Murine Coronavirus Delays Expression of a Subset of Interferon-Stimulated Genes[▽]

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The importance of the type I interferon (IFN-I) system in limiting coronavirus replication and dissemination has been unequivocally demonstrated by rapid lethality following infection of mice lacking the alpha/beta IFN (IFN- α/β) receptor with mouse hepatitis virus (MHV), a murine coronavirus. Interestingly, MHV has a cell-type-dependent ability to resist the antiviral effects of IFN- α/β . In primary bone-marrow-derived macrophages and mouse embryonic fibroblasts, MHV replication was significantly reduced by the IFN- α/β -induced antiviral state, whereas IFN treatment of cell lines (L2 and 293T) has only minor effects on replication (K. M. Rose and S. R. Weiss, *Viruses* 1:689–712, 2009). Replication of other RNA viruses, including Theiler's murine encephalitis virus (TMEV), vesicular stomatitis virus (VSV), Sindbis virus, Newcastle disease virus (NDV), and Sendai virus (SeV), was significantly inhibited in L2 cells treated with IFN- α/β , and MHV had the ability to rescue only SeV replication. We present evidence that MHV infection can delay interferon-stimulated gene (ISG) induction mediated by both SeV and IFN- β but only when MHV infection precedes SeV or IFN- β exposure. Curiously, we observed no block in the well-defined IFN- β signaling pathway that leads to STAT1-STAT2 phosphorylation and translocation to the nucleus in cultures infected with MHV. This observation suggests that MHV must inhibit an alternative IFN-induced pathway that is essential for early induction of ISGs. The ability of MHV to delay SeV-mediated ISG production may partially involve limiting the ability of IFN regulatory factor 3 (IRF-3) to function as a transcription factor. Transcription from an IRF-3-responsive promoter was partially inhibited by MHV; however, IRF-3 was transported to the nucleus and bound DNA in MHV-infected cells superinfected with SeV.

Mouse hepatitis virus (MHV), a prototype group II coronavirus, has been widely used to study coronavirus replication and dissemination and virus-induced disease pathogenesis. In a strain- and host-specific manner, MHV infection induces both acute and chronic disease in the mouse central nervous system (CNS) and various degrees of hepatitis. A reverse-genetics system for MHV has provided a platform for identifying virulence factors involved in developing encephalitis and demyelinating diseases such as multiple sclerosis and hepatitis. Human coronaviruses (HCoV) are associated with 5 to 30% of moderate (HCoV-229E and HCoV-OC43) respiratory illness and with respiratory diseases of greater seriousness, such as the 2002 outbreak of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) that resulted in approximately 750 deaths (37, 38, 68). The identification of additional HCoV strains (HCoV-NL63 and HCoV-HKU1) associated with respiratory diseases has added to the need to further our understanding of coronavirus pathogenesis.

Induction of the type I interferon (IFN) response, consisting primarily of the cytokines alpha/beta IFN (IFN- α/β), encom-

passes an essential component of the innate response to pathogen infection. Single- and double-stranded RNAs (ssRNAs and dsRNAs) produced during viral replication represent pathogen-associated molecular patterns (PAMPs) that are detected in the infected cell by pattern recognition receptors (PRR), Toll-like receptors (TLR) located on the surface of the cell (TLR-2 and TLR-4) and of endosomal membranes (TLR-3, TLR-7, and TLR-8), and cytoplasmic receptors in the family of RIG-I like helicases (RIG-I and MDA5) (5). Sensing of RNA by PRR leads to activation and nuclear localization of transcription factors (TFs) (including IFN regulatory factor 3 [IRF-3] and IRF-7) to induce IFN- α/β transcription and protein production. In addition, virus infection directly promotes expression of many other genes with antiviral, antiproliferative, or proinflammatory properties. The IFN-stimulated response elements (ISREs) found in the enhancer and promoter regions of these IFN-stimulated genes (ISGs) are bound by a complement of transcription factors, including IRF-3 or IRF-7, resulting in an increase in their expression (65). In addition, IFN- β secreted from infected cells binds to the IFN- α/β receptor (IFNAR) on the cell surface, resulting in activation of Jak and Tyk kinases that phosphorylate STAT1 and STAT2, which in turn form an oligomeric complex with IRF-9 called the ISGF3 complex (reviewed in reference 39). ISGF3 translocates to the nucleus and binds the ISRE to induce hundreds of ISGs, which work together to limit or prevent establishment of virus infection.

The importance of the type I IFN response in control of MHV replication *in vivo* is substantiated by observations in

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studies of IFNAR knockout mice (IFNAR^{-/-}). Regardless of the route of inoculation, MHV infection led to increased mortality of mice and release of tropism barriers, as high titers of virus were found in many other organs in addition to the usual target organs (brain and liver) when IFN- α/β signaling was eliminated (25, 45). Furthermore, intravenous administration or exogenous expression of IFN- α or - β in the liver prior to MHV challenge limits viral replication and hepatitis and prolongs survival of animals (3, 21, 28, 33, 52). Using the intranasal (IN) route of inoculation to compare MHV infection of wild-type mice and mice with cell-type-specific abrogation of IFNAR, Cervantes-Barragán et al. demonstrated that loss of IFNAR signaling in LysM⁺ macrophages, CD11c⁺ dendritic cells (DCs), CD19⁺ B cells, or CD4⁺ T cells had no significant effect on replication in the brain despite the limitation of replication in peripheral organs (8). Since IFNAR signaling is essential for control of the spread of MHV following intracranial (IC) inoculation (25, 45), these data imply that IFN signaling in parenchymal cells may have a greater impact on the spread of virus within the brain, depending on the route of inoculation and the initial cell types infected (14).

Significant amounts of IFN- β are produced in the brain and liver of MHV-infected mice (25, 42, 43, 45), although primary cultures of neurons, astrocytes, and hepatocytes that are productively infected by MHV express undetectable levels of IFN- β mRNA (45). This is in contrast to infection of these primary cells with other RNA viruses, which leads to significant IFN production. Thus far, plasmacytoid dendritic cells (pDCs), macrophages, and microglia appear to be the major contributors to the IFN- α and - β production observed in response to MHV infection *in vivo* (9, 45). In similarity to brain and liver parenchymal cells, MHV induces only low levels of IFN- β mRNA at late times postinfection and no IFN- β protein in cultured cells (46, 60, 69). Interestingly, other IFN agonists induce IFN- β in MHV-infected cell cultures, which suggests that MHV does not dominantly block IFN production (46, 60, 69). Taken together, these observations led to the suggestion that MHV evades detection by sequestering MHV dsRNA in a location that is inaccessible to PRR (60). In 293T cells, however, transfection of RNA from MHV-infected cells does not induce IFN- β whereas RNA from SeV- or rabies virus-infected cells induces significant amounts of IFN- β (44). Thus, 293T cells are unable to specifically detect MHV RNA, even when viral RNA is introduced directly into the cytoplasm. Further research would be necessary to determine the cell-type-dependent variables that enable only a small subset of cells to generate a type I IFN response to MHV infection.

Also unique to MHV infection is the cell-type-dependent ability to resist the antiviral effects of IFN- α/β -induced gene expression. MHV replication is restricted in IFN- β -treated mouse embryonic fibroblasts (MEF) and bone-marrow-derived macrophages (44, 70). In contrast, MHV replication in mouse L2 and 17Cl-1 and human 293T cultures is minimally inhibited by the antiviral state induced by prior treatment of the cells with IFN- α or - β (44, 46, 64). Since replication of many other RNA viruses, including Sendai virus (SeV), Newcastle disease virus (NDV), Theiler's murine encephalitis virus (TMEV), Sindbis virus, and vesicular stomatitis virus (VSV), is almost completely inhibited at a lesser or equal dose of IFN- β (data

not shown), L2 and 293T cells appear to have an intact IFN response.

These observations led us to investigate the mechanism by which MHV was able to resist IFN antiviral activities in L2 and 293T cells. We observed that MHV can rescue SeV from the antiviral effects of IFN- α/β only when MHV infection has been established prior to IFN- α/β treatment and subsequent SeV infection of cultures. Furthermore, MHV is able to inhibit synthesis of a subset of ISGs induced in both an IFN-dependent and SeV-mediated IFN-independent fashion. We suggest that the ability of MHV to block ISG expression allows SeV to replicate in cultures treated with IFN- β .

MATERIALS AND METHODS

Cells lines, plasmids, and viruses. Murine L2, human 293T, and African green monkey Vero E6 fibroblast cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM HEPES, and 1% penicillin-streptomycin. The pRL-SV40 and pRL-TK plasmids express renilla luciferase from SV40 and thymidine kinase (TK) constitutive promoters, respectively (Promega, Madison, WI). Several reporter constructs used to express firefly luciferase under the control of different promoters were as follows: pElu-ISRE with the interferon-stimulated response element (63), pLuc-(PRDII)₂ containing two copies of an NF- κ B-responsive element, pLuc-(PRDIII-I)₃ with three copies of an IRF-3-responsive element (18), and p55C1B-luc with a unique IRF-3-responsive element (32) (all have been previously described). The MHV receptor (MHVR) CEACAM-1 was expressed using the pCI-neo1a[1-4] plasmid, kindly provided by Kathryn Holmes (University of Colorado Health Sciences Center, Aurora, CO). Wild-type recombinant MHV strain A59 (rA59) and rA59 containing the spike from MHV-2 and expressing enhanced green fluorescent protein (EGFP) (rA59/S_{MHV-2}-EGFP) have been previously described (12) and were grown in 17Cl-1 cells. In the text and figure legends, rA59 is referred to as MHV. Sendai virus (SeV) strain Cantell (4) was grown in 10-day-old embryonated eggs. The Daniels (DA) strain of Theiler's murine encephalitis virus (TMEV) (a kind gift from Julian Leibowitz), vesicular stomatitis virus expressing GFP (VSV-GFP) (56), and recombinant Newcastle disease virus expressing monomeric red fluorescent protein (rNDV-mRFP) (35) were used for infection at a multiplicity of infection (MOI) of 1. Sindbis virus expressing GFP from a subgenomic promoter was used for infection at 50 PFU/cell and was a kind gift from Sara Cherry (University of Pennsylvania, Philadelphia, PA) (20).

Luciferase assays. 293T cells were seeded in 12-well plates and transfected using 0.4 μ g of pCI-neo1a[1-4], 60 ng of pRL-SV40, and 0.6 μ g of pElu-ISRE, pLuc-(PRDII)₂, pLuc-(PRDIII-I)₃, or p55C1B and Eugene 6 transfection reagent (Roche, Mannheim, Germany). At 24 h posttransfection, cells were mock infected with lysate from 17Cl-1 cells or infected with MHV at an MOI of 1. Following 3 h of infection with MHV, cells were treated with 1,000 U/ml recombinant human IFN- β (Calbiochem, La Jolla, CA) or 1,000 U/ml universal human IFN- α/β (PBL Interferon Source, Piscataway, NJ), superinfected with SeV at an MOI of 1, or left untreated. At 8 or 15 h after treatment or after infection, cells were analyzed for luciferase expression by the use of a dual luciferase reporter assay (Promega) according to the manufacturer's instructions.

qRT-PCR. 293T cells were seeded in 12-well plates and transfected with 0.4 μ g pCI-neo1a[1-4] and with 1 μ g of either pCAGGS parental vector or pCAGGS expressing the V protein from simian virus 5 (SV5) [pCAGGS-(V)SV5] (51). At 24 h posttransfection, cells were mock infected with lysate from 17Cl-1 cells or infected with MHV at an MOI of 1. Following 3 h of infection with MHV, cells were treated with 1,000 U/ml recombinant IFN- β or IFN- α , superinfected with SeV at an MOI of 1, or left untreated. Alternatively, cells were coinfect with MHV and SeV at an MOI of 1. At 8 or 15 h after treatment or infection, RNA was isolated from 293T cells by the use of an RNeasy mini kit (Qiagen, Valencia, CA). Quantitative reverse transcription-PCR (qRT-PCR) was performed using an iQ5 iCycler (Bio-Rad, Hercules, CA) as previously described (46). Cycle threshold (C_T) values were normalized to β -actin levels [$\Delta C_T = C_{T(\text{ISG})} - C_{T(\beta\text{-actin})}$]; ISG expression levels were calculated as fold change relative to mock infection levels using the formula $2^{-\Delta\Delta C_T}$. Primers were designed with Primer3 software (Massachusetts Institute of Technology [http://frodo.wi.mit.edu/primer3/]). Primer sequences are available upon request.

Immunofluorescence. For MHV rescue of SeV from IFN- β , L2 cells were seeded in a 24-well plate as described above. Briefly, L2 cells were mock infected or infected with rA59/S_{MHV-2}-EGFP at an MOI of 1 followed by treatment with

1,000 U/ml recombinant mouse IFN- β 3 h later. After 3 h of incubation with IFN- β , cells were infected with SeV (MOI = 1) in the presence of IFN- β for the indicated time periods. Cells were then fixed in 3% paraformaldehyde and 0.02% glutaraldehyde for 10 min and permeabilized in 0.5% Triton X-100. For translocation assays, 293T cells were seeded in 24-well plates and transfected using Eugene6 transfection reagent with 0.4 μ g of pCI-neo1a[1–4] and 1 μ g of either pCAGGS parental vector (for endogenous IRF-3 staining) or pCAGGS expressing STAT1 fused to EGFP (pCAGGS-STAT1-GFP) (41). Infections, IFN- β treatment, and fixation were performed as described above. For STAT1 translocation, cells were probed with a monoclonal antibody (Mab) directed against the MHV nucleocapsid (N) protein (Mab 1.16.1; kindly provided by Julian Leibowitz, Texas A&M University, College Station, TX) at a dilution of 1:500. As a secondary antibody, Alexa Fluor 594 goat anti-mouse antibody (Molecular Probes, Eugene, OR) was used at a dilution of 1:400. For IRF-3 translocation, MHV was detected with a rabbit polyclonal antibody at a dilution of 1:100 and endogenous IRF-3 with a mouse monoclonal antibody (BD Pharmingen, San Jose, CA) at a dilution of 1:100. In rescue experiments, SeV was detected with a SeV-specific monoclonal antibody (Mab 5F5; kindly provided by Carolina López, Mount Sinai School of Medicine, New York, NY) at 1 μ g/ml. As secondary antibodies, Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes, Eugene, OR) was used at a dilution of 1:50 and Alexa Fluor 594 goat anti-mouse antibody (Molecular Probes, Eugene, OR) was used at a dilution of 1:400. All antibodies were diluted in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 2% bovine serum albumin. Samples were examined under a Nikon Eclipse 2000E-U fluorescence microscope (Nikon Inc., Melville, NY).

Chromatin immunoprecipitation. 293T cells (1×10^6) were seeded in 10-cm-diameter dishes and transfected using Eugene6 reagent with 8 μ g of pCI-neo1a[1–4] and with 6 μ g of pCAGGS parental vector or pCAGGS expressing the NS1 protein from influenza A virus/PR/8/34 (48). At 24 h posttransfection, cells were infected with MHV at an MOI of 1 for 3 h followed by SeV infection. Chromatin immunoprecipitation (ChIP) assays were conducted as described by Impey et al. (24). Briefly, chromatin in formaldehyde-fixed cell lysates was sonicated to an average size of 600 bp by using a Braun-sonic probe sonicator (four times at 10 s each time on ice, using 40- to 50-W pulses with 1-min rest intervals). Lysates were clarified by centrifugation at $20,800 \times g$ for 10 min at 4°C and incubated with primary antibody overnight at 4°C. Antibodies used for ChIP included 2 μ g of rabbit polyclonal IRF-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 2 μ g of normal rabbit IgG (Cell Signaling Technology, Danvers, MA). Immunocomplexes were captured with salmon sperm DNA-protein A agarose (Millipore, Temecula, CA) and washed, and the bead pellet was resuspended in 100 μ l of Tris-EDTA (TE) buffer (pH 8.0). RNA was digested for 30 min at 37°C with 50 μ g of RNase A (Roche, Indianapolis, IN). Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.25%, and proteins were digested with 250 μ g of proteinase K (Roche) for 12 h at 37°C. Formaldehyde cross-links were reversed at 65°C for 6 h. Samples were subjected to phenol-chloroform extraction, and the DNA was precipitated in 100% ethanol. DNA fragments were quantified by qRT-PCR with primers designed using sequences within 500 bp of the promoter region of the gene. Reactions were run on a Bio-Rad iQ5 thermocycler (Bio-Rad) for one cycle at 95°C for 3 min and for 40 cycles at 95°C for 15 s and 67°C for 30 s. ChIP data are presented as percent input as calculated by dividing the values obtained from total input and multiplying by 100. Primer sequences are available upon request.

RESULTS

MHV coinfection rescues Sendai virus from the antiviral effects of interferon. Interferon- α or - β treatment of many cell types leads to activation of a signaling cascade that induces expression of hundreds of genes, many of which have direct or indirect antiviral properties (39). Numerous groups have shown that replication of RNA viruses, including SeV, VSV, NDV, Sindbis virus, and TMEV, in addition to many other viruses, is inhibited in IFN- α - or - β -treated cultures (15, 46, 59). Pretreatment of L2 fibroblasts with IFN- α or - β at 3 to 16 h prior to infection severely inhibits replication of SeV (Fig. 1A) (46), VSV, NDV, Sindbis virus, and TMEV (data not shown). The recombinant rA59/S_{MHV-2} MHV strain used in the experiments and represented in Fig. 1 contains the spike from MHV-2 and all other genes from the recombinant A59

(rA59) strain of MHV (11). This virus was used because the MHV-2 spike does not induce cell-cell fusion; thus, individual infected cells rather than large syncytia could be visualized. As previously shown for the A59 strain of MHV, replication of recombinant MHV expressing the spike gene of MHV-2 (rA59/S_{MHV-2}) is unaffected by pretreatment of cells with a high concentration (1,000 U/ml) of recombinant mouse IFN- α (Fig. 1B) or IFN- β (44, 46).

We hypothesized that MHV may prevent the expression of antiviral genes or inhibit functions of antiviral proteins that allow the virus to replicate in the presence of high concentrations of type I IFN in a manner similar to that described for other viruses. In fact, MHV-encoded nucleocapsid protein was shown to inhibit RNase L activity in the context of a recombinant vaccinia virus infection (64). L2 cells were treated for 3 h with IFN- α or - β followed by coinfection with MHV (rA59/S_{MHV-2}) and SeV (Fig. 1B, top panels), VSV, NDV, Sindbis virus, or TMEV (data not shown).

rA59/S_{MHV-2} coinfection was unable to successfully rescue any of these IFN-sensitive viruses (Fig. 1B, top panels, and data not shown). We reasoned that an established MHV infection might be more successful at blocking IFN signaling. To test this hypothesis, we attempted an alternative rescue protocol whereby cells were infected with rA59/S_{MHV-2} 3 h prior to IFN- α / β treatment followed by coinfection with an IFN-sensitive virus 3 h post IFN- α or - β treatment and replication of both viruses was evaluated 16 h after superinfection. SeV replication was rescued when rA59/S_{MHV-2} infection was established in coinfecting cultures 3 h prior to IFN treatment (Fig. 1B, middle panels). Interestingly, MHV preinfection was unable to recover replication of other IFN- β -sensitive viruses (TMEV, VSV, Sindbis virus, NDV) even at lower doses of IFN- β (data not shown). This result was not due to an inability to achieve coinfection, as MHV readily infected the same cells as NDV, TMEV, VSV, and Sindbis virus (reference 46 and data not shown). These data suggest that MHV infection changes the environment of the infected cell to limit the antiviral potential of IFN- α / β -transduced signals in a highly selective and non-broad-based manner, since not all viruses are rescued. SeV and rA59/S_{MHV-2} were able to replicate to some extent in the same cells whether in the absence (Fig. 1B, bottom panels) or presence (Fig. 1B, top and middle panels) of IFN- α ; however, not all cells were coinfecting. These results may have been a consequence of limited replication of both viruses in coinfecting cells, which would result in decreased antigen expression and in a reduced ability to detect virus replication by immunofluorescence.

MHV inhibits induction of the interferon-stimulated response element by IFN- α / β . The observation that MHV has the ability to rescue SeV from the antiviral effects of IFN only when MHV infection is established before cultures are exposed to IFN indicates that MHV must suppress mRNA expression or the activity of protein(s) that are induced by IFN- α / β and would otherwise constrain SeV replication. To investigate this assertion, we transfected 293T cells with plasmids encoding the MHV receptor (CEACAM1a) and a reporter plasmid with firefly luciferase expression driven by the IFN-stimulated response element (ISRE) from ISG54. (The remaining experiments were performed using 293T cells, since L2 cells are not readily transfectable and MHV-in-

FIG. 1. MHV rescues SeV from the antiviral effects of IFN when MHV infection is established before IFN treatment. (A) L2 fibroblasts cultures were mock treated or treated with 1,000 U/ml recombinant mouse IFN- α 3 h prior to SeV infection at an MOI of 1. At 16 h postinfection, SeV-infected cells were stained with SeV-specific monoclonal antibody and DAPI (4',6-diamidino-2-phenylindole) to determine the level of infection. (B) L2 cells were infected with MHV (rA59/S_{MHV-2}-EGFP) at an MOI of 1 for 3 h prior to treatment with 0 U/ml (bottom panels) or 1,000 U/ml (top and middle panels) recombinant mouse IFN- α . Following 3 h of IFN- α treatment, cultures were infected with SeV at an MOI of 1, fixed at 16 h postinfection, and subsequently stained with SeV-specific antibody. rA59/S_{MHV-2}-EGFP infection was monitored by measuring levels of GFP expression. Data are representative of the results of four independent experiments.

fecting 293T cells display resistance similar to that seen with IFN- α/β -induced antiviral effects [44]). MHV infection could not prevent activation of the ISRE when transfected 293T cells transiently expressing the MHV receptor were treated with IFN- α/β at the same time as infection with MHV (Fig. 2A). Consistent with the results shown in Fig. 1, MHV infection established 3 h prior to treatment with IFN- α/β successfully blocked induction of the ISRE-luciferase reporter by IFN to a lesser extent than the well-characterized antagonist of IFN signal transduction, the V protein from simian virus 5 (2) (Fig. 2B).

MHV transiently inhibits the induction of a subset of ISGs by IFN- α/β . The ability of MHV to inhibit IFN- α/β -induced reporter gene expression from the ISRE promoter indicated that MHV infection could affect expression of ISGs in 293T cells. ISG induction was evaluated in the presence or absence of MHV infection by the use of quantitative reverse transcription-PCR (qRT-PCR) to assess ISG mRNA levels in total RNA isolated from 293T cells following IFN- β treatment. For the remaining experiments, we evaluated only the effects of MHV on IFN- β signaling, since the results obtained with IFN- α and - β -treated cells in previous assays were similar. As expected, based on the ability of MHV to inhibit the induction of the ISRE reporter construct, cells infected with MHV prior to 8 h of treatment with IFN- β accumulate significantly less

ISG54 as well as ISG56, MDA5, and RIG-I mRNAs ($P \leq 0.05$) (Fig. 3A). Interestingly, ISG15 induction was unaffected by MHV infection and tumor necrosis factor alpha (TNF- α) and IRF-7 and IRF9-27 were not induced in 293T cells at 8 h post IFN- β treatment (Fig. 3A). TNF- α was included as a negative control, since TNF- α mRNA expression is not directly induced by IFN- β . In this culture system, MHV was unable to achieve complete inhibition of ISG expression, since only 40 to 50% of cells were transiently expressing receptor and susceptible to MHV infection, while presumably all cells in the culture retained the ability to respond to IFN- β , resulting in increases of ISG mRNA levels. Furthermore, we observed that the ability of MHV to suppress induction of a subset of ISGs appeared to be transient. At 15 h post IFN- β treatment, ISG mRNAs regulated by MHV at 8 h postinfection were no longer negatively regulated. This observation was not a result of the death of infected cells, since up to 50% of the cells were infected at 15 h postinfection, as demonstrated by MHV antigen detection by immunofluorescence (data not shown) and high (10^7 PFU/ml) MHV titers that were released from infected cultures up to 24 h postinfection (44). In contrast, expression of some ISGs (ISG56, ISG54, MDA5, and RIG-I) and TNF- α was augmented in the presence of MHV (Fig. 3B). ISGs not induced at 8 h posttreatment (IRF-7 and IRF9-27), however, have significantly elevated mRNA levels at 15 h post IFN- β treat-

FIG. 2. MHV infection prior to IFN- α/β treatment inhibits activation of the ISRE. 293T cells were transiently transfected with expression vector for the MHV receptor CEACAM1a, an ISRE reporter construct linked to firefly luciferase (pElu-ISRE), and a renilla expression vector driven by the SV40 promoter (pRL-SV40). At 24 h post-transfection, the cultures were treated with 1,000 U/ml recombinant human IFN- α or IFN- β and simultaneously mock infected or infected with MHV at an MOI of 1 (A) or infected with MHV and treated with 1,000 U/ml IFN- α or - β 3 h later (B). Alternatively, cells were transfected with an expression vector for the V protein of simian virus 5 (V) for 24 h and then treated with 1,000 U/ml IFN- β . Cell lysates were collected 8 h posttreatment and assayed for firefly and renilla luciferase expression levels. Firefly luciferase values are normalized to renilla luciferase values. IFN- β -induced luciferase expression was set to 100%, and all other values were calculated as percentages of the IFN- β signal. Statistical significance was determined by Student's *t* test (**, $P < 0.005$). Data are representative of the results of four independent experiments.

ment, and expression of IRF-7 and IRF9-27 is negatively regulated by MHV preinfection (Fig. 3B). These observations indicate that two distinct groups of ISGs (ISG56, ISG54, RIG-I, and MDA5 versus IRF-7 and -9 to -27) may share common transcriptional regulatory units that can be affected by MHV infection. We propose that suppression of early induction of some ISGs enables the virus to accomplish at least a single cycle of replication, allowing the virus to better overcome the inhibitory effects of IFN- α/β . In the case of TNF- α , we observed late (15 h postinfection) induction of mRNA only in the presence of MHV infection (Fig. 3B). Interestingly, MHV infection alone does not induce expression of TNF- α or of any of the ISGs we evaluated up to 18 h postinfection (Fig. 3). The unique regulation of TNF- α expression in the context of MHV and IFN- β is addressed further in the Discussion.

Activation of STAT1 by IFN-induced signaling is not inhibited by MHV infection. MHV inhibition of IFN-induced ISG mRNA production focused our investigation on the possible mechanism(s) employed by MHV to resist the effects of the

presence of IFN- α/β in 293T cells. Many viruses encode antagonists that inhibit various points in the IFN signaling pathway, ultimately preventing synthesis of ISGs (31). The activation state of STAT1, the downstream transcriptional activator, reflects signal transduction from the alpha interferon receptor complex consisting of two subunits (IFNAR1 and -2). STAT1 translocates to the nucleus following phosphorylation of Tyr701 and oligomerization of STAT1, and phosphorylated STAT2 and IRF-9 form the ISGF3 transcriptional unit. In addition to this well-characterized ISGF3 complex, activated STAT1 may form a homodimer or interact with other STATs and IRFs to form alternative functional transcription factor (TF) complexes (6, 55). 293T cells express low levels of endogenous STAT1 that are undetectable by immunofluorescent staining and barely detectable by Western blot analysis (Fig. 4B) (61). Thus, transient transfection and overexpression of STAT1-GFP was necessary to enable the detection of the STAT1 activation state following MHV infection and IFN- β treatment in immunofluorescence assays (see Fig. 4A). 293T cells transiently expressing the MHV receptor and STAT1-GFP were infected with MHV 3 h prior to IFN- β exposure. At 6 h post IFN- β treatment, STAT1-GFP was localized to the nucleus in most cells (Fig. 4A, middle panels) and this activation of STAT1 was unaffected by MHV infection (Fig. 4A, bottom panels). We took into consideration the possibility that overexpression of STAT1-GFP could result in saturation in these cells and might mask the identification of potential MHV antagonists in this assay. Thus, we analyzed endogenous STAT1 and STAT2 phosphorylation by Western blotting with phosphospecific antibodies to monitor STAT activation. We observed no differences in the extent of IFN- β -induced STAT1 phosphorylation in MHV-infected 293T cultures (as early as 30 min postinfection; data not shown) at either Tyr701 or Ser727, which are both necessary for STAT1 activation (6) (Fig. 4B). Similarly, STAT2 interacts with the SH2 domain of IFNAR1 and, upon IFN engagement, STAT2 becomes activated by phosphorylation on Tyr690 by the tyrosine kinase Tyk. STAT2 phosphorylation is essential for oligomerization with STAT1 and IRF-9 (6) but is unaffected by MHV infection (Fig. 4B). Consistent with observations that MHV does not induce expression of IFN- β protein in 293T cells (K. Rose, unpublished observation), MHV infection alone did not induce phosphorylation of STAT1 or STAT2 (Fig. 4B). STAT1 and STAT2 genes are transcriptionally activated by IFN signaling; however, basal or induced levels of these proteins are undetectable by Western blot analysis (Fig. 4B); thus, α -tubulin levels were used as a means to ensure equal loads of protein in the wells.

Direct virus-mediated synthesis of ISGs is inhibited by MHV infection. Recognition of PAMPs by PRR on the surface of cells or in the cytoplasm activates signals leading to the production of a number of genes with indirect and direct antiviral properties, including IFN- β (49). Genes induced through PRR have a high degree of overlap with those genes stimulated by IFN- β . Previously published data revealed that MHV was unable to induce IFN- β mRNA or protein in L2, L929, and 17Cl-1 mouse fibroblast cell lines (46, 60, 69). In addition, coinfection of MHV was unable to prevent IFN agonists [SeV and poly(I:C)] from stimulating IFN- α/β production (46, 69). Based in part on the data presented above, we predicted that if MHV infection were established in a culture

FIG. 3. MHV infection delays expression of a subset of ISGs induced by IFN- β . 293T cells were transfected with an expression vector for the MHV receptor. At 24 h later, cells were mock infected or infected with MHV at an MOI of 1. Cultures were treated with 0 U/ml (mock and MHV) or 1,000 U/ml recombinant human IFN- β 3 h post MHV or mock infection (MHVpre or mock pre). Total RNA was isolated from cells 8 h (A) or 15 h (B) post IFN- β treatment and analyzed for expression of indicated ISGs by performing qRT-PCR with gene-specific primers. Data are representative of the results of three independent experiments. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.005$).

before introduction of an IFN agonist, MHV could put blocks in place to prevent induction of ISGs. Using the ISRE-luciferase reporter to assay induction of ISGs in MHV-infected 293T that were coinfecting with SeV at the same time as MHV (MHV + SeV) or infected with SeV after MHV (MHV pre), we observed that MHV was more effective at inhibition of the ISRE when 293T were infected with MHV prior to SeV (Fig. 5A and 5B). In fact, MHV was able to inhibit activation of the ISRE better when MHV infection was established 3 h before SeV inoculation versus only 1 h before (Fig. 5A). Since SeV stimulates production of large amounts of IFN- α/β that could contribute to the activation of the ISRE reporter, we eliminated the production of IFN- α/β in this assay by using Vero E6 cells, which are unable to produce IFN- α/β (17). Consistent with results in Fig. 5B, the ISRE-luciferase reporter was partially inhibited in Vero E6 cells, transiently expressing the MHV receptor, only when MHV infection was established at least 3 h prior to SeV infection (Fig. 5C), confirming that MHV inhibits ISG induction that results from SeV recognition by PRR that is independent of IFN- α/β production. As seen before (Fig. 2B), preinfection with mock cell lysate was unable to affect transcription from an ISRE (Fig. 5).

To determine the extent to which MHV can prevent transcription of ISGs in response to SeV infection, 293T cells transiently expressing the MHV receptor were infected with MHV followed by SeV 3 h later. In this assay, MHV significantly ($P \leq 0.05$) reduced mRNA induction of some ISGs (including ISG56, RIG-I, and IFN- β) and TNF- α while exhibiting no effect on others (MDA5 and ISG54) as evaluated 8 h post SeV infection (Fig. 6A). MDA5 and ISG54 mRNA induction, however, was restricted by MHV infection as assayed at 15 h post SeV infection (Fig. 6B), suggesting that the ISGs interrogated in this assay are dynamically controlled through unique transcriptional programs. As we observed when using IFN- β to activate ISG synthesis, ISG15 mRNA expression was unaffected by MHV infection at either early (8 h) or later (15

h) times post SeV infection (Fig. 6). Again, MHV infection of 293T, as monitored by immunofluorescence staining with an MHV-nucleocapsid-specific antibody, suggests that only 30 to 40% of the cells in the culture were infected (data not shown). This likely explains the less than complete inhibition of ISG production by MHV, since not all SeV-infected cells have the potential to be coinfecting.

The observation that MHV infection must be established before IFN- β -induced signaling or SeV-mediated expression of ISGs suggests that MHV does not enter the cell with the immediate capability to limit ISG synthesis and must require a period of time to manipulate early induction of some ISGs. To address whether *de novo* protein synthesis was a necessary prerequisite for the ability of MHV to delay ISG expression, we analyzed IFN-induced or SeV-mediated ISG mRNA accumulation in MHV-infected cultures that were treated with the translational elongation inhibitor cycloheximide 1 h before MHV infection. Synthesis of ISGs in response to IFN- β or SeV in cycloheximide-treated cells, however, was inhibited independently of MHV infection (K. Rose, unpublished data); thus, we were unable to determine whether protein synthesis was required for MHV-induced inhibition of ISG expression.

MHV limits SeV-mediated transcription from IRF-3- and NF- κ B-responsive promoters. Recognition and binding of viral RNA by PRR initiates a signal transduction cascade, leading to nuclear translocation of the transcription factor IRF-3 (65). Cervantes-Barragan et al. (9) reported that TLR-7 located in endosomal membranes was necessary for detection of MHV in pDCs, and we identified MDA5 as a factor contributing to the recognition of MHV in bone-marrow-derived macrophages (44, 45). Similarly, defective interfering particles produced during early SeV infection are sensed by MDA5. Alternatively, recognition of RNA produced as a result of SeV replication requires RIG-I (66). Through the activity of either helicase, SeV readily induces IRF-3 activation by phosphorylation followed by translocation of IRF-3 to the nucleus. We performed

FIG. 4. MHV does not alter STAT1 translocation to the nucleus or STAT1 and STAT2 phosphorylation. (A) An expression vector for STAT1 fused to GFP (STAT1-GFP) was transfected along with a plasmid expressing the MHV receptor into 293T cells. Cultures were then mock infected or infected with MHV at an MOI of 1 24 h posttransfection. MHV infection was allowed to proceed for 3 h before treatment of cultures with 0 U/ml (top panels) or 1,000 U/ml (middle and bottom panels) recombinant human IFN- β . Following 6 h of IFN- β treatment, cells were fixed in paraformaldehyde and stained with an antibody that specifically detects MHV nucleocapsid protein. (B) 293T cells transiently transfected with MHV receptor were treated as described above, and cell lysates were collected at the indicated times post IFN- β treatment. Protein levels were normalized, and lysates were processed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for Western blot analysis with antibodies to detect STAT1 and STAT2 and phosphorylated forms of both proteins. Levels of α -tubulin were used to ensure equal levels of loading. Data are representative of the results of two independent experiments.

assays with several widely used IRF-3-responsive promoters (PRDIII-I from the IFN- β promoter [18] and p55C1B [32]) to evaluate SeV-mediated reporter expression. Activation of both IRF-3-responsive promoters was reduced when cells were infected with MHV prior to SeV infection, with expression from the p55C1B reporter (Fig. 7A) inhibited to a greater extent

than from the PRDIII-1 reporter (Fig. 7B) at 8 h post SeV infection. In support of the data presented in Fig. 6, the IRF-3-responsive promoter was inhibited by MHV at early times post SeV infection (8 h; Fig. 7B) but at later times (15 h; Fig. 7C) MHV augmented the IRF-3-mediated activation of the promoter following SeV infection. Overexpression of influenza

FIG. 5. Preinfection of 293T with MHV delays ISG induction mediated directly by SeV. (A to C) 293T or Vero E6 cultures were transiently transfected with MHV receptor, pSV40-RL, and ISRE-luciferase reporter construct. (A) At 24 h later, cultures were left untreated (UT), infected with MHV (MHV), or mock infected (mock). Following MHV infection for 1 h (1h pre), 2 h (2h pre), or 3 h (3h pre), cultures were infected with SeV at an MOI of 1. Cell lysates were collected 8 h postinfection and analyzed for luciferase expression. Following 24 h transfection, 293T (B) or Vero E6 (C) cultures were left untreated, mock infected (mock pre), or infected with MHV (MHV pre). At 3 h post MHV infection, cultures were superinfected with SeV or a combination of MHV and SeV (MHV + SeV). Cells were lysed 8 h later and analyzed for firefly and renilla luciferase expression. Firefly luciferase levels were normalized to renilla luciferase levels. SeV-induced luciferase expression was set to 100%, and all other values were calculated as percentages of the SeV signal. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.005$). Data represent the results of at least three independent experiments.

virus A/PR/8/34-encoded NS1 protein was used as a positive control, since the ability of NS1 to block IRF-3 has previously been well documented (57) (Fig. 7B and C). Although IRF-3 is essential for activation of many ISGs (1), NF- κ B is an important transcriptional cofactor for synthesis of IFN- β and other ISGs (65). Using the NF- κ B-responsive element (PRDII) cloned from the IFN- β promoter, we ascertained the effect of MHV preinfection on the ability of NF- κ B to be activated by

either of two distinct pathways, namely, TNF- α or SeV infection. While MHV had no effect on the ability of TNF- α to activate NF- κ B, MHV significantly reduced activation of NF- κ B at 8 h post SeV infection (Fig. 7D). Again, in similarity to the influence of MHV on the transcriptional activity of IRF-3, the block to NF- κ B transcriptional activation was released at later times post SeV infection (15 h; Fig. 7E) and the presence of MHV added to the NF- κ B response. Interestingly, MHV infection blocks the ability of TNF- α to drive expression from the NF- κ B-responsive promoter at 15 h posttreatment by an unknown mechanism (Fig. 7E). Overexpression of nsp1 encoded in both SARS-CoV (36) and MHV (70) has been shown to degrade cellular RNA and reduce expression from constitutive reporters. We wanted to ensure that the effect MHV exerted on the IRF-3 and NF- κ B promoters was specific. Thus, the ability of MHV infection to inhibit induction of luciferase driven by two constitutive promoters, thymidine kinase (TK) and SV40, was evaluated. MHV infection of 293T cells did not change the level of expression induced by either constitutive promoter, suggesting that nsp1 expressed in the context of the virus does not significantly degrade 293T cellular RNA (Fig. 7F). In addition, SeV infection did not alter transcription by either the TK or SV40 promoter (Fig. 7F).

MHV is unable to prevent SeV-induced binding of IRF-3 to ISREs. Our data suggest that MHV is able to antagonize SeV-mediated transcriptional activation of the ISRE by interfering to some extent with the activity of IRF-3 and NF- κ B (Fig. 7); therefore, we performed several assays to determine the level at which MHV was able to antagonize activation of IRF-3. As observed previously in studies of Vero E6 and 17Cl-1 cells (46, 69), MHV does not induce IRF-3 translocation in 293T cells expressing an MHV receptor (Fig. 8A, top panels). By immunofluorescence imaging of 293T cells, we observed nuclear translocation of endogenous IRF-3 in response to SeV infection (Fig. 8A, middle panels). Preinfection of 293T cultures with MHV, however, was unable to inhibit SeV-induced translocation of IRF-3 (Fig. 8A, bottom panels).

Although MHV does not prevent IRF-3 from translocating to the nucleus, the possibility remained that MHV could inhibit the ability of IRF-3 to function as a transcription factor. Several recent reports presented evidence that dimerization, coactivator association, and nuclear translocation of IRF-3 are not directly correlated with its ability to induce transcription (10, 54) and are instead markers of a hyperactive and unstable form of IRF-3 (10). Thus, nucleus-localized IRF-3 might be prevented from association with DNA. To evaluate this possibility, we performed chromatin immunoprecipitation (ChIP) using an IRF-3-specific antibody and assayed the precipitated chromatin fragments by qRT-PCR using primers specific to ISGs. Using IgG serum as an isotype control for ChIP, we found that MHV infection did not change the binding of IRF-3 to the response elements of ISG54 or RIG-I, two genes whose induction was inhibited by prior MHV infection (Fig. 8B) at 8 h post SeV infection. Calreticulin (CALR) and 9-27, genes not induced by SeV infection, and ISG15, a gene whose induction was not changed by MHV infection, were used as negative controls. As a positive control for the IRF-3 ChIP assay, we transiently expressed the influenza virus A/PR/8/34 NS1 protein in 293T cells, a treatment that has been previously demonstrated to inhibit IRF-3 (57), before infection with SeV. NS1

FIG. 6. MHV delays induction of ISGs mediated by SeV. 293T cells were transfected with an expression vector for the MHV receptor. At 24 h later, cells were mock infected (mock pre) or infected with MHV (MHV pre) at an MOI of 1. Following 3 h of incubation with MHV, cultures were mock infected or infected with SeV (MOI = 1). Total RNA was isolated from cells 8 h (A) or 15 h (B) post SeV infection and analyzed for expression of indicated ISGs by performing qRT-PCR with gene-specific primers. The experiments were performed in triplicate, and statistical significance was determined by Student's *t* test (* $P < 0.05$; **, $P < 0.005$). Data represent the results of three independent experiments.

expression significantly inhibited binding of IRF-3 to the response element of ISG15 and RIG-I (Fig. 8B).

DISCUSSION

We present evidence that MHV is refractory to the antiviral effects of IFN- α/β in specific cell types (L2 and 293T). Yet IFN- α or - β treatment of these cells significantly limited replication of all other RNA viruses evaluated, indicating that IFN signaling is intact in L2 and 293T cells and that MHV has a distinct ability to resist IFN-induced antiviral properties. MHV is able to confer this resistance to SeV only when MHV infection is established prior to IFN- α/β treatment and subsequent SeV infection. Since other RNA viruses were not rescued in a similar manner, we hypothesize that SeV and MHV may be sensitive to some of the same antiviral pathways. Furthermore, these observations suggest a unique relationship that enables MHV to resist IFN- α/β in certain transformed cell types (L2, HeLa, and 293T), since we have not observed similar immune evasion capabilities in primary cell cultures (MEF and bone-marrow-derived macrophages) (44, 46, 64).

We present evidence that MHV delays mRNA induction of some but not all of a group of ISGs in 293T cells following IFN- β exposure. MHV may suppress early ISG expression to allow at least a single cycle of replication to overcome the antiviral effects of IFN. Induction of these ISGs at later times postinfection may reflect a release of the block imposed by MHV, or these ISGs may be induced by an alternative pathway at later times post IFN- β treatment. In the case of TNF- α , however, we observed that induction of TNF- α following IFN- β treatment was dependent on MHV infection. This result may reflect the ability of MHV to induce expression of a molecule that was essential for IFN- β -induced TNF- α transcription that was not basally expressed in 293T cells (29).

Our observation that SeV was selectively rescued by MHV

may reflect the fact that MHV and SeV are sensitive to antiviral properties of common ISGs. Scant information is available on the direct antiviral effects of any single ISG on a specified virus. IFN-induced Mx GTPases selectively inhibit influenza virus, Thogoto virus, and bunyavirus replication (22), while ISG20, viperin, and dsRNA-dependent protein kinase R (PKR) inhibit hepatitis C virus replicons (26). Investigations are currently under way in our laboratory to identify ISGs that have antiviral potential against MHV.

Although several alternative pathways are activated when IFN- α/β engages the IFNAR1/IFNAR2 complex, many viruses have evolved mechanisms to block the predominant pathway leading to STAT1 and STAT2 activation and oligomerization with IRF-9 (58). Our data clearly show that MHV does not influence STAT1 or STAT2 induction, phosphorylation status, or translocation to the nucleus following IFN- β treatment. Complex mammalian promoters, however, contain potential binding sites for several transcription factors (TFs). Not surprisingly, several groups have demonstrated that ISREs differ in their requirements for binding of a combination of seven different STATs (STAT1-7) and nine unique IRFs (IRF1-9), in addition to other transcriptional regulators, for the induction of mRNA synthesis (58). Thus, by regulating the bioavailability or activity of specific transcription factors or coactivators, MHV may regulate expression of a group of ISGs. Furthermore, IFN-induced transcription relies on chromatin-remodeling complexes, which presents another level at which MHV could be manipulating gene expression (34). Given the vast array of control points at which MHV may exert its regulatory effects, we can conclude only that MHV does not inhibit activation of STAT1 and STAT2.

Viruses employ multiple strategies not only to inhibit IFN- β signaling but also to prevent IFN- β production. Not surprisingly, we observed that MHV infection of cultures prior to SeV infection can also reduce direct virus-mediated induction of

FIG. 7. MHV reduces the ability of SeV to activate IRF-3 and NF- κ B. 293T cells were transfected with expression plasmid for MHV receptor, pRL-SV40, and either of two IRF-3-responsive promoters, p55C1B (A) or pLuc-(PRDIII-I)₃ (B and C), with or without pCAGGS-NS1. Alternatively, cells were transfected with pRL-SV40 and an NF- κ B responsive promoter, pLuc-(PRDII)₂ (D and E). At 24 h posttransfection, cultures were mock infected (mock pre) or infected with MHV (MHV pre) and then infected 3 h later with SeV (MOI = 1), treated with 1,000 U/ml TNF- α , or left untreated. Cells were lysed, and renilla expression levels were determined 8 h (A, B, and D) or 15 h (C and E) postinfection or posttreatment. (F) 293T cells were transiently transfected with pElu-ISRE and either pRL-SV40 or pRL-TK expressing renilla luciferase under the control of SV40 or thymidine kinase promoters, respectively. At 24 h posttransfection, cultures were left untreated (–) or infected with SeV or MHV and cell lysates were collected for analysis of luciferase expression 8 h postinfection. (A to E) Values represent percentages of firefly expression relative to SeV-treated culture values after normalization to renilla expression levels. Statistical significance was determined by Student's *t* test (*, *P* < 0.05; **, *P* < 0.005). Data represent the results of three independent experiments.

IFN- β and other ISGs in an IFN-independent fashion. We observed no difference in the ability of SeV to induce IRF-3 translocation in cultures where MHV infection was established 3 h prior to SeV. In similar experiments, 17Cl-1 and Vero E6 cells infected with MHV in combination with poly(I:C) and SeV, respectively, demonstrated that MHV lacked the ability to prevent IRF-3 translocation (46, 69). Commonplace assays used to monitor IRF-3 activation following virus infection include IRF-3 phosphorylation, homodimerization, and translocation to the nucleus. In addition, multiple studies have suggested that IRF-3 must undergo phosphorylation at several sites (30, 50) and deglutathionylation (40) to induce conformational changes permitting interaction with and acetylation by CBP/p300, which unmask the DNA binding domain in IRF-3 (23). Using two well-characterized IRF-3 reporter constructs and an NF- κ B reporter, we provide evidence that MHV is able to partially inhibit SeV-mediated expression of these reporters in a specific manner, since expression from constitutive SV40 or TK promoters is not significantly altered. Interestingly, ChIP assays with an IRF-3-specific antibody suggested that the presence of MHV does not significantly affect the

ability of IRF-3 to bind chromatin of MHV-regulated ISGs. In accordance with the data presented in Fig. 6 to 8, IRF-3 translocates to the nucleus and interacts with ISG DNA. Thus, it appears that MHV influences a step further downstream to affect accumulation of certain ISG mRNAs as follows: (i) MHV may affect the dynamics of chromatin remodeling post IRF-3 binding, which limits accessibility for essential transcriptional coactivators; (ii) MHV blocks recruitment or bioavailability of other TFs or transcriptional coactivators that are essential for expression of a subset of ISGs; and (iii) stability of certain ISG mRNAs is altered during early MHV infection.

Taken together, our data show that MHV delays transcription of a subset of ISGs without inhibiting activation of the major TFs involved in IFN- β -induced or SeV-mediated induction of ISGs. Further investigations encompassing the expression profile of a larger group of ISGs may reveal common features within these groups of genes and uncover possible targets of MHV antagonism. As demonstrated by Wei et al. (62), ISGs that are coregulated share common TF binding sites that can be identified by applying mathematical algorithms. Such a computational method may reveal pathways, in addition

FIG. 8. MHV does not prevent SeV-mediated IRF-3 translocation and DNA binding. 293T cells were transfected with an expression vector for the MHV receptor. (A) At 24 h posttransfection, cells were either left untreated or inoculated with MHV (top and bottom panels) at an MOI of 1. Following 3 h of incubation with MHV, cultures were mock infected (top panels) or infected with SeV (middle and bottom panels) at an MOI of 1. At 6 h post SeV infection, cells were fixed in paraformaldehyde and analyzed for immunofluorescence detection of IRF-3 and MHV nucleocapsid by the use of specific antibodies. (B) 293T cells seeded in 10-cm-diameter dishes were transfected with an expression vector for the MHV receptor and either empty control vector or pCAGGS-NS1. At 24 h posttransfection, cultures were either mock infected, infected with MHV (MOI = 1), or left untreated (NS1). At 3 h later, cells were inoculated with SeV (MOI = 1) and SeV infection was allowed to proceed for 6 h before cells were fixed for ChIP assays using an IRF-3-specific antibody or an IgG isotype control. qPCR analysis of precipitated DNA fragments by the use of primers designed within the promoter of specific genes was used to quantify IRF-3 binding to specific genes. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.005$). Data represent the results of two independent experiments.

tion to the well-characterized ones investigated thus far, that are targeted by MHV in a cell-type-specific manner.

The ability of MHV to resist the antiviral state induced by IFN- β in a cell-type-specific manner suggests several strategies

that are not mutually exclusive. First, ISGs that have antiviral activity against MHV are induced by IFN- β only in certain cell types; this may be due to differences in the basal expression levels of TFs or signaling molecules or in the accessibility of

chromatin surrounding promoters of ISGs. Second, MHV may, in some cell types, replicate at a privileged site that is inaccessible to potential inhibitors. This possibility seems unlikely, since it would require a location in the cell that would not be affected in some way by the hundreds of ISGs. Alternatively, MHV may encode antagonists that interfere with IFN- β signaling or inhibit the function of antiviral proteins stimulated by IFN- β . The ability of MHV to antagonize the IFN pathway may depend on the relative levels of an MHV antagonist and its cellular target, which may differ from one cell type to another.

Several groups have reported the ability of MHV-encoded proteins to act as IFN antagonists. Ye et al. used a recombinant vaccinia virus-based system to demonstrate that the MHV nucleocapsid is able to inhibit RNase L activity in 17Cl-1 and HeLa cells (64). RNase L degrades both viral and cellular RNA and is a downstream target of 2'-5' oligoadenylate synthetase (OAS), an enzyme that is activated upon binding of dsRNA (49). Of note, MHV alone does not activate RNase L in 17Cl-1 or HeLa cells (64). Several groups investigated the potential of MHV-encoded papain-like protease 2 (PLP2) to inhibit IFN- α/β induction, with conflicting conclusions (19, 67). Zheng et al. concluded that the deubiquitinase domain of MHV PLP is essential for preventing IRF-3 translocation to the nucleus and IFN- α/β production (67). In contrast, Frieman et al. demonstrated that overexpression of MHV PLP2 was unable to inhibit IFN- α/β production, even though this group and others have shown that PLP from the closely related SARS virus prevented IRF-3-dependent production of IFN- α/β in similar assays (16, 19). Also, a mutant of MHV with a 99-nucleotide deletion in the C-terminal portion of nsp1 (MHV-nsp1 Δ 99) was attenuated in its ability to replicate in the liver and the spleen (70). There was a small but statistically significant reduction in the replication of MHV-nsp1 Δ 99 compared to wild-type virus in macrophage cultures, but not conventional dendritic cell cultures, pretreated with IFN- α (70). Using an MHV mutant with a slightly larger C-terminal deletion in nsp1 (nsp1 Δ C) (7), however, we observed no significant differences in titers of virus released from IFN- β -treated versus mock-treated 293T cells (K. Rose, unpublished data). In addition, nsp1 of several coronaviruses, including MHV, has been shown to inhibit cellular mRNA synthesis (27, 70): in the case of SARS nsp1, by inducing degradation of mRNA (27). Since the induction of ISG15 is unaffected by MHV preinfection and MHV does not prevent further induction of ISGs at 15 h postinfection (Fig. 3 and 6), it is unlikely that general host mRNA degradation explains the rescue of SeV by MHV. In addition, MHV inhibition of reporter expression is specific, as expression from a TK or an SV40 constitutive promoter is unaffected (Fig. 7F). These results imply that the nsp1 C terminus is not essential for MHV resistance to IFN- β in 293T cells.

Overexpression of the MHV-encoded proteins (nucleocapsid and membrane) that have been reported to antagonize IFN (64) and to promote IFN induction (13), respectively, did not significantly affect ISRE-luciferase expression in assays similar to those represented in Fig. 2 and 5 (K. Rose, unpublished data). We evaluated several MHV mutant viruses that are attenuated *in vivo*, including viruses with point mutations in ORF2a and nsp14 (53), viruses with mutations in the catalytic

domain of ns2 that significantly attenuate replication in the liver (47), and nsp1 Δ C, in an attempt to identify a virus that had lost the ability to antagonize IFN. Although these mutations affect MHV pathogenesis *in vivo*, replication of these mutant viruses was minimally altered in the presence of IFN- β and all mutants could delay transcription of the ISRE reporter in 293T cells as well as wild-type MHV. We conclude that either there may be more than one protein that acts to prevent early ISG expression, making it difficult to identify using mutants with only one protein rendered nonfunctional, or else other structural or nonstructural proteins, not assayed above, function alone or in concert to prevent early ISG induction in 293T cells. Future studies will be aimed at identifying MHV antagonists of IFN signaling and evaluating their role in limiting the antiviral effects in specific cell types that are targets of MHV infection *in vivo*.

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