular sensor RIG-I presumably recognises dsRNA and 5’triphosphorylated RNAs and leads to IFNβ activation. The viral RNA products that are detected by RIG-I upon SeV infection are still to be discovered: the trailer RNAs carrying 5’ppp ends remain good candidates. In this study, we also tested whether the overexpression of the viral C or V proteins could prevent the induction of IFNβ (via RIG-I) upon both SeV GFP (+/-) and DI-H4 infections.

In this study, we demonstrated that SeV-GFP(+/-) and SeV-DI-H4 infections as well as transfections of the trailer pppRNAs or dsRNA (polyI/C) induced IFNβ activation in MEFs cells in a RIG-I dependent manner (not Mda-5). This suggests that RIG-I presumably detects both the 5’triphosphorylated RNAs and dsRNA. Moreover, these data showed that in infected or transfected MEF cells, the expression of SeV C protein had a much greater effect in counteracting RIG-I dependent IFNβ activation than the SeV V protein; and that the C\textsuperscript{24-204} region was sufficient for this function.
Activation of the Beta Interferon Promoter by Unnatural Sendai Virus Infection Requires RIG-I and Is Inhibited by Viral C Proteins

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As infection with wild-type (wt) Sendai virus (SeV) normally activates beta interferon (IFN-β) very poorly, two unnatural SeV infections were used to study virus-induced IFN-β activation in mouse embryonic fibroblasts: (i) SeV-DI-H4, which is composed mostly of small, copyback defective interfering (DI) genomes and whose infection overproduces short 5′-triphosphorylated trailer RNAs (pppRNAs) and underproduces viral V and C proteins, and (ii) SeV-GFP(+/-), a coinfection that produces wt amounts of viral gene products but that also produces both green fluorescent protein (GFP) mRNA and its complement, which can form double-stranded RNA (dsRNA) with capped 5′ ends. We found that (i) virus-induced signaling to IFN-β depended predominantly on RIG-I (as opposed to mda-5) for both SeV infections, i.e., that RIG-I senses both pppRNAs and dsRNA without 5′-triphosphorylated ends, and (ii) it is the viral C protein (as opposed to V) that is primarily responsible for countering RIG-I-dependent signaling to IFN-β. Nondefective SeV that cannot specifically express C proteins not only cannot prevent the effects of transfected poly(I-C) or pppRNAs on IFN-β activation but also synergistically enhances these effects. SeV-V-minus infection, in contrast, behaves mostly like wt SeV and counteracts the effects of transfected poly(I-C) or pppRNAs.

All viruses evade the cellular innate immune system in part by expressing gene products that interfere with the ability of the host cell to establish an antiviral state (6). In the case of the Paramyxovirinae, this anti-host-defense activity is due mostly to viral C and V proteins (15, 27, 31). The C and V proteins are encoded by separate alternate open reading frames (ORFs), which both overlap that of the P protein. V and C are also referred to as accessory gene products, as not all members of this virus subfamily express one or the other. More specifically, rubulavirus and avulavirus express V but do not express C proteins, and human parainfluenza virus type 1 (PIV1), a respirovirus most closely related to Sendai virus (SeV), expresses C but does not express a V protein (16, 20).

Paramyxovirus V and C proteins antagonize interferon (IFN) signaling by various mechanisms, and they also target the production of type I IFN (15, 31). Beta IFN (IFN-β) production is one of the earliest events in the cellular innate immune response, which leads to the establishment of an antiviral state. IFN-β production requires the coordinated activation of several transcription factors, including NF-κB and IRF3 (15, 29). For intracellular RNA virus replication, the signaling pathway that leads to IRF3 activation starts with mda-5 and RIG-I, two cytoplasmic DExH/D-box helicases with N-terminal CARD domains. These helicases respond to double-stranded RNA (dsRNA) and, at least for RIG-I, to 5′-triphosphorylated single-stranded RNA (ssRNA) (pppRNA), which are generated in the cytoplasm during RNA virus replication (9, 11, 25). Upon the detection of these viral RNAs, the CARD domains of these helicases interact with IPS-1/Cardif/MAVS/VISA, which is present in the mitochondrial membrane, and this CARD-CARD interaction is thought to lead to the recruitment and activation of TBK1, IKKε, and other IKK kinases that activate NF-κB and IRF3, thereby activating the IFN-β promoter (8). The production of these early IFNs initiates autocrine and paracrine signal amplifications via the Jak/Stat pathway to produce a generalized antiviral state and also assists in the subsequent activation of adaptive immune responses.

The role of mda-5 in sensing RNA virus infection was uncovered because mda-5 was found to bind to the PIV5 V protein and other paramyxovirus V proteins, including SeV V. These V-protein–mda-5 interactions, moreover, prevented IFN-β activation in response to transfected poly(I-C) (1). On the other hand, other studies found that RIG-I and not mda-5 acts as the sensor of paramyxovirus infection (13, 28). This paper provides evidence that for SeV infection of mouse embryonic fibroblasts (MEFs), it is the C protein (and not V) that is primarily responsible for this effect and that C acts by countering RIG-I (and not mda-5). Independent expression of C was found to inhibit RIG-I-dependent signaling to the IFN-β promoter induced by either pppRNAs or dsRNAs. Moreover, SeV that cannot specifically express C proteins was unable to counteract poly(I-C)- or pppRNA-induced IFN-β activation, whereas SeV that cannot express V behaved mostly like wild-type (wt) SeV.

MATERIALS AND METHODS

Cells, viruses, and antibodies. MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. SeV stocks were grown in the allantoic cavities of 9-day-old embryonated chicken eggs for 3 days at 33°C. For nondefective stocks (109 PFU/ml), 0.1 ml of

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a 10° dilution (ca. 1,000 PFU) was inoculated per egg. In the case of DI stocks, 0.1 ml of a 10° dilution was used. In all cases, the amount of viral proteins present in the resulting allantoic fluid was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Comassie blue staining of pelleted virus. Viruses were determined by plaquing on LLC-MK2 cells.

SeV-GFP(+) , which expresses green fluorescent protein (GFP) from a trans- gene between the M and F genes, and SeV-GFP(- ) or SeV-RFP, which ex- presses antisense GFP mRNA or red fluorescent protein (RFU) (dReD) from similarly located transgenes, were prepared as previously described (31). DI-H4 stocks were described previously (30).

Primary antibodies used included rabbit anti-RFP (AB216; Chemicon); anti- actin monoclonal antibody (324b; Chemicon); rabbit anti-GFP (632460; BD biosciences); rabbit anti-SeV-PCV (homemade); anti-hemagglutinin (HA) MAb (16B12; BABCO); anti-Flag MAb (F1004; Sigma), rabbit anti-mda-5 (J. Tschopp, Lausanne, Switzerland), and rabbit anti-RIG-I (T. Fujiya, Kyoto, Japan).

Plasmids, transient transfections, infections, inductions, luciferase assay, and fluorescence-activated cell sorter analysis. EBS plasmids (3) expressed viral and fluorescent proteins and were constructed by standard methods; precise detail can be obtained from the authors. NS1 (residues 1 to 77 [NS1(1-77)] (from Jacques Perreault) and E3L (residues 100 to 190 [E3L(100-190)]) (from Bertram Jacobs), were HA tagged and cloned into pBluescript-SK(+). The second way is to infect the cells with an SeV stock that contains a well-characterized copyback DI genome (H4) (30). The third way is to coinfect cells with SeV- GFP(+), which expresses a GFP transgene, and SeV-GFP(-), which expresses mRNA containing the complement of the GFP ORF, as recently described for vesicular stomatitis virus (VSV) (24). As shown in Fig. 1A, infection with increasing amounts of SeV-GFP(+) alone leads to increasing GFP expression. Coinfection of 20 PFU/cell of SeV-GFP(+) with increasing amounts of SeV-GFP(-) leads to the gradual decrease of GFP expression (top). At 20 PFU/cell of SeV- GFP(-), there are roughly equal amounts of GFP and anti-GFP mRNAs intracellularly (by strand-specific quantitative RT-PCR) (Fig. 1B and see Materials and Methods), and there is a 90% loss of GFP expression (Fig. 1A, top). This loss of transfection with 1.5 g of plasmids expressing V (whose promoter expressing a luci- ferase reporter gene in MEFs (see Fig. 2A). The first is to simply transfect a synthetic dsRNA, poly(I):poly(C) [poly(I- C)], into the cells. The second way is to infect the cells with an SeV stock that contains a well-characterized copyback DI genome (H4) (30). The third way is to coinfect cells with SeV- GFP(+), which expresses a GFP transgene, and SeV-GFP(-), which expresses mRNA containing the complement of the GFP ORF, as recently described for vesicular stomatitis virus (VSV) (24). As shown in Fig. 1A, infection with increasing amounts of SeV-GFP(+) alone leads to increasing GFP expression. Coinfection of 20 PFU/cell of SeV-GFP(+) with increasing amounts of SeV-GFP(-) leads to the gradual decrease of GFP expression (top). At 20 PFU/cell of SeV- GFP(-), there are roughly equal amounts of GFP and anti-GFP mRNAs intracellularly (by strand-specific quantitative RT-PCR) (Fig. 1B and see Materials and Methods), and there is a 90% loss of GFP expression (Fig. 1A, top). This loss of expression cannot be accounted for by the reduced level of GFP mRNA (Fig. 1B). In contrast, coinfection with increasing amounts of SeV expressing RFP as a neutral control (SeV- RFP) has an increased ability to interfere with GFP expression (Fig. 1A). More importantly, whereas infection with SeV-GFP(+) alone or its coinfection with SeV-RFP leads to little

FIG. 1. IFN-β induction induced by SeV-GFP(+/−) infections. (A) Parallel cultures of MEFs were first transfected with plIFNB-luc and pTK-luc and then infected with increasing amounts of either SeV-GFP(+) (alone which expresses a GFP mRNA from a transgene between the M and F genes) or 20 PFU/cell of SeV-GFP(+) plus increasing amounts of either SeV-GFP(-) (which expresses an anti-GFP mRNA from a transgene in the same location) or SeV-RFP (which expresses an RFP mRNA from a transgene in the same location), as indicated. GFP expression was monitored by fluorescence-activated cell sorter analysis at 20 hpi. Cell extracts were prepared at 20 hpi, and equal amounts were used to determine luciferase activities (below). These transfections were carried out three times with independent virus stocks, with similar results. (B) Cytoplasmic extracts were centrifuged on CsCl density gradients to isolate nonencapsidated (pellet) RNAs. The levels of GFP and anti-GFP mRNAs in 15 µg of CsCl pellet RNA were determined using sense- and antisense-specific primers for RT, followed by quantitative PCR (TaqMan) (see Materials and Methods). (C) Parallel cultures of MEFs were first transfected with the luciferase reporter plasmids plus either an empty vector, one expressing wt E3L(100-190), or one expressing mutant E3L(100-190) (E3L-mut.) and then infected with increasing amounts of SeV-GFP(+) and SeV-GFP(-) as indicated. Cell extracts were prepared at 20 hpi, and equal amounts were used to determine luciferase activities. Equal amounts of cell extracts were also Western blotted using anti-N and anti-HA (below).
or no activation of IFN-β (Fig. 1A), coinfection with SeV-GFP(−) clearly activates the IFN-β promoter (Fig. 1A, bottom). This IFN-β activation is inhibited by the coexpression of the dsRNA-binding domain of the vaccinia virus E3L protein, whereas this activation is unaffected by a mutant form of E3L containing two point mutations that eliminate the binding of dsRNA (10) (Fig. 1C). Taken together, our results show that only SeV coinfections that can form GFP dsRNA induce IFN-β activation.

The two SeV infections that activate IFN-β differ from each other in several respects. First, DI-H4 genomes are of the copyback variety and contain the strong antigenomic replica-}


or no activation of IFN-β (Fig. 1A), coinfection with SeV-GFP(−) clearly activates the IFN-β promoter (Fig. 1A, bottom). This IFN-β activation is inhibited by the coexpression of the dsRNA-binding domain of the vaccinia virus E3L protein, whereas this activation is unaffected by a mutant form of E3L containing two point mutations that eliminate the binding of dsRNA (10) (Fig. 1C). Taken together, our results show that only SeV coinfections that can form GFP dsRNA induce IFN-β activation.

The two SeV infections that activate IFN-β differ from each other in several respects. First, DI-H4 genomes are of the copyback variety and contain the strong antigenomic replication promoter at their 3′ ends. DI-H4 genomes thus have a strong competitive advantage in replication over nondefective (ND) genomes, and this sometimes leads to less viral structural proteins like N and P being present intracellularly (see, e.g., P protein in Fig. 3A), but sometimes, this difference is minimal (see, e.g., Fig. 2A). However, in either case, viral V and C proteins are almost entirely absent in these DI-H4-infected cells, whereas V and C are found at wild-type levels in GFP(+/−) infections (Fig. 2 and data not shown). Both of these viral proteins are thought to limit IFN-β activation due to virus infection (15, 31). Second, the DI genome replication promoters, like those of the ND genomes, are always active in the presence of viral polymerase, and short 5′-triphosphorylated trailer RNAs (rather than full-length DI genomes) are transcribed by a relatively nonprocessive polymerase, especially when the N protein is limiting. Unlike genome synthesis that is dependent on ongoing (N) protein synthesis, that of trailer RNA actually increases when translation is blocked with cycloheximide (18). SeV trailer RNAs are known to specifically bind to TIAR, a cellular RNA binding protein of the ELAV family (4), and to prevent virus-induced apoptosis (7, 12). DI-H4 infections are expected to overproduce trailer RNAs, that is dependent on ongoing (N) protein synthesis, that of trailer RNA actually increases when translation is blocked with cycloheximide (18). SeV trailer RNAs are known to specifically bind to TIAR, a cellular RNA binding protein of the ELAV family (4), and to prevent virus-induced apoptosis (7, 12).

Relative contributions of mda-5 and RIG-I in sensing SeV infections in MEFs, mda-5 signaling to IFN-β was discovered that the V–mda-5 interaction is a general property of paramyxovirus V proteins, including that of SeV (1, 5). Nevertheless, several groups have now found that SeV infection is sensed by RIG-I (and not mda-5) (13). To determine whether RIG-I was also responsible for signaling to the IFN-β promoter in our MEFs, we examined the effect of expressing a dominant-negative form of RIG-I (RIG-I(ΔCARDs)), whose N-terminal CARD domains are deleted (Fig. 2A). MEFs were first transfected with pIFNβ-luc6, pTK-luc2, and plasmids expressing either RIG-I(ΔCARDs) or an empty vector as a negative control. After 24 h, the cells were either transfected with poly(I-C) or infected with either SeV-DI-H4 or SeV-GFP(+/−), and luciferase levels were determined 24 h later. As shown in Fig. 2A, both SeV infections strongly activated the IFN-β promoter, whereas transfected poly(I-C) had a more modest effect in this experiment. RIG-I(ΔCARDs) coexpression did not affect the levels of P, V, and C proteins found intracellularly (bottom), but this coexpression reduced IFN-β activation to background levels in all three cases.

To determine whether the loss of IFN-β activation by RIG-I(ΔCARDs) coexpression was due to its ability to also inhibit mda-5 signaling, e.g., by sequestering cytoplasmic viral RNAs, we examined whether RIG-I(ΔCARDs) could inhibit mda-5 signaling. As mda-5 and RIG-I can be activated by simple overexpression, we examined the effect of RIG-I(ΔCARDs) expression on IFN-β activation due to the overexpression of these two helicases. As shown in Fig. 2B, IFN-β activation clearly occurred upon exogenous mda-5 or RIG-I expression. Moreover, whereas RIG-I(ΔCARDs) coexpression completely inhibited activation due to exogenous RIG-I, RIG-I(ΔCARDs) coexpression had little effect in countering IFN-β activation due to exogenous mda-5. In contrast, the coexpression of a dominant-negative form of mda-5 completely inhibited IFN-β activation due to mda-5 overexpression (Fig. 2B). Taken together, these results suggest that IFN-β activation in our MEFs in response to these SeV infections is predominantly, if not exclusively, due to the action of RIG-I.

The coexpression of either SeV V or C proteins strongly inhibited IFN-β activation due to RIG-I overexpression, whereas only the V protein strongly inhibited IFN-β activation due to mda-5 overexpression (Fig. 2B). The finding that SeV V inhibits RIG-I signaling as well as that of mda-5 is consistent with data from Childs et al. (5), who reported that SeV V was a possible exception to the rule that all V proteins inhibited mda-5 but not RIG-I. They reported that SeV V did in fact modestly inhibit RIG-I (35%), whereas all other V proteins had no effect. SeV V and C inhibition of SeV-DI-H4 and SeV-GFP(+/−) induced IFN-β activation. We next examined the abilities of
the SeV V and C proteins to inhibit IFN-β activation induced by SeV infections. As shown in Fig. 3A, exogenous expression of the SeV V protein did not affect the level of viral P, V, and C proteins in SeV infections, but it did reduce IFN-β activation due to DI-H4 infection by ~60%. Remarkably, SeV V overexpression did not inhibit IFN-β activation due to SeV-GFP(+)− infection. The coexpression of exogenous SeV C protein (actually C1–23-Tom-C24–204, which migrates just slightly slower than the viral P protein) similarly did not affect the level of viral P, V, and C proteins in SeV infections. C overexpression, however, more strongly inhibited IFN-β activation due to either SeV infection [DI-H4-induced activation was reduced by ~90%, and GFP(+)−-induced activation was reduced by ~75%]. Coexpression of the unmodified C protein produced similar results (Fig. 2B and data not shown). The ability of the GFP(+)− infection to activate IFN-β, despite
normal levels of expression of the V and C proteins, is presumably due to the early formation of GFP dsRNA. In this case, the SeV V protein is considerably less potent than C in preventing the response to this dsRNA.

As the DI-H4 infections accumulate so few V and C proteins, we compared the abilities of these proteins (expressed from plasmids) to inhibit IFN-β activation relative to RIG-I(ΔCARDs) and the dsRNA binding domain of the influenza A virus (IAV) NS1(1–73), another viral protein that inhibits RIG-I signaling (22, 25). As shown in Fig. 3B, the SeV C protein was as active as RIG-I(ΔCARDs) in combating an increasing dose of DI-H4 infection and almost as active as NS1. Consistent with above-described results (Fig. 3A), the SeV V protein was less active than C but was still able to inhibit most of the DI-H4-induced IFN-β activation.

The SeV C1–204 protein is composed of two domains: the N-terminal 23 amino acids (C1–23) which act as a plasma membrane (PM) targeting signal (19) and which is present in the longer (C’/C) but not in the shorter (Y1/Y2) “C” proteins, and C24–204, or the Y1 protein, which acts as a protein interaction domain. Whereas C24–204 (or Y1) is naturally expressed during infection, C1–23 is only found fused to Y1. In order to study the different contributions of these two domains to C-protein function, we have used tomato red fluorescent protein (Tom) in which C1–23 is fused to the N terminus of Tom and C24–204 is fused to its carboxy terminus as a carrier. The interposition of Tom between these two domains of C remarkably does not appear to affect any of the activities of C1–23 (19). MEFs were transfected with the luciferase reporter plasmids along with plasmids expressing various Tom constructs as indicated (Fig. 4). After 24 h, half of the cultures were infected with SeV-DI-H4 and luciferase levels were determined after a further 24 h. The expression of C1–22-Tom, which carries the wt PM anchor and is localized at the cell surface, or P8P9-Tom, which carries a mutant PM anchor and is distributed throughout the cytoplasm (19), had little or no effect on the DI-H4-induced IFN-β activation. In contrast, both C1–23-Tom-C24–204 and P8P9-Tom-C24–204 reduced IFN-β activation to near-background levels. C24–204 alone (Tom-C24–204), moreover, was still quite active in this respect (Fig. 4). Thus, the C24–204 or Y1 protein interaction domain appears to be responsible for inhibiting RIG-I-dependent IFN-β activation, and this inhibition is largely independent of whether C24–204 is localized at the PM.

SeV C protein inhibits IFN-β activation induced by transfected pppRNA. A general property of nonsegmented negative-strand RNA viruses is that short, promoter-proximal pppRNAs (leader and trailer RNAs) are transcribed from their replication promoters, especially when unassembled N protein is limiting (17, 18). The ability of SeV infections to induce IFN is essentially due to the presence of DI genomes that are present in their egg-grown stocks, especially those of the copyback variety (30). As mentioned above, copyback DI genomes have a strong replicative advantage because they contain strong replication promoters at the DI genome and antigenome 3’ ends. Copyback DI genome replication thus generates short trailer RNAs that are unmodified at either end and can be considered as unstable, abortive replication products (see Discussion).

To examine whether trailer RNAs act as pathogen-associated molecular patterns (PAMPs), we transfected trailer RNA made by T7 RNA polymerase in vitro into our MEFs and monitored the activation of IFN-β. As the ability of pppRNAs to induce IFN-β activation is not sequence dependent (11), we also examined model RNAs that were initiated with GTP but then treated with phosphatase or those initiated with the dinucleotide GpC rather than pppG (23). The transfections of all
FIG. 5. pppRNA-induced activation of IFN-β. (A) Parallel cultures of MEFs were transfected with pIFN-β-luc and pTK-luc, and pRIG-I was also transfected in some cultures, as indicated. After 24 h, the cells were transfected for 3 h with increasing amounts (1 or 3 μg) of either pppGGG/RNA1, phosphatase-treated GGG/RNA1, pppGCA/RNA1, or ohGCA/RNA1, as indicated. Cytoplasmic extracts were prepared 18 h post-RNA transfection and used to determine luciferase levels. (B) Parallel cultures of MEFs were transfected with pIFN-β-luc, pTK-luc, and plasmids expressing Tom, RIG-I(ΔCARDs), IAV NS1(1–73), C1–23-Tom-C24–204 (C*), or V, as indicated. After 24 h, the cells were transfected with increasing amounts (1 μg and 3 μg) of pppGGG/RNA1, as indicated. Cytoplasmic extracts were prepared after 3 h of RNA transfection and used to determine luciferase levels. Rel., relative. (C) Same as above (B), except that the cells were transfected with 3 μg of ppptrailer RNA.
three 5′-triphosphorylated ssRNAs clearly led to IFN-β activation, whereas both RNAs that contained 5′-hydroxyl ends had essentially lost their ability to activate IFN-β in parallel transfections (Fig. 5A and C), confirming previously reported results (11, 25, 26). We then examined the ability of the SeV C and V proteins to inhibit 5′-pppRNA-dependent activation of IFN-β compared to RIG-I(CARDs) and IAV NS1(1–73). MEFs were first transfected with plasmids expressing various viral inhibitory proteins or an empty plasmid as a negative control and then transfected with increasing amounts of pppRNAs. IFN-β activation was monitored after a further 18 h. As shown in Fig. 5B, expression of the SeV C protein was as active as RIG-I(CARDs) in inhibiting IFN-β activation at all amounts of pppRNAs transfected although not quite as active as NS1(1–73). Expression of SeV V was again the least inhibitory; in fact, significant inhibition occurred only at the lowest amount of pppRNA. Thus, short 5′-triphosphorylated ssRNAs such as trailer RNA are potent stimulators of IFN-β when transfected into our MEFs, and expression of the SeV C protein (but not the SeV V protein) can effectively inhibit this stimulation (Fig. 5B and C).

Relative importance of C and V in inhibiting RIG-I-dependent signaling to IFN-β. Another way to investigate the relative importance of the C and V proteins in inhibiting RIG-I-dependent signaling to IFN-β is to compare the relative abilities of SeV infections that cannot specifically express the C or V proteins to affect pppRNA- or poly(I-C)-induced IFN-β activation. MEFs were therefore first infected with 20 PFU/ml of either wt SeV, SeV-Vminus (containing a stop codon in the V ORF just downstream of the mRNA editing site, which produces a W-like protein instead of V), or SeV-Cminus (containing three stop codons in the C ORF downstream of the Y2 initiation codon). The infected cells were then transfected (at 24 hpi) with pIFN-β-luc plus either pppRNA, poly(I-C), or no RNA and then harvested after 18 h to determine reporter gene activity. As shown in Fig. 6, these three SeVs replicate to clearly different levels in our highly IFN-competent MEFs (even though they replicate similarly in BSR T7, 293T, and Vero cells), highlighting the essential functions that these accessory proteins play in countering the innate immune response. Nevertheless, in the absence of transfected RNA, only SeV-Cminus infection activates IFN-β to any extent or increases RIG-I levels; RIG-I is an IFN-stimulated gene, and its level reflects that of the antiviral state (Fig. 6, bottom). Transfections of either pppRNA or poly(I-C) strongly activated the reporter gene and increased RIG-I levels. Prior infection with either SeV wt or SeV-Vminus reduced transfected RNA stimulation of the reporter gene and prevented the increase in RIG-I levels (Fig. 6). SeV-Vminus was only slightly less effective than wt SeV in this respect. In sharp contrast, prior infection with SeV-Cminus not only did not prevent the increase in RIG-I levels but also acted synergistically with either pppRNA or poly(I-C) transfection to increase reporter gene activity by increasing the level of RIG-I. These results reinforce the view...
that it is primarily the SeV C protein (and not V) that inhibits pppRNA- and dsRNA-induced signaling to the IFN-β promoter via RIG-I during SeV infection.

DISCUSSION

SeV has been one of the most extensively used model viruses to investigate IFN induction in infected cells. Most of this work has used the commercially available Cantell strain of SeV, whose ability to induce IFN, like that of other SeV strains, is due to the presence of DI genomes in egg-grown stocks. Nondefective SeVs that are plaque purified from these stocks, including that of the Cantell strain, do not induce IFN unless cellular RIG-I levels are artificially increased (21, 30). For nondefective SeV infection, the expression of the C and V proteins is apparently sufficient to prevent IFN-β activation under normal conditions. Measles virus infection, in contrast, can apparently induce IFN in the absence of DI genomes, and evidence that this induction is due to the synthesis of leader pppRNAs has recently been provided (26). Leader and trailer RNAs, which are unmodified at either end, are unstable in infected cells unless they are encapsidated with the N protein (presumably after their synthesis as free RNAs) (2). Free leader and trailer RNAs are more easily detected in VSV infections, which synthesize larger amounts of viral RNAs over a shorter period of time. For nondefective SeV infections, eight times as many trailer RNAs/antigenome template are found as leader RNAs/genome template, consistent with the relative strengths of their respective replication promoters. For VSV copyback DI infections, there are 40 times as many trailer RNAs/ template (nondefective antigenome plus DI genome) as leader RNAs/genome template, presumably reflecting the increased strength of the copyback DI replication promoters. The VSV polymerase clearly has a strong preference for initiating RNA synthesis at the 3′ ends of copyback DI genomes over both ND genomes and antigenomes (17).

We previously noted that not all SeV stocks that contain DI genomes strongly induce IFN; this ability appears to be restricted to stocks containing relatively small copyback DI genomes (the smaller the DI genomes, the more moles of ends are present for a given weight). The commercially available Cantell strain contains a copyback DI genome of only 546 nucleotides in length, the smallest SeV DI genome described to date (30). SeV copyback DI-H4 genomes (1,410 nucleotides) have the same strong replicative advantage as their VSV counterparts because they also contain strong replication promoters at both their genome and antigenome 3′ ends. Thus, SeV copyback DI infections presumably synthesize considerably more pppRNAs than standard virus infections. We also previously noted that when cytoplasmic extracts of DI-H4-infected cells are centrifuged on CsCl density gradients, small amounts of DI genome RNA are found in the pellet fraction (30). This indicates that this RNA is not encapsidated with the N protein and therefore forms dsRNA panhandles in a concentration-independent manner. Thus, copyback DI-H4 infections apparently produce considerably more of both known PAMPs of RNA virus infection than do standard virus infections. Coupled with their strongly reduced accumulation of the viral C and V proteins, it is easy to see why these copyback DI infections are such potent inducers of IFN-β.

All paramyxoviruses express either C or V proteins, and many viruses express both. In viruses that express only C or V, we presume that either viral protein alone counteracts the innate immune response of the host to aid virus replication. The C and V proteins, which bear no sequence similarity, likely target different key elements of the host response. Viruses that express both C and V presumably have more diverse ways of countering innate immunity. In support of this notion, SeV infections that cannot specifically express either the C or V protein contain increased levels of IFN-β and interleukin-8 mRNAs relative to wt SeV infections (31), and the independent expression of the C or V protein will inhibit poly(I-C)- or Newcastle disease virus-dependent activation of IRF-3 (15). Previous work has identified mda-5 as being a key target of paramyxovirus V proteins in countering the innate immune response (1). This paper provides evidence that for SeV infection of MEFs, it is the C protein (and not V) that is primarily responsible for this effect and that C acts by countering RIG-I-dependent signaling to IFN-β. For example, the independent expression of either C or V inhibited IFN-β activation due to RIG-I overexpression (Fig. 2B). Also, both proteins inhibited IFN-β activation due to DI-H4 infection, although C was always more effective here than V (Fig. 3). However, only C expression effectively inhibited IFN-β activation due to GFP(+/-) infection (Fig. 3) or transfected poly(I-C) or pppRNAs (Fig. 5). Perhaps the strongest indication that the C proteins are primarily responsible for countering the innate immune response comes from experiments with SeV that cannot specifically express the C or V proteins. SeV-Cminus infection not only cannot prevent the effects of transfected poly(I-C) or pppRNAs on IFN-β activation but also synergistically enhances these effects. SeV-Vminus infection, in contrast, behaves mostly like wt SeV infection and counteracts the effects of transfected poly(I-C) or pppRNAs (Fig. 6).

Finally, we note that poly(I-C) (made with polynucleotide phosphorylase that generates 5′-diphosphate ends and which is transfected into cells) and the presumed GFP dsRNA (that is directly generated in the cytoplasm via the viral transcriptase and which contains capped 5′ ends) both activate IFN-β via RIG-I in MEFs. Thus, the ability of dsRNA to induce RIG-I signaling does not depend on the manner in which it is introduced into this cell compartment, nor is it peculiar to the presence of 5′-diphosphorylated ends that are not normally found in cells and could theoretically act as PAMPs. Moreover, in either case, the activation of IFN-β by these dsRNAs is inhibited by the SeV C protein and not V, presumably because this signaling passes through RIG-I and not mda-5. It appears that our MEFs contain insufficient amounts of mda-5 to sense SeV infection, as these MEFs respond well to the expression of plasmid-derived mda-5 (Fig. 2B). This conclusion is also consistent with our finding that three different rubulavirus V proteins that are known to counteract poly(I-C)-induced mda-5 signaling were unable to inhibit IFN-β activation in response to SeV-DI-H4 infection (data not shown). We expect that the SeV V protein will be more important in countering the innate immune response in other cells in which mda-5 functions as a PAMP recognition receptor.
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REFERENCES


V. ADDITIONAL DATA

“THE IMPLICATION OF SEV NUCLEOCAPSID IN THE INDUCTION OF THE IFN-β”
Additional Data
The implication of SeV nucleocapsid in the induction of the IFN-β

The success of the innate cellular defense to viral infection is dependent on the capacity of the host to detect the presence of the invading pathogen. Upon infection, many cellular sensors recognise different components of the virus and initiate in turn signal transduction cascades (such as IRF-3) producing in the end cytokines, including IFNs. The RNA helicase RIG-1 has been recently discovered to recognise viral RNA. RNA recognition leads to IRF-3 and NF-κB activation and finally to the induction of type I IFNs and the antiviral state of the cell. To circumvent the detection of their own RNA genome, the mononegalevirales possess the nucleocapsid (NCs) that surrounds completely the RNA viral genomes (and antigenomes). It is likely that their nucleocapsid never disassemble during genome expression. This protection seems to be quite strong considering that the RNA genomes (within the nucleocapsid) are resistant to nuclease attack at any salt concentration and that the NC is very stable, as it withstands the high salt and gravity forces of cesium chloride density gradient centrifugation (Lamb and Kolakofsky, 1996). Further more, the N proteins are believed to be “sticky”, in the way that they are tightly bound to the viral RNA genomes and can also encapsidate free RNA independently of whether it comes from the virus or the cell.

It has been shown in 2002 that MeV nucleocapsid (NC) protein was the major component of IRF-3 activation and triggered the induction of IFN (Tenoever et al., 2002). The ability to activate IRF-3 during the course of infection has also been observed in other single stranded, enveloped RNA viruses such as RSV, NDV, VSV and SeV (Casola et al., 2001; Servant et al., 2001; Sundstrom et al., 2001). This suggests that the IRF-3 cascade could be involved in the viral NC detection. Since NCs are the first viral elements that enter the cell, it is logical to make the hypothesis that, like MeV, SeV NCs act as PAMPs and consequently induce IFNβ activation upon SeV infection.

1) **SeV N protein expressed alone does not activate IFNβ.** When N is expressed alone (in the absence of the viral P protein which forms a complex with N and prevent “illegitimate” non-specific binding to cellular RNA), it is often found in high number and aggregates together with non genome RNAs, which represent a bogus NCs. To analyze the direct effect of the SeV N protein alone, MEF cells were first transfected
with a plasmid containing a luciferase reporter gene under the control of the IFNβ promoter and 24h later with a plasmid expressing SeV N protein (Fig. A). We have examined the ability of the N protein to induce IFNβ activation after transfection and compared the level of IFNβ activation with those of the copyback DI-H4 infection that we used as a positive control. As shown in figure A, the expression of N protein alone does not induce IFNβ activation. By contrast, DI-H4 infection activates strongly the activation of the IFNβ in this experiment. These results show that under these conditions, SeV viral N protein does not induce IFNβ activation.

![Graph showing relative luciferase activity](image)

**Figure A**: Transfected SeV N proteins do not induce IFNβ activation.

Parallel cultures of MEFs were first transfected with pIFNβ-luc and pTK-luc. After 24h, the cells were either infected with SeV-DI-H4, or transfected with plasmids expressing SeV N protein (pEBS_N). Cytoplasmic extracts were prepared after 20h of incubation, and used to determine firefly and renilla luciferase levels.

2) **Transfected encapsidated SeV (NCs) genomes induce IFNβ activation.** To further investigate the role of the NCs, 24 hours SeV infected cells were collected and the NCs purified by CsCl centrifugation (cf. M&M). Two doses of purified SeV NCs (or PolyI/C) were transfected into MEF cells and tested for their ability to activate IFNβ by using the reporter plasmid in which the IFNβ promoter expresses a luciferase reporter gene. Surprisingly, we observed that transfected purified NCs induce strongly
IFNβ activation and that the transfection of 10μl of purified NCs (corresponding to 250000 infected cells) is almost as efficient as a DI-H4 infection. Moreover, we can see that NCs transfection is more competent than the synthetic dsRNA (poly I/C) treatment in activating IFNβ. These results suggest that induction of IFNβ requires the presence of the NCs complex (fig. B).

**Figure B:** Transfected NCs induce IFNβ activation.

Parallel cultures of MEFs were first transfected with pIFNβ-luc\textsuperscript{ff} and pTK-luc\textsuperscript{f}. After 24h, the cells were either infected with SeV-DI-H4, or transfected either with poly I/C (5ug) or purified SeV NCs (5-10ul) isolated by CsCl density gradients centrifugation (cf.M&M). Cell extracts were prepared after further 20h of incubation, and equal amounts were used to determine luciferase activities.

3) **SeV C and V proteins expression decreases the IFNβ activation upon NCs transfection.** Since SeV C and V proteins are responsible of counteracting the host innate immune response and because we have shown in paper three that IFNβ activation upon DI-H4 infection was in part due to a downregulation of the viral C and V proteins, we decided to over-express the V or C proteins in NCs-transfected MEF cells. We observed that IFNβ activation was strongly reduced by C expression (4 folds), and more modestly by V expression (2 folds) (Fig C). Thus, the SeV V and C proteins inhibit IFNβ activation induced by NCs transfection.
Figure C: Over-expression of viral C and V proteins inhibits IFNβ activation induced by SeV NCs transfection. Parallel cultures of MEFs were transfected with pIFNβ-luc and pTK-luc and plasmids expressing the SeV V protein, or the SeV C protein. 24h post-transfection, the cells were transfected with purified SeV NCs (5ul). Cytoplasmic extracts were prepared after further 20h of incubation, and used to determine luciferase levels.

4) NCs from either SeV or DI-H4 infections induce IFNβ at the same level. Because DI-H4 infection induces more strongly IFNβ activation compared to a ND SeV infection, we decided to compare NCs coming from both infections and their ability to induce IFNβ activation. Prior to transfection, the purified NCs were charged on a gel and stained with Commassie Blue in order to compare the amount of N proteins from each infection (SeV or DI-H4) (Fig D.). Because DI-H4 genome is 10 times smaller than the ND genome (and thus contains 10 times more moles of ends), we asked whether DI-H4 NCs could induce more IFNβ than a SeV NCs. Two different concentrations of NCs were transfected (for both types of infection) into MEF cells and IFNβ activation was monitored using the reporter plasmid carrying the IFNβ promoter expressing the luciferase gene (Fig D). We observed that the level of IFNβ activation is the same in both the SeV and the DI-H4 NCs transfections. These data suggest that the IFNβ activation is not dependent on the number of moles of genome. Furthermore, because the number of mole correlates with the number of 5'
triphosphorylated extremities of the genome, we can suggest that the IFNβ activation upon NCs transfection is not dependent on the number of 5’ppp ends of each genome. To confirm this idea, we decided to remove the eventual triphosphates at the ends of the genomes by treating the purified NCs with phosphatase (CIP) (cf.M&M). The NCs were then transfected in MEF cells and the IFNβ activation was analyzed. Interestingly, transfected NCs previously treated with CIP had no effect on the level of IFNβ activation. This data suggest that 5’tri-phophorylated ends of the genomes presumably do not act as a viral inducer of the IFNβ upon NCs transfection (Fig. E).

**Figure D:** Transfections of purified NCs from SeV or DI-H4 infections induce IFNβ at the same level. Parallel cultures of MEFs were first transfected with pIFNβ-luc and pTK-luc. After 24h, the cells were transfected with the same amount of NCs (2.5-5ul) purified from either SeV or DI-H4 infections. Cytoplasmic extracts were prepared after 20h of incubation, and used to determine luciferase levels. The amount of viral N proteins after two CsCl gradient centrifugations was determined by SDS-PAGE and Coomassie-blue staining and is represented in the upper panel.

5) **RNase treatment shuts down the induction of IFNβ.** In order to rule out the possible RNA contamination of our purified NCs, we decided to treat the viral NCs with the RNases T and A. After RNase treatment, the cells were transfected as usual and challenged for the IFNβ activation. Unexpectedly, we can observe no more IFNβ activation, suggesting that RNA products are presumably the ones responsible for the strong induction of IFNβ upon NCs transfection (Fig. E).
**Figure E**: Effect of transfected NCs on the IFNβ activation after CIP and RNase treatment

Parallel cultures of MEFs were transfected with pIFNβ-luc and pTK-luc. 24h post-transfection, the cells were transfected with purified SeV NCs (5ul) or with SeV NCs that were previously treated with CIP or RNase. Cytoplasmic extracts were prepared after further 20h of incubation, and used to determine luciferase levels. The amount of viral N proteins after two CsCl gradient centrifugations was determined by SDS-PAGE and Coomassie-blue staining and is represented in the upper panel.

**CONCLUSION**

We first wanted to determine whether viral NCs themselves could act as PAMPs. We found that bona fide NCs (as opposed to N protein aggregates that form upon N expression in the absence of viral infection) did indeed appear to activate IFNβ. This activation could be inhibited by the viral C and V proteins, and did not appear to be influenced by the number of 5’ppp ends present in the preparation; and this for two reasons: first, the transfections of the NCs from both the ND and DI-H4 did not show any difference in the level of IFNβ activation and that phosphatase treatment had also no effect. Unexpectedly, we treated the NCs with RNase as a control for the phosphatase treatment, and found that the IFNβ activation was shut down. These results suggest that it is (cellular or viral) RNA trapped in the NCs that is responsible for the IFNβ activation and not the NC itself.
MATERIALS AND METHODS

Cells and viruses
Mouse embryo fibroblast cells (MEFs) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

SeV stocks were grown in the allantoic cavities of 9-days-old embryonated chicken eggs for 3 days at 33 °C. For nondefective stocks (109 pfu/ml), 0.1 ml of a 105 dilution (ca. 1000 pfu) was inoculated per egg. In the case of DI stocks, 0.1 ml of a 103 dilution was used. In all cases, the amount of viral proteins present in the resulting allantoic fluid was analyzed by SDS-PAGE and Coomassie-blue staining of pelleted virus. Virus titers were determined by plaquing on LLC-MK2 cells.

Plasmids, transient transfections, infections, luciferase assay
EBS plasmids expressing viral proteins were constructed by standard methods (Bontron et al., 1997); the precise detail can be obtained from the authors.

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). pTK-rl-lucter, used as a transfection standard, contains the herpes simplex virus TK promoter region upstream of the renilla luciferase gene (Promega).

For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1.5 μg of pβ-IFN-fl-lucter, 0.5 μg of pTK-rl-lucter, 1.5 μg of plasmids (EBS) expressing N. At 24 h post-transfection, the cells were (or not) infected with DI-H4 stocks or transfected with 5 μg of poly-I/C or with 5-10μl of purified SeV or DI-H4 NCs. (cf. M&M Purification and analysis of nucleocapsid RNAs) using TransMessenger transfection reagent (QIAGEN).

20hrs 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.

Purification of nucleocapsid RNAs and RNase/phosphatase treatments
Confluent MEFs in 10 cm Petri dishes (10⁷ cells) were infected with 20 pfu/cell of SeV Z or SeV-DI-H4. 24hpi, the cells were collected and lysed in 300 ul of NP40 lysis buffer. Cytoplasmic extracts were then centrifuged in a 20–40% (w/w) CsCl density gradient (16h/35,000 rpm/12°C) for purification. The bands containing the encapsidated RNAs resulting from replication (SeV Z or SeV-DI-H4 genomes) were extracted, pelleted in TNE
1X (45min/35000 rpm/4°C) and resuspended in 40ul TE 0.1% SDS, 10% glycerol. This step was repeated twice in the case of the infections.

Encapsidated RNAs from the SeV infection were treated (or not) with 60U of calf intestine alkaline phosphatase (CIP) in a buffer (10X) containing 5M Tris-HCl, 1mM EDTA (Roche), or with 50U RNase T1 (Roche) and 1ug RNase A (Roche) for 30min at 37°C. CIP and RNAse treatments were performed between the two CsCl density gradients.
VI. GENERAL DISCUSSION
General Discussion

The ability of the host cell to fight viral infection requires recognition of the invading viral pathogen as such and subsequent induction of signaling cascades that lead to the production of innate and adaptive antiviral responses. IFNβ plays a crucial role in priming neighbouring cells for possible infection and is also involved (with other IFN-stimulated genes) in recruiting cells of the host immune system to the site of infection, providing a way to eliminate infected cells. In the present study, we investigated the mechanisms used by the host cell to recognize SeV infection, and those used by SeV to counteract the cellular innate immune system.

By using different SeV stocks carrying mutations either in the promoter region or in the C proteins, we demonstrated that the presence of the leader RNA transcript (or the overexpression of the trailer) as well as the expression of the viral C and V proteins have an important role in counteracting the IFNβ activation and the expression of the chemokine IL-8 (c.f. Paper 1). As opposed to the V protein and the leader RNA, a number of investigations have been made on the SeV C protein. As mentioned in the first paper, this particular protein is in charge of many functions during infection: (i) it stimulates viral RNA synthesis early in infection (Latorre et al., 1998a); (ii) it inhibits viral RNA synthesis late in infection by binding to the viral polymerase (Cadd et al., 1996b); (iii) it has a role in the virus particle budding, which is facilitated by its binding to Alix, and in virion assembly, possibly by interaction with the matrix (M) protein (Mottet et al., 1996; Sakaguchi et al., 2005); (iv) The C protein interacts with Stat1 in two ways: counteracting IFN signaling and inducing Stat1 instability (Garcin et al., 2003). (v) Finally, the C protein inhibits the IRF-3-dependent activation of IFNβ as well as the activation of IL-8 expression (Strahle et al., 2003). These multiple functions of SeV C protein during infection fit well with its property to interact with various viral and cellular proteins, such as Stat1, Alix and Rig-I (unpublished). However, these interactions remain to be closely analyzed. Additionally, the reason why IL-8 expression has been only recently discovered could be explained by the fact that standard SeV induces very little IL-8. Indeed IL-8 is only expressed upon infection of SeV bearing mutations in the C and V proteins, and in the leader region. Moreover, it has been already reported that SeV and MeV infections induce an other CC-chemokine RANTES via the activation of IRF-3 (Genin et al., 2000; Tenoever et al., 2002). These data confirm the results suggesting that SeV targets the inflammatory and adaptive immune responses (IL-6 and IL-8) as well as the IFN-induced intracellular antiviral state (IFNβ and STAT1).
SeV is commonly used by many laboratories for its capacity to strongly induce IFN. This ability was long known to be associated with the presence of DI genomes in the SeV stock. The third paper provides evidence that the strong induction of IFNβ activation upon SeV infection (SeV stock containing DI genomes) is mainly due to the presence of copyback DI genomes. Moreover, the level of IFNβ activation was found to be proportional to that of DI genome replication. Apparently, the ability of DI-H4 to induce IFNβ is not only related to its strong interference with the helper, which leads to lower levels of V and C proteins intracellularly but also to the relative content of copyback DI genomes, which can presumably form dsRNA. (cf. paper 2).

It is important to emphasize the difference between a standard SeV-WT infection from an infection of SeV that contains DI genomes. In the case of a SeV-WT infection, the RNA genomes and antigenomes are tightly assembled into NCs, meaning that they are never free to anneal (like other NNV). Furthermore, Weber et al. have shown recently that no detectable amounts of dsRNA produced by NNV have been found in infected cells (Weber et al., 2006). This result can be explained by the fact that the amount of dsRNA produced by NNV are below their detection limit, since one molecule of dsRNA per cell can be effective in triggering an antiviral response. If it is the case, we can imagine two different ways for SeV-WT (devoided of DI genomes) to produce dsRNA: 1) During SeV replication, the polymerase ignores occasionally the stop signal at the end of the trailer template, which results in the extension of the trailer beyond the trailer/L gene junction. This event will produce a long trailer whose extended 3’sequences can anneal to those of the L gene (Vidal and Kolakofsky, 1989). 2) dsRNA can also be the result of a readthrough by the transcriptase of the L gene-end site until the end of the genome (an event that can happen at 5% of the time at all gene junctions). In this case the genome 5’end can anneal to the trailer RNAs (Le et al., 2002). Finally, if we consider that there is a small amount of dsRNA that is generated in SeV infection, it is evident that the presence of dsRNA will be dampen by the SeV C and V proteins expressed during ND genome replication and that the expression of IFNβ will consequently be blocked.

In the case of copyback DI infections, it is a different story. Because DI-H4 contains two antigenomic promoters located at both the genomes and antigenomes ends, it has strong replicative advantage over the standard virus. Consequently, a huge amount of DI genomes is rapidly generated and the level of viral proteins (including the level of the nucleocapsid protein) is also strongly attenuated. This rapid generation of DI genomes and the low level of
NC proteins could explain why the genomes and antigenomes may not assemble into nucleocapsids and would consequently form dsRNA. If DI-H4 genomes and antigenomes are not encapsidated during infection, two forms of dsRNA can be found (in addition to those mentioned for a SeV infection). First, the extremities of the DI genome (complementary on 110 nucleotides) can self anneal in a concentration independent manner and form dsRNA, resulting in the shape of panhandles. Secondly, the genome and the antigenome can anneal together, which remains unlikely since this would require the melting of intramolecular secondary structure.

Recent evidence suggests that NNV infections could activate the innate immune response by producing viral signatures other than dsRNA, namely 5’tri-phosphorylated single-stranded RNA transcripts (5’pppRNAs). This discovery suggests that dsRNA may not always be implicated in the IFNβ induction upon viral infection and that there is probably more than one factor able to induce this primary cellular induction. Two cytoplasmic helicases, namely RIG-I and Mda-5, have been recently found to respond to dsRNA and, at least for RIG-I, to 5’pppRNAs. These RNAs are generated in the cytoplasm during RNA virus replication. Upon detection of specific viral RNAs RIG-I and Mda-5 interact with Cardif which is present in the mitochondrial membrane, and this interaction is thought to lead to the recruitment and activation of the TBK1, IKKe and other IKK kinases that activate NF-kB and IRF3, thereby activating the IFNβ promoter.

In the third paper, we investigated different potential inducers of IFNβ, including the synthetic dsRNA (polyI/C) and 5’pppRNAs and the involvement of RIG-I in these inductions. We showed evidences that i) transfection of poly-I/C and ii) a SeV coinfection that can produce GFP dsRNA, as well as iii) transfections of small 5’pppRNAs can all induce IFNβ. In all cases, the IFNβ induction was dependent on RIG-I. Not all 5’pppRNAs generated by SeV can act as PAMPs: 1) SeV mRNAs carry 5’ ppp ends, but because they are capped, these mRNAs cannot be potential targets. 2) SeV genomes and antigenomes also carry triphosphorylated 5’ends, but because the RNA is tightly and entirely encapsidated, it cannot be seen. 3) Finally, leader and trailer RNA transcript are the most susceptible to induce IFNβ, because they are short unencapsidated 5’pppRNAs that are independent on on-going (N) protein synthesis. For this reason, we suspect that, in the case of DI-H4 infection, there is a real overproduction of pppRNAs (trailer) resulting in the induction of the IFNβ activation. Alternatively, other viral structures may take the role of RNA as a danger signal for the host cell. Indeed, it was previously shown that the NCs from VSV and MeV are capable of
triggering IFN induction. It was interesting to observe in our experiments that NCs purified from SeV infection could strongly induce IFNβ. However, RNase treatment revealed that it was probably not the NCs that were responsible for this activation, but more likely RNA products that have contaminated the CsCl banded NCs. Because the NC is the first viral element that enters the cell, one can imagine that viruses have evolved to avoid its detection. Thus, it is reasonable to think that NCs do not act as PAMPs. Concerning MeV, it was shown recently that the NCs were not responsible for the IFNβ activation after all and that it was the leader transcript of MeV that was responsible for the activation of IFNβ via the RIG-I pathway (Helin et al., 2001; Plumet et al., 2007). This again comforts the idea that SeV leader and trailer RNAs are more likely to be the main PAMPs responsible for triggering the antiviral responses upon SeV infection.

Regarding the C and V proteins, we presented evidence that for SeV infection of MEFs, it is the C protein (and not V) that is primarily responsible for this effect, and that C acts by countering RIG-I dependent signaling to IFNβ. For example, independent expression of either the C or the V proteins inhibited IFNβ activation due to RIG-I over-expression. In addition to this, both proteins inhibited IFNβ activation due to DI-H4 infection, although C was always more effective than V (in our experiments). However, only C expression effectively inhibited IFNβ activation due to GFP(+/−) infection, or transfected poly-I/C or pppRNAs (cf paper 3).

These new findings, suggesting that the 5′pppRNA is a PAMP specific to the NNV infections, lead us to reconsider the way the cell could detect the presence of viruses. However, no proof has been found concerning the role of 5′pppRNA products in the activation of IFNβ in SeV infection. Further analyses of the precise RNAs involved in the IFNβ activation need to be done. Additionally, more investigations are necessary to determine how these PAMPs are detected and the mechanisms that drive cellular responses. Finally, learning more about the protein targets of SeV proteins also appear important in order to understand the details of the IFN suppressive activities of SeV and virus infection in general.
VIII. Reference List


Sendai Virus Targets Inflammatory Responses, as Well as the Interferon-Induced Antiviral State, in a Multifaceted Manner

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We have used cDNA arrays to compare the activation of various cellular genes in response to infection with Sendai viruses (SeV) that contain specific mutations. Three groups of cellular genes activated by mutant SeV infection, not by wild-type SeV, were identified in this way. While some of these genes are well-known interferon (IFN)-stimulated genes, others, such as those for interleukin-6 (IL-6) and IL-8, are not directly induced by IFN. The genes for beta IFN (IFN-β), which is critical for initiating an antiviral response, was also specifically activated in mutant SeV infections. The SeV-induced activation of IFN-β was found to depend on IFN regulatory factor 3, and the activation of all three cellular genes was independent of IFN signaling. Mutations that disrupt four distinct elements in the SeV genome (the leader RNA, two regions of the C protein, and the V protein) all lead to enhanced levels of IFN-β mRNA, and at least three of these viral genes also appear to be involved in preventing activation of IL-8. Our results suggest that SeV targets the inflammatory and adaptive immune responses as well as the IFN-induced intracellular antiviral state by using a multifaceted approach.

Alpha/beta interferons (IFN-α/β) are cytokines that act in a pleiotropic manner to limit viral replication and spread (2, 57). In fibroblasts (e.g., the bronchial epithelial target of many paramyxoviruses), the product of the single IFN-β gene is directly induced by viral infection, and IFN-β feeds back onto cells in an autocrine manner to induce multiple IFN-α genes and in a paracrine manner to prime neighboring cells for their possible infection (60). Since most viruses induce IFN-β to some extent, intracellular double-stranded RNA (dsRNA) generated from the viral genome is traditionally assumed to be the common signature of virus replication that sets the IFN system in motion (22, 32). dsRNA is thought to induce the formation of an enhancosome at the IFN-β promoter that includes IFN regulatory factor 3 (IRF-3) and NF-κB (among other transcription factors) (65). IFNs induce a cellular state that is nonconducive for viral replication by signaling through their cell surface receptor, leading to the phosphorylation of cytoplasmic STAT proteins and their nuclear translocation. IFN-α/β responses are regulated primarily via IFN-stimulated gene (ISG) factor 3, a heterotrimeric transcription factor composed of STAT1, STAT2, and IRF-9 (p48). ISG factor 3 binds to a DNA element (IFN-stimulated response element) in the promoters of ISGs and activates their expression (7).

The extravasation of neutrophils, eosinophils, basophils, and mononuclear cells is the salient feature of the innate response to microorganisms in the lung. Localized and systemic pro- and anti-inflammatory cytokines thus also play an important role in the outcome of viral infection and pathogenicity of this organ (58). The CC chemokine interleukin-8 (IL-8) is secreted from epithelial surfaces in a polar fashion during infection with pathogenic bacteria such as Salmonella enterica serovar Typhimurium and sets up a subepithelial chemotactic gradient directing neutrophils and other immune cells to the site of infection (27). In polarized epithelial monolayers, S. enterica serovar Typhimurium-induced IL-8 expression is controlled via the activation of the mitogen-activated protein kinase cascade and IκBα kinase, followed by NF-κB translocation to the nucleus and production of IL-8 mRNA. IL-8 secretion by primary human monocytes in response to dengue virus infection is also tightly linked to NF-κB activation (3). Sendai virus (SeV) infection of human embryonic kidney 293 cells induces the expression of the CXC chemokine RANTES in an IRF-3- and NF-κB-dependent manner (23, 41). NF-κB, like IRF-3, is found in the cytoplasm of unstimulated cells, retained in a complex with the inhibitory IκB proteins. Upon stimulation with many inducers, including dsRNA and virus infection, IκB is rapidly phosphorylated and degraded, resulting in NF-κB release and translocation to the nucleus (30, 33).

Given the importance of the host innate immune response to virus infection, viruses have, during their coevolution with cells, developed strategies to regulate cytokine synthesis and action. SeV, a model paramyxovirus and respiratory pathogen of mice, is known to use its C protein to evade the host interferon response by at least two mechanisms. (i) C binds STAT1, preventing its activation in response to IFN, and the carboxyl part of the C protein (i.e., residues 24 to 204, or the Y proteins) is sufficient for this activity. A phenylalanine at position 170 of C is also critical for blocking STAT1 activation (18, 59). (ii) C also targets STAT1 for degradation, and the amino-terminal residues of the C proteins (residues 1 to 23, which are absent and not directly induced by IFN) feeds back onto IFN. The gene for beta IFN (IFN-β), which is critical for initiating an antiviral response, was also specifically activated in mutant SeV infections. The SeV-induced activation of IFN-β was found to depend on IFN regulatory factor 3, and the activation of all three cellular genes was independent of IFN signaling. Mutations that disrupt four distinct elements in the SeV genome (the leader RNA, two regions of the C protein, and the V protein) all lead to enhanced levels of IFN-β mRNA, and at least three of these viral genes also appear to be involved in preventing activation of IL-8. Our results suggest that SeV targets the inflammatory and adaptive immune responses as well as the IFN-induced intracellular antiviral state by using a multifaceted approach.

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in the Y proteins) are essential for this activity (reference 17 and references therein).

This paper reports that SeVs carrying specific mutations in the C gene, in contrast to wild-type SeV (SeV-wt), activate IL-8 and IFN-β expression as well as that of several ISGs. Our results suggest that the products of virtually all of the viral accessory genes (C and V proteins and leader RNA) act to prevent the expression of these cellular genes that are active to the overall host antiviral response.

**MATERIALS AND METHODS**

Cells and viruses. 2C4 cells (39), 2TGH cells (48), and their derived cell lines U3A (45) and USA and USA-IIFNAR (43) were obtained from IM Kerr (Imperial Cancer Research Fund, London, United Kingdom) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in the presence of the relevant maintenance drug (hydrocortisone at 250 μg/ml or G418 at 400 μg/ml). The generation of recombinant SeV (SeV-rex) expressing alternate C and V (and P) proteins is described elsewhere (8, 19, 37, 38). All SeV stocks were grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Virus preparations from the allantoic fluid were analyzed by sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis and Coomassie blue staining after virus pelleting. Virus titers were determined by plaqueing on LLC-MK2 cells.

**Virus infections.** Cells were infected at a multiplicity of infection of 20 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After an absorption period of 1 to 2 h, the inoculum was removed and replaced with fresh medium.

**Plasmids, transient transfections, and luciferase assay.** IRF-3 (54), IFN-βN (41), and IRF-3-ribosome (67) were obtained from John Hiscott and Paula Pitha. pDsRed2, expressing red fluorescent protein (RFP), was from Clontech. The reporter plasmid with the firefly luciferase gene under the control of the human IFN-β promoter was designed by King and Goodbourn (35) and is referred to here as pIFN-β-luciferase, pTK-rluciferase, used as a transfection standard, contains the herpes simplex virus thymidine kinase promoter region upstream of the Renilla luciferase gene (Promega). For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pIFN-β-luciferase, 0.3 μg of pTK-rluciferase, 1 μg of IRF-3-expressing plasmid, and 6.9 μl of Fugene (Roche) according to the manufacturer’s instructions. At 24 h posttransfection, the cells were (or were not) infected with SeV recombinants or treated with 50 μg of pol(y)-luciferase (Sigma, St. Louis, Mo.) per ml. Twenty hours later, cells were harvested and assayed for firefly and Renilla luciferase activities (dual-luciferase reporter assay system, Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of Renilla luciferase.

**RNA extraction and quantification.** Total RNA was extracted with Trizol reagent (Invitrogen), and two dilutions were electrophoresed on agarose-HCHO gels. The gels were stained with ethidium bromide, and the intensities of the 18S and 28S rRNA bands were quantified by using the ChemiDoc System (Bio-Rad) and One-D-scan software. All samples were diluted to a final concentration of 1 μg/ml so that their subsequent transcription into DNA, if not quantitative, would be little influenced by this parameter.

**RT and real-time PCR via TaqMan.** Ten microliters of total RNA was mixed with 0.5 μg of random hexamer primer (Promega) and subjected to a reverse transcription (RT) reaction with Superscript enzyme (Gibco), as described by the manufacturer, in a total volume of 50 μl. Two microliters of each cDNA was then combined with 1 μl of internal control (either 20× ribosomal 18S or human glyceraldehyde-3-phosphate dehydrogenase [GAPDH] [Applied Biosystems], 11 μl of 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: for the IFN-β gene, 5′-CGAACATTTTTCGTGCAGAAGC-5′ (forward), 5′-TCTACTGTGCTTGAAGCGC-3′ (reverse), and 5′-CTGGTGAATTGGAAGCTTG-3′ (probe); for the IL-8 gene, 5′-CGGTTGCTGCTTGGAGCA-3′ (forward), 5′-TTAGCCTCCTGGGCAAAT-3′ (reverse), and 5′-GGCTTGCTGCTGGAAGCTC-3′ (probe); for the IL-6 gene, 5′-GTCCATGATGCTAGTGTGAA-3′ (forward), 5′-CACCATGCGAAGCGC-3′ (reverse), and 5′-GACCGCAGGATGAACTGTC-3′ (probe); for the V gene of SeV, 5′-GCGAAATCGGCTCTGATGTTG-3′ (forward), 5′-ATAGCGAGATGTACCAAAGC-3′ (reverse), and 5′-GCAAATGCGATGCTGATGCTG-3′ (probe); and for the N gene of SeV, 5′-CGAAGGAGGATGCCCAGCG-3′ (forward), 5′-GAGCTCACCAGCCAGAGC-3′ (reverse), and 5′-CCAGGATGCAGCTGCTGCA-3′ (probe).

**RESULTS**

In our experiments we have used two strains (or lineages) of SeV whose history is relevant to studies of virulence. The natural host of SeV has not been clearly identified, and this virus is sometimes referred to as murine parainfluenza virus type I because it efficiently infects mice, causes disease, and spreads readily to uninfected animals. However, there is no virologic or serologic evidence of SeV in wild mouse populations (29). There are two known lineages of SeV, Z/H/Fushimi and Ohita M/Hamanatsu (12, 31, 53, 62). The nucleotide sequences within each lineage are 99% identical, and they are 89% identical between lineages. Z/H/Fushimi comes from viruses isolated in the early 1950s after an epidemic of pneumonitis of newborn infants in Sendai, Japan (29, 56). These SeVs have been passaged extensively in eggs in various labs since the 1950s, and they are moderately virulent for mice (50% lethal dose [LD₅₀] = 10⁵ to 10⁶ PFU). All of the SeV² strains used in this study (including the wt) are recombinants.

Ohita M (SeV²M) and Hamanatsu, in contrast, are highly virulent (LD₅₀ < 10⁴), low-egg-passage (nonrecombinant) viruses isolated from two completely separate, very severe epidemics of animal houses in Japan. This lineage is presumably
closer to the virus in its natural (unknown) host, and it is known that SeV passage in eggs attenuates its virulence in mice. SeV M grew poorly in cell culture, and a clear-plaque variant emerged that was avirulent (LD50 > 10^5) and contained only two amino acid substitutions, C170S and L120S (31). When placed in the rSeV background, the C170S mutation was found to account for most or all of the loss of virulence (15). This virus is referred to here as SeVM-C170S. SeVM-C170S appeared to initiate the infection of mice normally, but the infection was limited to the first day. This was the first evidence that the C gene, like the SeV V gene (34), was involved in countering host innate defenses. Although SeV Z-wt is attenuated relative to SeVM-wt in laboratory mice, rSeV Z-wt still replicates as efficiently in the monkey and chimpanzee models of human respiratory disease as human parainfluenza virus type 1 (56), the virus which is most closely related to SeV and which is endemic in children.

We have used a cDNA array designed to study the human cell response to IFN-α/β (55) to monitor the effects of various SeV infections on host mRNA levels. Around 150 genes of interest were selected from the UniGene database. These genes comprise known ISGs and genes of intrinsic interest which might or might not be induced by IFNs in different cell systems. They include genes involved in cell proliferation, immune responses, and the responses to a variety of cytokines (see Table 1 of reference 55). We compared matched sets of SeV carrying two different mutations in the C gene (SeVM, C170S and SeV Z, C10-15), whose products interact with STAT1 in different ways (to interfere with IFN signaling and to induce STAT1 instability, respectively) (Fig. 1D). We also used matched sets of SeV carrying mutations in the viral replication promoters (SeV Z, GP1 and -GP31-42) that prevent apoptosis and lead to persistent infections (the numbers refer to the nucleotides of the genomic promoter that have been replaced with the equivalent sequences of the antigenomic promoter) (16, 20). Promoter mutations are thought to act via mutant leader RNAs that are abundantly transcribed from the genomic replication promoter and which bind to cellular RNA-binding proteins that regulate mRNA fate (28).

Upon infection with SeVM-wt or SeV Z-wt, many of these mRNA levels remain unchanged (Fig. 1A to C). This lack of response is presumably due in part to active SeV countermeasures that neutralize the cell’s antiviral response (24). Upon infection with the mutant viruses, the mRNA levels of 15 of the 150 genes examined were elevated, and three patterns of gene activation were seen (all values are triplicates, and a twofold difference is very significant [55]). One series of genes (group 1, nine genes) (Fig. 1A) is activated by SeV C170S infection alone; these mRNA levels are unchanged in SeV Z, C10-15 and SeV Z, GP1 and -GP31-42 infections. The C170S substitution inactivates the ability of all four C proteins (C’, C, Y1, and Y2) to stably bind STAT1 and to inhibit IFN signaling (18, 59). According to this view, any of the four C proteins may function to prevent these mRNA levels from increasing during SeV infection (Fig. 1D) (14). The IL-6 gene is the sole representative of group 2; it is activated by SeV Z, C10-15 as well as SeV C170S, but not by the promoter mutants or the wt viruses (Fig. 1B). According to this view, a second function of the C gene, specific to the NH2-terminal 23 amino acids present only in the longer C proteins, is also required for SeV to prevent IL-6 activation. The third group, consisting of five genes, is activated by SeV Z, C10-15 and SeV Z, GP1 and -GP31-42 as well as SeV C170S but not by SeV Z, GP1 and -GP31-42 or SeV Z-wt infections (Fig. 1C). Apparently, a third function provided specifically by the first 30 nucleotides (nt) of the genomic promoter (or leader RNA) is also required to prevent activation of genes such as that of IL-8 (Fig. 1C). This third function is not the ability of mutant leader RNA to bind TIAR, a host RNA-binding protein important for virus-induced apoptosis, as this occurs with SeV Z, GP1 and -GP31-42 as well (28). In summary, comparative analysis of host gene activation with SeV with specific mutations has identified three groups of cellular genes that respond differently to SeV infection.

**Real-time RT-PCR estimations of mRNA levels.** IFN signaling through the JAK/STAT pathway activates many ISGs (such as 6-16, PKR, etc.) that collectively contribute to the cellular antiviral response. SeVs that interdict IFN signaling would therefore also prevent the activation of these ISGs. While many of the genes activated by the mutant SeVs are well known ISGs, IL-6 and IL-8 are known to be non-ISGs; treatment of 2TGH cells with 1,000 IU of IFN-α does not increase IL-6 or IL-8 mRNA levels over those of the untreated control (55). We therefore continued our study of selected host genes activated by SeV by real-time RT-PCR (TaqMan), a method that is more quantitative than DNA arrays. We first examined IL-8 (a chemokine) and IFN-β, an early host response protein whose gene was absent in the DNA array. 2C4 cells (a 2TGH-derived cell line) were infected with 20 PFU of the various SeVs per cell, and the levels of various host mRNAs were determined, including that of GADPH as an internal control (see Materials and Methods). All four SeVs (SeVM-wt, C170S, SeV Z-wt, and C10-15) grow relatively well in 2C4 cells, as indicated by their accumulated N mRNAs (Fig. 2 and 3) or N proteins (data not shown); however, the SeVM-wt infections accumulated ca. 2-fold less N mRNA than the three other SeV infections (Fig. 2C). The relative levels of virus replication are presumably important in stimulating the host response, and SeVM-wt infections might therefore be expected to activate IL-8 and IFN-β less strongly than the other SeVs for this reason alone. We found that IL-8 and IFN-β mRNA levels were elevated >20-fold in SeVM C170S versus SeVM M-wt infections. A strong difference was also found between SeV Z, C10-15 and SeV Z, C-wt infections, where N mRNAs had accumulated identically (Fig. 2). Moreover, IL-8 and IFN-β mRNA levels were also elevated in other SeV M C170S versus SeVM M, C-wt infections of 2C4 cells, where N mRNAs had accumulated identically (Fig. 3). Thus, specific mutations in two different regions of the SeV C proteins lead to increased activation of IL-8 and IFN-β. In all cases, IL-8 and IFN-β mRNA levels were more strongly increased by SeVM C170S than by SeV Z, C10-15.

We also examined the effects of various SeV infections of cells transfected with plasmids expressing IRF-3 (or RFP as a neutral control), and in some cases the cells were also treated with 50 μg of poly(I)-poly(C) (dsRNA) per ml. Elevated IRF-3 levels should enhance the antiviral response of the cells to SeV infection, and the combined treatment is thought to approximate a virus infection in itself and should potentiate the antiviral response. This appears to be so, as the levels of IL-6, IL-8,
FIG. 1. Comparison of host mRNA levels in 2fTGH cells infected with various SeVs. (A to C) Parallel cultures of 2fTGH cells were infected with 20 PFU of the various SeVs per cell. Total cytoplasmic RNA was prepared with Trizol at 24 hpi, and equal amounts (10 μg) were used as a template for oligo(dT)-primed [33P]cDNA synthesis. The [33P]cDNA was annealed to triplicate DNAs arrayed on nylon membranes, and the [33P]cDNA bound was quantitated in a PhosphorImager. The graphs show the fold increase in each mRNA relative to the mock control. (D) Schematic representation of the viral mutations and their effects on host gene activation. The C proteins are shown as two telescoping boxes representing the longer (C'6 and C) and shorter (Y1 and Y2) C proteins, whose activities during infection, and the mutations investigated, are indicated. The promoter mutation GP42 is thought to exert its effect via mutant leader (Le) RNA. The presumed requirement for the various wt genetic elements to prevent host gene activation is shown. The names of the mutant SeVs used are also indicated. Instab., instability; sig., signaling.
and IFN-β mRNAs induced by SeV infection in general increased with increasing proresponse treatment (i.e., RFP, IRF-3, and IRF-3 plus dsRNA) (Fig. 3). IRF-3 overexpression (Fig. 3A to C, IRF-3 versus RFP) enhanced IFN-β and IL-6 activation by SeV but did not enhance that of IL-8. The additional dsRNA treatment had little effect on further enhancing IFN-β and IL-6 mRNA levels but strongly enhanced IL-8 mRNA levels. These differences in the enhancement of IL-6 and -8 and IFN-β activation upon treatment with IRF-3 with or without dsRNA presumably reflect different activation pathways in response to SeV infection. It is possible that IL-8 activation requires both IRF-3 and dsRNA, whereas IRF-3 is sufficient for IL-6 and IFN-β activation.

SeV activation of IL-6, IL-8, and IFN-β is independent of IFN signaling. 2fTGH human fibrosarcoma cells were chosen for these experiments because sublines defective in specific components of the IFN signaling system have been generated from these cells by X irradiation (48). U5A cells, for example, are defective in the IFN-α/β receptor 2 chain, which is essential for IFN-α/β signaling, and these cells have been restored to IFN sensitivity by complementation with the IFNAR2 gene (U5A+IFNAR2 cells) (43). Even though IL-6 and -8 are not activated upon simple IFN treatment of uninfected cells, IFN secreted during SeV infection may act differently, as additional signaling pathways are being induced by the virus infection. Moreover, C<sup>F<sub>170S</sub></sup> (in contrast to C<sup>Δ10-15</sup>) does not prevent IFN signaling, and it is important to know whether this phenotype is responsible for the activation of IL-6 and -8. We therefore examined the various SeV infections of U5A as well U5A+IFNAR2 cells to determine whether activation of IL-6 and IFN-β by SeV-C<sup>F<sub>170S</sub></sup> and SeV-C<sup>Δ10-15</sup> required IFN signaling. We also examined the activation of the 6-16 gene, a known ISG, as a positive control (10). As shown in Fig. 4, both SeV-C<sup>F<sub>170S</sub></sup> and SeV-C<sup>Δ10-15</sup> activated IL-6, IL-8, and IFN-β in U5A cells relative to SeV-wt infection. The IFNAR2-complemented cell line yielded similar results, except that the activation of these genes was paradoxically reduced in U5A+IFNAR2 cells relative to U5A cells. In contrast to the case for IL-6, IL-8, and IFN-β, little or no activation of 6-16 occurred in SeV-C mutant-infected U5A cells, whereas a modest activation was evident in U5A+IFNAR2 cells. Moreover, 6-16 was the only mRNA whose levels in U5A+IFNAR2 cells exceeded those in U5A cells. The activation of IL-6, IL-8, and IFN-β during SeV infection, in contrast to that of 6-16, is thus largely independent of IFN signaling.

STAT1-defective U3A cells. The SeV C proteins interact with STAT1 in two ways (Fig. 1D). C and STAT1 form a stable complex in vitro and during SeV infection, and this complex is associated with a loss of IFN signaling. These events are blocked by the C<sup>F<sub>170S</sub></sup> mutation but not by C<sup>Δ10-15</sup>. The shorter Y proteins are also active in this respect. The longer C proteins alone also induce STAT1 instability, and in contrast to their effects on IFN signaling, this effect does not require F170 (Fig. 1D). To examine whether SeV-C mutant-induced activation of IL-6, IL-8, and IFN-β requires STAT1, U3A cells, which are known to be defective for STAT1, were examined (45). However, we were unable to examine the companion U3A+STAT1 cells, as these cells were found to have lost STAT1 expression. Moreover, attempts to recomplement U3A cells with STAT1 failed (data not shown).

The results of the U3A cell infections are shown in Fig. 5. As before, IL-6 and -8 and IFN-β mRNA levels were all clearly increased in SeV-C<sup>F<sub>170S</sub></sup> infections relative to SeV-wt infections. However, in contrast to the case for U5A and 2fTGH cells, SeV-C<sup>Δ10-15</sup> infections did not contain enhanced mRNA levels relative to SeV-wt infections. Given that STAT1 is probably not the only gene that has been lost in U3A cells that have been X irradiated (which randomly destroys chromosomal DNA), we can conclude only that enhanced mRNA levels due to SeV-C<sup>Δ10-15</sup> infection require STAT1 and/or...
another, unknown gene, whereas activation due to SeV-C<sup>F170S</sup> requires neither STAT1 nor any other gene destroyed in U3A cells.

**IL-8 gene activation and IL-8 secretion.** IL-8 is not known to act intracellularly. We therefore examined whether the SeV-induced IL-8 gene activation in U5A/U5A<sub>IFNAR2</sub> cells also led to increased IL-8 protein synthesis and secretion. When IL-8 protein levels in cytoplasmic extracts of the various SeV-infected cells were examined, they were found to roughly mirror the mRNA levels (Fig. 4E). However, when the culture supernatants were examined, a somewhat different result was found (Fig. 4F). Whereas the increased IL-8 mRNA level of SeV-CΔ10-15-infected U5A cells was accompanied by strongly increased IL-8 secretion, that of U5A+IFNAR2 cells led to only a modest increase in IL-8 secretion. Moreover, the in-
Increased IL-8 mRNA levels in either SeV-C$^{F170S}$-infected cell line did not lead to clearly increased IL-8 secretion.

IL-8 expression can be controlled at both the transcriptional and posttranscriptional levels. In polarized epithelial monolayers, *S. enterica* serovar Typhimurium-induced IL-8 secretion requires not only the activation of NF-$\kappa$B and production of IL-8 mRNA but also the activation of the small, Rho family GTPases Cdc42 and Rac1, which regulate endocytic protein traffic from the Golgi network to the basolateral surface of the cell. In the absence of Cdc42 or Rac1 function, IL-8 mRNA levels increase in response to bacterial infection but IL-8 is not secreted (3), similar to the case for our SeV-C$^{F170S}$-infected

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**FIG. 4.** SeV infection of cells defective for the IFN receptor and their complemented pseudo-wt derivatives. (A to D) Parallel cultures of U5A or U5A + IFNAR2 cells were infected (or not) in duplicate with 20 PFU of the various SeVs per cell for 24 h. The relative amounts of IFN-$\beta$, IL-6, IL-8, and 6-16 mRNAs present were determined as described for Fig. 2. (E and F) Equal samples of the culture supernatants (F) or cytoplasmic extracts of the cultures (E) were analyzed for IL-8 protein levels by enzyme-linked immunosorbent assay.
U5A cells. The requirement for Cdc42 and Rac1 activation, moreover, is cell type dependent (9). The C^{F170S} and C^{Δ10-15} mutations thus appear to affect IL-8 secretion differently.

SeV prevents IFN-β gene activation in several ways. The manner in which IFN-β transcription is induced by virus infection is well studied, and the activation of IRF-3 is central to this process. IRF-3 is expressed constitutively and is found in the cytoplasm in an inactive, unphosphorylated state. Upon virus infection or dsRNA treatment of cells, IRF-3 is phosphorylated by an unknown kinase and translocates to the nucleus.

where, together with other transcription factors such as NF-κB (which is itself also directly activated by virus infection or dsRNA), it activates IFN-β transcription (41, 65, 68). Phosphorylation of IRF-3 after viral infection is the first step in the activation of a gene program that includes a positive feedback loop of IFN-α/β and IRF family members (60). The results described above suggest that the SeV C gene encodes functions that prevent virus-induced IFN-β transcription (directly or indirectly). During the course of this work, it was reported that the SeV V protein, as well as the V proteins of the rubulaviruses SV5 and hPIV2, also prevented IFN-β transcription (26, 50, 64).

Given that SeV appears to use two viral genes (C and V) to neutralize IFN-β expression, we have examined a broader panel of mutant rSeV infections for their relative activation of the IFN-β promoter compared to that of dsRNA treatment. Besides SeV-C^{F170S} and C^{Δ10-15}, we examined two promoter mutants, SeV-AGP55, in which the first 55 nt of the antigenomic promoter is replaced with the equivalent leader sequences of the genomic promoter (38). SeV-AGP55 transcribes leader RNA from both promoters (and no trailer RNA). The converse SeV-GP48 has the first 48 nt of the genomic promoter replaced with the equivalent trailer sequences, and SeV-GP48 transcribes basically trailer RNA from both promoters (and no leader RNA; GP48 and GP1-42 are identical in this respect [data not shown]) (19, 20). Finally, we examined SeV-V^-W^+, which contains a stop codon at the beginning of the V open reading frame (ORF), such that edited V mRNAs are translated into W-like proteins, and specifically no V protein is expressed (8).

2fTGH cells were transfected with a reporter plasmid in which luciferase is controlled by the IFN-β promoter (pIFN-β-luciferase). To determine whether virus-induced IFN-β transcription required IRF-3 activation, the cells were cotransfected with either a dominant-negative mutant of IRF-3 (IRF-3N), an anti-IRF-3 ribozyme, or an empty control plasmid (see Materials and Methods). The transfected cultures were then infected (or not) with the various SeVs (or treated with dsRNA) 24 h later and were harvested at 20 h postinfection (hpi). As shown in Fig. 6, with the notable exception of SeV-AGP55, all of the mutant SeV infections induced the reporter more strongly than SeV-wt and as well as dsRNA treatment. In all cases, the coexpression of IRF-3ΔΔN or an anti-IRF-3 ribozyme prevented the SeV-induced expression of the reporter. Thus, mutations in two regions of the C protein that carry out different functions, as well as the loss of leader RNA expression or the expression of the V protein, all lead to IFN-β promoter activation in an IRF-3-dependent manner. Overexpression of the W protein cannot compensate for the lack of V protein, so the highly conserved Cys-rich carboxyl domain of V is specifically required. Only the loss of trailer RNA expression (SeV-AGP55) did not result in IFN-β activation.

Leader and trailer RNAs, the promoter-proximal products of viral RNA synthesis, are AU rich and are thought to bind to cellular RNA-binding proteins that bind AU-rich elements (28). In contrast to leader RNA, trailer RNA is expressed relatively late in infection (upon antigenome accumulation) and would not be expected to counteract immediate-early response genes. However, many of the other viral products that do not have a dedicated essential role in the replication ma-
the only one of the four mutant SeVs not to have lost virulence to SeV-wt, and consistent with this failure, SeV-GP31-42 was mutation failed to activate any of the cellular genes relative the mutant SeV examined by DNA arrays, only the leader(31-

DISCUSSION

We have used cDNA arrays to compare the activation of various cellular genes in response to infection with SeVs that contain mutations in their C proteins or leader regions. Three groups of cellular genes were identified in this way (Fig. 1). Of the mutant SeV examined by DNA arrays, only the leader(31-42) mutation failed to activate any of the cellular genes relative to SeV-wt, and consistent with this failure, SeV-GP31-42 was the only one of the four mutant SeVs not to have lost virulence in mice. The C^F170S mutation is associated with a ca. 5-log-unit increase in LD_50 (31), and this mutation appears to be the most important in cell culture infections as well as in mice (15). This mutation activates IL-6, IL-8, and IFN-β more strongly than CΔ10-15, and only this mutation activates all three groups of cellular genes that respond to SeV infection.

The IFN-β gene is both a primary response gene and an ISG, and it plays a central role in initiating the IFN-induced antiviral response. This is the first report that the SeV C proteins and leader RNA counteract the expression of this key primary response gene, and it confirms that the report of Poole et al. (50) that the product of our hemagglutinin-tagged V gene is active in this respect. As expected, activation of IFN-β required activation of IRF-3 (Fig. 6) and was independent of IFN signaling (Fig. 4), but the details of how this occurs remain to be elucidated. We have also provided evidence that the SeV C proteins and leader RNA counteract the expression of the chemokine IL-8. Infections by other viruses, e.g., respiratory syncytial virus (RSV) (44), dengue virus (3), hepatitis C virus (49), and human immunodeficiency virus type 1 (46, 52), are known to induce IL-8 secretion, as do infections by bacteria and parasites, including Mycobacterium tuberculosis (66). In a related vein, cytomegalovirus, a large DNA virus, encodes a chemokine receptor that may facilitate virus replication (13), and human herpesvirus 8/Kaposi sarcoma virus carries four ORFs whose products are related to chemokines (42). Viral modulation of chemokine expression presumably represents one aspect of the continuous battle between viral parasites and antiviral, inflammatory, and immune responses of the host. SeV infection has been reported to induce the CXC chemokine RANTES via the activation of IRF-3 and NF-κB (23, 40). IL-8 may not have been noticed in these earlier studies, because SeV-wt induces very little IL-8 (Fig. 2). Our results suggest that SeV targets the inflammatory and adaptive immune responses (IL-6 and IL-8) as well as the IFN-induced intracellular antiviral state (IFN-β and STAT1). As IFN-β and IL-8 transcription both depend on NF-κB activation, SeV may target this key transcription factor as well.

The leader(1-42), CΔΔ10-15, C^F170S, and V^W^+^+^ mutations appear to disrupt four distinct elements in the SeV program to counteract the cellular antiviral response. The facts that they all lead to enhanced levels of IFN-β mRNA and that at least three of them increase IL-8 mRNA levels suggest that SeV employs a multifaceted approach to inhibit viral clearance by inflammatory cells as well as to prevent the IFN-induced antiviral state, sometimes using the same viral macromolecules due to its limited coding capacity. The best-studied example of paramyxovirus-induced activation of IL-8 is that of RSV (21, 69). The IL-8 promoter in A549 cells can be induced by RSV infection in at least three distinct pathways: via tumor necrosis factor alpha (which requires only an intact NF-κB binding site), directly by intracellular RSV replication (which also requires other transcription factor binding sites) (5), and via the interaction of the viral F protein with Toll-like receptor 4 (in which IRF-3 plays an important role) (36). Measles virus H protein interaction with Toll-like receptor 2 also activates IL-8 (1). If all three parallel cellular pathways for IL-8 expression operate during SeV infection of 2TGH cells, several different SeV products will be required to effectively prevent IL-8 activation (Fig. 1D).

Rubulaviruses do not express C proteins, but their V proteins have recently been found to prevent IFN-β expression by preventing the activation of IRF-3 and NF-κB, as well as in-
ducing the degradation of STAT1 or STAT2 (26, 50). The Rubulavirus V proteins thus also counteract more than one arm of the innate antiviral response. The versatility of these viral gene products continues to surprise us. The SeV C proteins have been more intensively studied than SeV V protein or leader RNA. Like the influenza A virus NS1 protein (6, 63) and hepatitis C virus NS5A protein (49), the SeV C proteins are pleiotropic polypeptides that have multiple activities during infection, presumably due to their interaction with various viral and cellular proteins. Their multiple functions, deciphered in large part via C gene mutations, include (i) stimulation of viral RNA synthesis early in infection (SeV-C/C’ infections exhibit a 10-h delay in the accumulation of viral products) (37); (ii) inhibition of viral RNA synthesis in a promotor-specific manner late in infection, by interacting with the P4-L vRdRP (this selective inhibition may promote the switch from mRNA synthesis to genome replication and increase the fidelity of vRdRP promoter recognition) (4, 61); (iii) a role in virion assembly, possibly by interaction with the matrix (M) protein (SeV-4C’ particles are poorly infectious and amorphic) (25); (iv) interaction with STAT1 in two separate ways, to inhibit IFN signaling and to induce STAT1 instability (17); and (v) inhibition of the IRF-3-dependent activation of IFN-β and the activation of IL-8 expression in an IFN signaling-independent manner (this work).

How C interacts specifically with all of its viral and cellular partners remains an enigma and is reminiscent of acidic activation domains of transcription factors that interact with multiple partners. Acidic activation domains are “natively disordered” (11, 51), and this property apparently allows them to bind different surfaces with high specificity (multiple induced fits) and limited stability. The NH2-terminal portion of the measles virus P protein that contains the overlapping C protein ORF is, in fact, a recent example of such natively disordered proteins, in accordance with the prediction of algorithms that detect unstructured regions (47). By using the same algorithms (PONDR), the SeV C protein is strongly predicted to be natively disordered, and this property is shared with the common NH2-terminal portions of rubulavirus V, I, and P proteins (17, 26). The CA10-15 deletion, moreover, is in a region of C with the highest prediction of disorder. It will be of interest to examine whether purified SeV C proteins are indeed natively disordered.

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ADDITIONAL PROOF

Fujii et al. (Y. Fujii, T. Sakaguchi, K. Kiyotani, C. Huang, N. Fukushima, Y. Egi, and T. Yoshida, J. Virol. 76:8540–8547, 2002) have shown that mutations in the leader region specifically attenuate virus virulence in mice.

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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.

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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...
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 IFN-β promoter (Poole et al., 2002) as infection with SeV that cannot specifically express the V protein strongly activates IFN-β (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 IFN-β. 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 IFN-β 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, IFN-β activation.

The IFN-β 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 IFN-β 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 (CEF)s 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 IFN-β promoter.

Results

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

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 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
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
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 embryonated 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

![Graph showing reporter gene (GFP) levels](image)

**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 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.
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.

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 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, RIG-I, ISG15 and ISG56 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 ambimRNA 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 and the dsRNA potential of copyback DI genomes is always greater than that of the ND genomes of SeV stocks containing copyback DI genomes,ambi-mRNA promoter will largely eliminate this potential. 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 ambimRNAs.

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 (109 pfu/ml), 0.1 ml of a 105 dilution (ca. 1000 pfu) was inoculated per egg. In the case of DI stocks, 0.1 ml of a 102 to 103 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 pellet virus. Virus titers were determined by plaqueing 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 Hirotsuki University School of Medicine.

Plasmids, transient transfections, luciferase assay and FACS

pβ-IFN-fl-luc, 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)luc, referred to here as pISRE-fl-luc, contains four tandem repeats of the IFN-inducible gene 9–27 ISRE fused to the firefly luciferase gene (Didcock et al., 1999). pTK-rl-luc 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-luc or plISRE-fl-luc, 0.3 μg of pTK-rl-luc, 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-luc into pEGFP-N1 (BD Biosciences Clontech), between the Astel and HindIII sites. For transfections, 100,000 cells were plated in six-well plates 20 h before transfection with 1 μg of pIFNβ-GFP and CaPO4 (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/Cstop (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 specific 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 (Eurogentec), 20 pmol (each) of forward and reverse primers μl MasterMix (Gibco) in a total volume of 50 μl. Two microliters of each cDNA was then combined with 1 μl of Human GAPDH (Applied Biosystems), 11 cDNA was then combined with 1 μl of 5′-GCAATACCGGTTGACGACTACG-3′ (Fwd); 5′-TGCTGACGGCGATCCG-3′ (Rev); 5′-CGAGAAGATGACGATCCGACGACTACG-3′ (Probe). YFP gene: 5′-CCGACAACCACCTAACCCTAGCTA-3′ (Fwd); 5′-GAACCTACGCAAGGGACCTTG-3′ (Rev); 5′-AAAGACCCCAAACGAGCCG-3′ (Probe). Real-time PCR was carried out in the 7700 Sequence Detector (Applied Biosystems).

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**References**


Activation of the Beta Interferon Promoter by Unnatural Sendai Virus Infection Requires RIG-I and Is Inhibited by Viral C Proteins

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As infection with wild-type (wt) Sendai virus (SeV) normally activates beta interferon (IFN-β) very poorly, two unnatural SeV infections were used to study virus-induced IFN-β activation in mouse embryonic fibroblasts: (i) SeV-DI-H4, which is composed mostly of small, copyback defective interfering (DI) genomes and whose infection overproduces short 5’-triphosphorylated trailer RNAs (pppRNAs) and underproduces viral V and C proteins, and (ii) SeV-GFP(+/-), a coinfection that produces wt amounts of viral gene products but that also produces both green fluorescent protein (GFP) mRNA and its complement, which can form double-stranded RNA (dsRNA) with capped 5’ ends. We found that (i) virus-induced signaling to IFN-β depended predominantly on RIG-I (as opposed to mda-5) for both SeV infections, i.e., that RIG-I senses both pppRNAs and dsRNA without 5’-triphosphorylated ends, and (ii) it is the viral C protein (as opposed to V) that is primarily responsible for countering RIG-I-dependent signaling to IFN-β. Nondefective SeV that cannot specifically express C proteins not only cannot prevent the effects of transfected poly(I-C) or pppRNAs on IFN-β activation but also synergistically enhances these effects. SeV-Vminus infection, in contrast, behaves mostly like wt SeV and counteracts the effects of transfected poly(I-C) or pppRNAs.

All viruses evade the cellular innate immune system in part by expressing gene products that interfere with the ability of the host cell to establish an antiviral state (6). In the case of the Paramyxovirinae, this anti-host-defense activity is due mostly to viral C and V proteins (15, 27, 31). The C and V proteins are encoded by separate alternate open reading frames (ORFs), which both overlap that of the P protein. V and C are also referred to as accessory gene products, as not all members of this virus subfamily express one or the other. More specifically, rubulavirus and avulavirus express V but do not express C proteins, and human parainfluenza virus type 1 (PIV1), a respirovirus most closely related to Sendai virus (SeV), expresses C but does not express a V protein (16, 20).

Paramyxovirus V and C proteins antagonize interferon (IFN) signaling by various mechanisms, and they also target the production of type I IFN (15, 31). Beta IFN (IFN-β) production is one of the earliest events in the cellular innate immune response, which leads to the establishment of an antiviral state. IFN-β production requires the coordinated activation of several transcription factors, including NF-κB and IRF3 (15, 29). For intracellular RNA virus replication, the signaling pathway that leads to IRF3 activation starts with mda-5 and RIG-I, two cytoplasmic DExH/D-box helicases with N-terminal CARD domains. These helicases respond to double-stranded RNA (dsRNA) and, at least for RIG-I, to 5’-triphosphorylated single-stranded RNA (ssRNA) (pppRNA), which are generated in the cytoplasm during RNA virus replication (9, 11, 25). Upon the detection of these viral RNAs, the CARD domains of these helicases interact with IPS-1/Cardif/MAVS/VISA, which is present in the mitochondrial membrane, and this CARD-CARD interaction is thought to lead to the recruitment and activation of TBK1, IKKe, and other IKK kinases that activate NF-κB and IRF3, thereby activating the IFN-β promoter (8). The production of these early IFNs initiates autocrine and paracrine signal amplifications via the Jak/Stat pathway to produce a generalized antiviral state and also assists in the subsequent activation of adaptive immune responses.

The role of mda-5 in sensing RNA virus infection was uncovered because mda-5 was found to bind to the PIV5 V protein and other paramyxovirus V proteins, including SeV V. These V-protein–mda-5 interactions, moreover, prevented IFN-β activation in response to transfected poly(I-C) (1). On the other hand, other studies found that RIG-I and not mda-5 acts as the sensor of paramyxovirus infection (13, 28). This paper provides evidence that for SeV infection of mouse embryonic fibroblasts (MEFs), it is the C protein (and not V) that is primarily responsible for this effect and that C acts by countering RIG-I (and not mda-5). Independent expression of C was found to inhibit RIG-I-dependent signaling to the IFN-β promoter induced by either pppRNAs or dsRNAs. Moreover, SeV that cannot specifically express C proteins was unable to counteract poly(I-C)- or pppRNA-induced IFN-β activation, whereas SeV that cannot express V behaved mostly like wild-type (wt) SeV.

MATERIALS AND METHODS

Cells, viruses, and antibodies. MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. SeV stocks were grown in the allantoic cavities of 9-day-old embryonated chicken eggs for 3 days at 33°C. For nondefective stocks (105 PFU/ml), 0.1 ml of

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A

![Graph A]

B

![Graph B]

C

![Graph C]
a 10^3 dilution (ca. 1,000 PFU) was inoculated per well. In the case of DI stocks, 0.1 ml of a 10^3 dilution was used. In all cases, the amount of viral protein present in the resulting allantoic fluid was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining of pelleted virus. Virus titers were determined by plaquing on LLC-MK2 cells.

SeV-GFP (+), which expresses green fluorescent protein (GFP) from a transgene between the M and F genes, and SeV-GFP(−) or SeV-RFP, which expresses antisense GFP mRNA or red fluorescent protein (RFP) (dsRRED) from similarly located transgenes, were prepared as previously described (31). DI-H4 stocks were described previously (30).

Primary antibodies used included rabbit anti-RFP (AB2162; Chemicon); anti-actin monoclonal antibody (MAB) (1501; Chemicon); rabbit anti-GFP (632460; BD biosciences); rabbit anti-SeV-PC/C (homemade); anti-hemagglutinin (HA) MAb (16B12; BABCO), anti-Flag MAb (F1804; Sigma), rabbit anti-mda-5 (J. Tschopp, Lausanne, Switzerland), and rabbit anti-RIG-I (T. Fujita, Kyoto, Japan).

Plasmids, transient transfections, infections, inductions, luciferase assay, and fluorescence-activated cell sorter analysis. EBS plasmids (3) expressed viral and fluorescent proteins and were constructed by standard methods; precise detail can be obtained from the authors. NS1 [residues 1 to 77 (NS1(1-77))] (from Jacques Perrault) and ELS (residues 100 to 190 (EL3[100-190]) (from Bertram Jacobs), were HA tagged and cloned into pEBS. Tagged RIG-I, RIG-I-C, or RIG-A-CARDS (dominant negative) and mouse mda-5 were obtained from Jurg Tschopp and Klaus Conzelmann.

pIFN-β-luc, which contains the firefly luciferase gene under the control of the human IFN-β promoter, was described previously (14). pTK-rl-luc, used as a transfection standard, contains the herpes simplex virus TK promoter region upstream of the Remilla luciferase gene (Promega).

For transfections, 100,000 cells were plated into six-well plates 20 h before transfection with 1.5 μg of pIFN-β-luc; 0.5 μg of pTK-r-luc; 0.5 μg of plasmids expressing RIG-I and MDA-5; 1.5 μg of plasmids expressing V (whose C ORF is cleaved with a stop codon), pC-β20 or pC-25, pTom-Cβ20 or (C'), NS1(1-77), or eIF-4E, mutant EL3(100-190), or R4-α proteins (as indicated); and TransIT-LT1 transfection reagent (Mirus). At 24 h posttransfection, the cells were either (or were not) infected with various SeV stocks or transfected with 5 μg of poly (I:C) using TransIT-LT1 transfection reagent. 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.

Immunoblotting. Cytoplasmic extracts were prepared using 0.5% NP-40 buffer. Equal amounts of total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes by semidy transfer. The secondary antibodies used were alkaline phosphatase-conjugated goat anti-rabbit (or mouse) immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Pierce) and analyzed in a Bio-Rad light detector using Quantity One software.

In vitro synthesis of RNA, purification, and transfection. DNA for T7 RNA polymerase-cleaved plasmids was linearized by PC using the following partially complementary primers: 5' CTAAGACTAGTCACTATA (ggg/gca) ACA CACCAACAAACCAACCACACAC3' (forward) and 5' GAAAGAAGGGTGTGGGTTGTTGGTGGTGGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTG3' (reverse). In vitro transcription was performed on 100 pmol of purified PCR product using T7 MiniA shuttlecl from Ambion according to the manufacturer's instructions. For RNA containing the usual eIF-4G start site, RNA was initiated with the dinucleotide 5' GAGC capCAG in a reaction without GTP. For RNA containing the usual 5' capGpG capCAG in a reaction without GTP. For RNA containing the usual 5' capGpG capCAG in a reaction without GTP.

RESULTS

Three ways to activate IFN-β. We have used three ways to induce the activation of an IFN-β promoter expressing a luciferase reporter gene in MEFs (see Fig. 2A). The first is to simply transfect a synthetic dsRNA, poly(I):poly(C) [poly(I-C)], into the cells. The second way is to infect the cells with an SeV stock that contains a well-characterized copyback DI genome (H4) (30). The third way is to coinfect cells with SeV-GFP (+), which expresses a GFP transgene, and SeV-GFP(−), which expresses mRNA containing the complement of the GFP ORF, as recently described for vesicular stomatitis virus (VSV) (24). As shown in Fig. 1A, infection with increasing amounts of SeV-GFP(+) alone leads to increasing GFP expression. Coinfection of 20 PFU/cell of SeV-GFP(+) with increasing amounts of SeV-GFP(−) leads to the gradual decrease of GFP expression (top). At 20 PFU/cell of SeV-GFP(−), there are roughly equal amounts of GFP and anti-GFP mRNAs intracellularly (by strand-specific quantitative RT-PCR) (Fig. 1B and see Materials and Methods), and there is a 90% loss of GFP expression (Fig. 1A, top). This loss of GFP expression cannot be accounted for by the reduced level of GFP mRNA (Fig. 1B). In contrast, coinfection with increasing amounts of SeV expressing RFP as a neutral control (SeV-RFP) has a reduced ability to interfere with GFP expression (Fig. 1A). More importantly, whereas infection with SeV-GFP(+) alone or its coinfection with SeV-RFP leads to little

FIG. 1. IFN-β activation induced by SeV-GFP(+/−) infections. (A) Parallel cultures of MEFs were first transfected with pIFNβ-luc and pTK-luc and then infected with increasing amounts of either SeV-GFP(+) (alone which expresses a GFP mRNA from a transgene between the M and F genes) or 20 PFU/cell of SeV-GFP(+) plus increasing amounts of either SeV-GFP(−) (which expresses an anti-GFP mRNA from a transgene in the same location) or SeV-RFP (which expresses an RFP mRNA from a transgene in the same location) and SeV-RFP (which expresses an RFP mRNA from a transgene in the same location), as indicated. GFP expression was monitored by fluorescence-activated cell sorter analysis at 20 hpi. Cell extracts were prepared at 20 hpi, and equal amounts were used to determine luciferase activities (below). These transfections were carried out three times with independent virus stocks, with similar results. (B) Cytoplasmic extracts were centrifuged on CsCl density gradients to isolate nonencapsidated (pellet) RNAs. The levels of GFP and anti-GFP mRNAs in 15 μg of CsCl pellet RNA were determined using sense- and antisense-specific primers for RT, followed by quantitative PCR (TaqMan) (see Materials and Methods). (C) Parallel cultures of MEFs were first transfected with the luciferase reporter plasmids plus either an empty vector, one expressing wild-type EL3(100-190), or one expressing mutant EL3(100-190) (EL3-mut.) and then infected with increasing amounts of SeV-GFP(+) and SeV-GFP(−) as indicated. Cell extracts were prepared at 20 hpi, and equal amounts were used to determine luciferase activities. Equal amounts of cell extracts were also Western blotted using anti-N and anti-HA (below).
or no activation of IFN-β (Fig. 1A), coinfection with SeV-GFP(−) clearly activates the IFN-β promoter (Fig. 1A, bottom). This IFN-β activation is inhibited by the coexpression of the dsRNA-binding domain of the vaccinia virus E3L protein, whereas this activation is unaffected by a mutant form of E3L containing two point mutations that eliminate the binding of dsRNA (10) (Fig. 1C). Taken together, our results show that only SeV coinfections that can form GFP dsRNA induce IFN-β activation.

The two SeV infections that activate IFN-β differ from each other in several respects. First, DI-H4 genomes are of the copyback variety and contain the strong antigenomic replica-
the SeV V and C proteins to inhibit IFN-β activation induced by SeV infections. As shown in Fig. 3A, exogenous expression of the SeV V protein did not affect the level of viral P, V, and C proteins in SeV infections, but it did reduce IFN-β activation due to DI-H4 infection (by ~60%). Remarkably, SeV V overexpression did not inhibit IFN-β activation due to SeV-GFP(+/-) infection. The coexpression of exogenous SeV C protein (actually C1–23-Tom-C24–204, which migrates just slightly slower than the viral P protein) similarly did not affect the level of viral P, V, and C proteins in SeV infections. C overexpression, however, more strongly inhibited IFN-β activation due to either SeV infection [DI-H4-induced activation was reduced by ~90%, and GFP(+/-)-induced activation was reduced by ~75%]. Coexpression of the unmodified C protein produced similar results (Fig. 2B and data not shown). The ability of the GFP(+/-) infection to activate IFN-β, despite
normal levels of expression of the V and C proteins, is presumably due to the early formation of GFP dsRNA. In this case, the SeV V protein is considerably less potent than C in preventing the activation of IFN-β.

As the DI-H4 infections accumulate so few V and C proteins, we compared the abilities of these proteins (expressed from plasmids) to inhibit IFN-β activation relative to RIG-I(ΔCARDs) and the dsRNA binding domain of the influenza A virus (IAV) NS1(1–73), another viral protein that inhibits RIG-I signaling (22, 25). As shown in Fig. 3B, the SeV C protein was as active as RIG-I(ΔCARDs) in combating an increasing dose of DI-H4 infection and almost as active as NS1. Consistent with above-described results (Fig. 3A), the SeV V protein was less active than C but was still able to inhibit most of the DI-H4-induced IFN-β activation. The SeV C1–204 protein is composed of two domains: the N-terminal 23 amino acids (C1–23) which act as a plasma membrane (PM) targeting signal (19) and which is present in the longer (C/C) but not in the shorter (Y1/Y2) “C” proteins, and C24–204, or the Y1 protein, which acts as a protein interaction domain. Whereas C24–204 (or Y1) is naturally expressed during infection, C1–23 is only found fused to Y1. In order to study the different contributions of these two domains to C-protein function, we have used tomato red fluorescent protein (Tom) in which C1–23 is fused to the N terminus of Tom and C24–204 is fused to its carboxy terminus as a carrier. The interposition of Tom between these two domains of C remarkably does not appear to affect any of the activities of C1–23 (19). MEFs were transfected with the luciferase reporter plasmids along with plasmids expressing various Tom constructs as indicated (Fig. 4). After 24 h, half of the cultures were infected with SeV-DI-H4 (as indicated). Cytoplasmic extracts were prepared after a further 24 h of incubation and used to determine luciferase levels. The relative levels of the various tomato constructs were determined by Western blotting with anti-dsRED (bottom). All transfections were carried out in duplicate, and the range of values obtained is indicated by error bars.

To examine whether trailer RNAs act as pathogen-associated molecular patterns (PAMPs), we transfected trailer RNA made by T7 RNA polymerase in vitro into our MEFs and monitored the activation of IFN-β. As the ability of pppRNAs to induce IFN-β activation is not sequence dependent (11), we also examined model RNAs that were initiated with GTP but then treated with phosphatase or those initiated with the dinucleotide GpC rather than pppG (23). The transfections of all
FIG. 5. pppRNA-induced activation of IFN-β. (A) Parallel cultures of MEFs were transfected with pIFNβ-lucΔ and pTK-lucΔ, and pRIG-I was also transfected in some cultures, as indicated. After 24 h, the cells were transfected for 3 h with increasing amounts (1 or 3 μg) of either pppGGG/RNA1, phosphatase-treated GGG/RNA1, pppGCA/RNA1, or ohGCA/RNA1, as indicated. Cytoplasmic extracts were prepared 18 h post-RNA transfection and used to determine luciferase levels. (B) Parallel cultures of MEFs were transfected with pIFNβ-lucΔ, pTK-lucΔ, and plasmids expressing Tom, RIG-I(ΔCARDs), IAV NS1(1–73), C1–23-Tom-C24–204 (C*), or V, as indicated. After 24 h, the cells were transfected with increasing amounts (1 μg and 3 μg) of pppGGG/RNA1, as indicated. Cytoplasmic extracts were prepared after 3 h of RNA transfection and used to determine luciferase levels. Rel., relative. (C) Same as above (B), except that the cells were transfected with 3 μg of ppptrailer RNA.
three 5'-triphosphorylated ssRNAs clearly led to IFN-β activation, whereas both RNAs that contained 5'-hydroxyl ends had essentially lost their ability to activate IFN-β in parallel transfections (Fig. 5A and C), confirming previously reported results (11, 25, 26). We then examined the ability of the SeV C and V proteins to inhibit 5′-pppRNA-dependent activation of IFN-β compared to RIG-I(ΔCARDs) and IAV NS1(1–73). MEFs were first transfected with plasmids expressing various viral inhibitory proteins or an empty plasmid as a negative control and then transfected with increasing amounts of pppRNAs. IFN-β activation was monitored after a further 18 h. As shown in Fig. 5B, expression of the SeV C protein was as active as RIG-I(ΔCARDs) in inhibiting IFN-β activation at all amounts of pppRNAs transfected although not quite as active as NS1(1–73). Expression of SeV V was again the least inhibitory; in fact, significant inhibition occurred only at the lowest amount of pppRNA. Thus, short 5′-triphosphorylated ssRNAs such as trailer RNA are potent stimulators of IFN-β when transfected into our MEFs, and expression of the SeV C protein (but not the SeV V protein) can effectively inhibit this stimulation (Fig. 5B and C).

Relative importance of C and V in inhibiting RIG-I-dependent signaling to IFN-β. Another way to investigate the relative importance of the C and V proteins in inhibiting RIG-I-dependent signaling to IFN-β is to compare the relative abilities of SeV infections that cannot specifically express the C or V proteins to affect pppRNA- or poly(I-C)-induced IFN-β activation. MEFs were therefore first infected with 20 PFU/ml of either wt SeV, SeV-Vminus (containing a stop codon in the V ORF just downstream of the mRNA editing site, which produces a W-like protein instead of V), or SeV-Cminus (containing three stop codons in the C ORF downstream of the Y2 initiation codon). The infected cells were then transfected (at 24 hpi) with pIFN-β-luc plus either pppRNA, poly(I-C), or no RNA and then harvested after 18 h to determine reporter gene activity. As shown in Fig. 6, these three SeVs replicate to clearly different levels in our highly IFN-competent MEFs (even though they replicate similarly in BSR T7, 293T, and Vero cells), highlighting the essential functions that these accessory proteins play in countering the innate immune response. Nevertheless, in the absence of transfected RNA, only SeV-Cminus infection activates IFN-β to any extent or increases RIG-I levels; RIG-I is an IFN-stimulated gene, and its level reflects that of the antiviral state (Fig. 6, bottom). Transfections of either pppRNA or poly(I-C) strongly activated the reporter gene and increased RIG-I levels. Prior infection with either SeV wt or SeV-Vminus reduced transfected RNA stimulation of the reporter gene and prevented the increase in RIG-I levels (Fig. 6). SeV-Vminus was only slightly less effective than wt SeV in this respect. In sharp contrast, prior infection with SeV-Cminus not only did not prevent the increase in RIG-I levels but also acted synergistically with either pppRNA or poly(I-C) transfection to increase reporter gene activity by increasing the level of RIG-I. These results reinforce the view...
that it is primarily the SeV C protein (and not V) that inhibits pppRNA- and dsRNA-induced signaling to the IFN-β promoter via RIG-I during SeV infection.

**DISCUSSION**

SeV has been one of the most extensively used model viruses to investigate IFN induction in infected cells. Most of this work has used the commercially available Cantell strain of SeV, whose ability to induce IFN, like that of other SeV strains, is due to the presence of DI genomes in egg-grown stocks. Nondefective SeVs that are plaque purified from these stocks, including that of the Cantell strain, do not induce IFN unless cellular RIG-I levels are artificially increased (21, 30). For nondefective SeV infection, the expression of the C and V proteins is apparently sufficient to prevent IFN-β activation under normal conditions. Measles virus infection, in contrast, can apparently induce IFN in the absence of DI genomes, and evidence that this induction is due to the synthesis of leader pppRNAs has recently been provided (26). Leader and trailer RNAs, which are unmodified at either end, are unstable in infected cells unless they are encapsidated with the N protein (presumably after their synthesis as free RNAs) (2). Free leader and trailer RNAs are more easily detected in VSV infections, which synthesize larger amounts of viral RNAs over a shorter period of time. For nondefective VSV infections, eight times as many trailer RNAs/antigenome template are found as leader RNAs/genome template, consistent with the relative strengths of their respective replication promoters. For VSV copyback DI infections, there are 40 times as many trailer RNAs/ genome template (nondefective antigenome plus DI genome) as leader RNAs/genome template, presumably reflecting the increased strength of the copyback DI replication promoters. The VSV polymerase clearly has a strong preference for initiating RNA synthesis at the 3′ ends of copyback DI genomes over both ND genomes and antigenomes (17).

We previously noted that not all SeV stocks that contain DI genomes strongly induce IFN; this ability appears to be restricted to stocks containing relatively small copyback DI genomes (the smaller the DI genomes, the more moles of ends are present for a given weight). The commercially available Cantell strain contains a copyback DI genome of only 546 nucleotides in length, the smallest SeV DI genome described to date (30). SeV copyback DI-H4 genomes (1,410 nucleotides) have the same strong replicative advantage as their VSV counterparts because they also contain strong replication promoters at both their genome and antigenome 3′ ends. Thus, SeV copyback DI infections presumably synthesize considerably more pppRNAs than standard virus infections. We also previously noted that when cytoplasmic extracts of DI-H4-infected cells are centrifuged on CsCl density gradients, small amounts of DI genome RNA are found in the pellet fraction (30). This indicates that this RNA is not encapsidated with the N protein and therefore forms dsRNA panhandles in a concentration-independent manner. Thus, copyback DI-H4 infections apparently produce considerably more of both known PAMPS of RNA virus infection than do standard virus infections. Coupled with their strongly reduced accumulation of the viral C and V proteins, it is easy to see why these copyback DI infections are such potent inducers of IFN-β.

All paramyxoviruses express either C or V proteins, and many viruses express both. In viruses that express only C or V, we presume that either viral protein alone counteracts the innate immune response of the host to aid virus replication. The C and V proteins, which bear no sequence similarity, likely target different key elements of the host response. Viruses that express both C and V presumably have more diverse ways of countering innate immunity. In support of this notion, SeV infections that cannot specifically express either the C or V protein contain increased levels of IFN-β and interleukin-8 mRNAs relative to wt SeV infections (31), and the independent expression of the C or V protein will inhibit poly(I-C)- or Newcastle disease virus-dependent activation of IRF-3 (15). Previous work has identified mda-5 as a key target of paramyxovirus V proteins in countering the innate immune response (1). This paper provides evidence that for SeV infections of MEFs, it is the C protein (and not V) that is primarily responsible for this effect and that C acts by countering RIG-I-dependent signaling to IFN-β. For example, the independent expression of either C or V inhibited IFN-β activation due to RIG-I overexpression (Fig. 2B). Also, both proteins inhibited IFN-β activation due to DI-H4 infection, although C was always more effective here than V (Fig. 3). However, only C expression effectively inhibited IFN-β activation due to GFP(+)− infection (Fig. 3) or transfected poly(I-C) or pppRNAs (Fig. 5). Perhaps the strongest indication that the C proteins are primarily responsible for countering the innate immune response comes from experiments with SeV that cannot specifically express the C or V proteins. SeV-C−/C− infection not only cannot prevent the effects of transfected poly(I-C) or pppRNAs on IFN-β activation but also synergistically enhances these effects. SeV-C−/C− infection, in contrast, behaves mostly like wt SeV infection and counteracts the effects of transfected poly(I-C) or pppRNAs (Fig. 6). Finally, we note that poly(I-C) (made with polyriboadenylic phosphorylase that generates 5′-diphosphate ends and which is transfected into cells) and the presumed GFP-dsRNA (that is directly generated in the cytoplasm via the viral transcriptase and which contains capped 5′ ends) both activate IFN-β via RIG-I in MEFs. Thus, the ability of dsRNA to induce RIG-I signaling does not depend on the manner in which it is introduced into this cell compartment, nor is it peculiar to the presence of 5′-diphosphorylated ends that are not normally found in cells and could theoretically act as PAMPs. Moreover, in either case, the activation of IFN-β by these dsRNAs is inhibited by the SeV C protein and not V, presumably because this signaling passes through RIG-I and not mda-5. It appears that our MEFs contain insufficient amounts of mda-5 to sense SeV infection, as these MEFs respond well to the expression of plasmid-derived mda-5 (Fig. 2B). This conclusion is also consistent with our finding that three different rubulavirus V proteins that are known to counteract poly(I-C)-induced mda-5 signaling were unable to inhibit IFN-β activation in response to SeV-DI-H4 infection (data not shown). We expect that the SeV V protein will be more important in countering the innate immune response in other cells in which mda-5 functions as a PAMP recognition receptor.
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