Accessory Protein 5a Is a Major Antagonist of the Antiviral Action of Interferon against Murine Coronavirus[▽]

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The type I interferon (IFN) response plays an essential role in the control of *in vivo* infection by the coronavirus mouse hepatitis virus (MHV). However, *in vitro*, most strains of MHV are largely resistant to the action of this cytokine, suggesting that MHV encodes one or more functions that antagonize or evade the IFN system. A particular strain of MHV, MHV-S, exhibited orders-of-magnitude higher sensitivity to IFN than prototype strain MHV-A59. Through construction of interstrain chimeric recombinants, the basis for the enhanced IFN sensitivity of MHV-S was found to map entirely to the region downstream of the spike gene, at the 3' end of the genome. Sequence analysis revealed that the major difference between the two strains in this region is the absence of gene 5a from MHV-S. Creation of a gene 5a knockout mutant of MHV-A59 demonstrated that a major component of IFN resistance maps to gene 5a. Conversely, insertion of gene 5a, or its homologs from related group 2 coronaviruses, at an upstream genomic position in an MHV-A59/S chimera restored IFN resistance. This is the first demonstration of a coronavirus gene product that can protect that same virus from the antiviral state induced by IFN. Neither protein kinase R, which phosphorylates eukaryotic initiation factor 2, nor oligoadenylate synthetase, which activates RNase L, was differentially activated in IFN-treated cells infected with MHV-A59 or MHV-S. Thus, the major IFN-induced antiviral activities that are specifically inhibited by MHV, and possibly by other coronaviruses, remain to be identified.

Type I interferons (alpha interferon [IFN- α] and IFN- β) are essential for host protection against the coronavirus mouse hepatitis virus (MHV) (3, 4, 16, 21, 36). Knockout mice lacking the IFN- α/β receptor rapidly succumb to MHV infection at doses that are controlled and cleared by wild-type mice (4, 16). On the other hand, most strains of MHV, including commonly studied laboratory strain A59, are poor inducers of IFN (13, 37, 49, 54, 61, 62) and are relatively insensitive to IFN in tissue culture (37, 58, 61). Such a pattern suggests that MHV has evolved mechanisms to evade or antagonize the action of this cytokine, even though IFN induction and responses by specialized classes of cells manage to ultimately overcome infection *in vivo* (3, 4, 34–37).

The type I IFN system functions at three levels to combat viral infections (19, 33, 40, 43). Initially, extracellular and intracellular pattern recognition receptors (PRRs) respond to the presence of individual classes of pathogen-associated molecular signatures displayed by viruses and other microorganisms. PRR stimulation triggers signaling events that give rise to the activation or recruitment of multiple transcription factors necessary for induction of the IFN- β gene. Most notable among these components are cytoplasmic IFN regulatory factor 3 (IRF-3) and NF- κ B, both of which must translocate to the nucleus to upregulate transcription. At the second level, IFN- β binds to the IFN- α/β receptor, leading to a second wave of signal transduction that culminates in the induction of numerous IFN-stimulated genes (ISGs). ISG expression, in turn, can amplify the first two levels of responses via various positive

feedback loops, one of which includes the induction of IFN- α . Finally, at the third level, some ISGs encode activities that collectively establish an antiviral state capable of acting against a broad range of RNA and DNA viruses.

To date, studies of the interaction of the innate immune system with MHV or other coronaviruses have focused almost entirely on either the mechanism of viral induction of IFN or the signal transduction events initiated by IFN (11, 36, 37, 62). Growing numbers of coronavirus gene products are reported to interfere at various critical points in these pathways. However, the furthest downstream level of the system—the nature of the antiviral activities contained among the ISG products and the means by which these activities are countered by coronaviruses—has received comparatively little analysis, except for one study that identified the MHV nucleocapsid (N) protein as a significant IFN antagonist (58). In the work presented here, we have mapped and analyzed the critical differences between two strains of MHV, one highly resistant and the other highly sensitive to IFN. Our results show that the product of accessory gene 5 is a potent antagonist of the antiviral action of IFN against MHV.

MATERIALS AND METHODS

Cells and viruses. Stocks of MHV strains and mutants were propagated in mouse 17 clone 1 (17Cl1) cells; plaque assays and plaque purifications of constructed mutants and recombinants were carried out with mouse L2 cells. The interspecies chimeric virus designated fMHV.v2 (14) was grown in feline FCWF cells. MHV strain S (MHV-S) (39) was originally obtained from John Parker (Microbiological Associates) and underwent two rounds of plaque purification prior to passaging for the stocks that were used in all subsequent experiments. For sequence determination, genomic RNA was isolated from a passage 3 virus stock that was purified by two cycles of equilibrium centrifugation on potassium tartrate-glycerol gradients as described previously (22). Reverse transcription (RT) of RNA was carried out with a random hexanucleotide primer and avian myeloblastosis virus reverse transcriptase (Life Sciences), and overlapping am-

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plicons of cDNA spanning the genome were produced with AmpliTaq polymerase (Roche). RT-PCR products were purified using QIAquick spin columns (Qiagen) prior to DNA sequencing. The 5' end of the genome was determined by direct dideoxy sequencing of genomic RNA.

IFN dose-response assay. Monolayers (20 cm²) of L2 or L929 cells, grown to 80% confluence, were incubated at 37°C in 5 ml of Dulbecco's minimal essential medium (MEM) containing 10% fetal bovine serum and 0, 1, 10, 100, or 1,000 U/ml recombinant mouse IFN- α A (Chemicon). Following 24 h of treatment, monolayers were washed three times with Dulbecco's MEM containing 10% fetal bovine serum and then infected with MHV at a multiplicity of 2.0 PFU per cell. After an absorption period of 2 h, the inoculum was removed and the monolayers were washed twice with Eagle's MEM containing 10% fetal bovine serum and then incubated in the same medium at 37°C. Released virus was harvested at 18 h postinfection, and infectious titers were determined in triplicate by plaque assay on L2 cells.

Construction of MHV chimeric recombinants and mutants. All MHV reverse genetics was carried out through targeted RNA recombination (28). The MHV-S/A59 chimeras were isolated by screening for large plaques on IFN-treated cells as described in Results. All other chimeras and mutants were isolated using the host range-based selection system described in detail previously (14, 24). In brief, monolayers of FCWF cells were infected with fMHV.v2 and then transfected with *in vitro*-synthesized donor RNAs. Harvested progeny virus was selected and purified on mouse L2 cells, and the presence of incorporated mutations or genes in candidate recombinants was confirmed by RT-PCR analysis of viral RNA.

Transcription vectors for the synthesis of donor RNAs were all derived from pMH54 (24), which encodes 5' elements of the MHV-A59 genome linked to the 3'-most 8.6 kb of the MHV-A59 genome, a region that encompasses all of the structural protein genes. Insertions, mutations, and rearrangements were generated, sometimes via subclone intermediates, by means of either direct PCR or splicing overlap extension-PCR (15). The vector for the MHV-A59/S chimera, pMHVS2A, was generated from pSG6, a plasmid identical to pMH54, except that pSG6 contains a coding-silent BspEI site spanning codons 444 to 446 of the N gene (14). MHV-S RNA was amplified by RT-PCR to produce a cDNA bounded by the SbfI site downstream of the S gene and the BspEI site at the end of the N gene, and this fragment was exchanged for the corresponding MHV-A59 sequence in pSG6. For pSGMH54 Δ 5a, the vector for the gene 5a-KO (knockout) mutant, the set of knockout mutations was created in a gene 4-gene 5 subclone, pLK63, which was a precursor of pMH54 (24). The SbfI-EagI fragment from the resulting plasmid, running from the S gene downstream boundary through the M gene, was then transferred to pMH54. The vector for the N gene chimera, pMH54-SN, was constructed by replacement of the pMH54 BssHII-NheI fragment, running from the M gene to the N gene, with MHV-S cDNA that had been amplified by RT-PCR. In the cDNA insert, the upstream BssHII site was changed to AscI because the MHV-S sequence has a second BssHII site in the corresponding interval; this change resulted in a coding-silent change in codon 189 of the MHV-A59 M gene. The 5a-KO and N chimera mutations were combined in a single transcription vector, pMH54-SN-5aKO, by replacement of the EagI-NheI fragment of pSGMH54 Δ 5a with the same fragment from pMH54-SN.

Vectors for mutants with gene 5a homologs substituting for MHV gene 2 were assembled by a strategy originating with pPM9, the construction of which will be described elsewhere. In brief, pPM9 contains an expansion of pMH54 to include the 3'-most 2.9 kb of gene 1b, downstream of which there is a deletion of transcription-regulating sequence 2 (TRS2), gene 2a, and almost all of the hemagglutinin-esterase (HE) gene; in place of the deletion, unique SalI and AscI sites were created. Vector pPM9 was used to generate the MHV-A59 Δ 2a-HE control virus; it should be noted that this virus differs from the one described by de Haan et al. (8). An MHV-S chimeric version of pPM9, pMHVS2PM9, was constructed by transfer of the XhoI-PacI fragment, running from the S gene to the end of the poly(A) tail, from pMHVS2A into pPM9. Each gene 5a homolog, preceded by a canonical TRS (AAUCUAAAC), was then inserted between the SalI and AscI sites. For pS5M, the vector for the MHV-A59/S chimera+5a(MHV) mutant, MHV gene 5a was constructed by PCR using pMH54 as the template. A silent change was made in codon 111 to eliminate the E gene AUG codon in the +1 reading frame. For pS5B, the vector for the MHV-A59/S chimera+5a(BCoV) mutant, the bovine coronavirus (BCoV) 12.7-kDa protein-encoding gene was synthesized from overlapping oligonucleotides by PCR. Silent changes were made in codons 62 to 64 to eliminate the internal TRS in that gene; additionally, silent changes were made in codons 106 and 108 to eliminate E gene AUG codons in the +1 reading frame. For pS5H, the vector for the MHV-A59/S chimera+5a(HKU1) mutant, human coronavirus HKU1 (HCoV-HKU1) gene 4 was synthesized from overlapping oligonucleotides by

PCR. A silent change was made in codon 108 to eliminate the E gene AUG codon in the +1 reading frame.

All DNA cloning and manipulations were carried out by standard techniques (42). Oligonucleotides for PCR, fragment construction, and DNA sequencing were obtained from Integrated DNA Technologies. The compositions of all plasmid constructs were initially verified by restriction analysis. Then, all PCR-generated segments, cloned cDNA precursors, and reconstructed or newly created junctions of each plasmid were confirmed by DNA sequencing.

Western blotting and RNA analysis. For cell lysate preparation, confluent monolayers (20 cm²) of L2 or 17C11 cells were treated and/or infected as described in Results for particular experiments. At harvest, monolayers were washed twice with phosphate-buffered saline and then lysed by addition of 500 μ l of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% Nonidet P-40, 0.7 μ g/ml pepstatin, 1.0 μ g/ml leupeptin, 1.0 μ g/ml aprotinin, and 0.5 mg/ml Pefabloc SC (Roche). Lysates were held for 15 min on ice and then clarified by centrifugation. For the analysis of RNA degradation, a parallel set of sample monolayers was washed twice with phosphate-buffered saline and lysed by a Nonidet P-40 gentile lysis procedure and RNA was isolated by two extractions each with phenol-chloroform and chloroform, followed by two ethanol precipitations (22).

For Western blot analysis, cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 10% (for N protein, eIF2 α , and PKR) or 15% (for E protein) polyacrylamide gels, with prestained protein standards (Invitrogen) in flanking lanes, and proteins were transferred to polyvinylidene difluoride membranes. Blots were then probed with antibodies, and bound primary antibodies were visualized using a chemiluminescence detection system (ECL; Amersham). The antibodies used were anti-N protein mouse monoclonal J.3.3 antibody (provided by John Fleming, University of Wisconsin, Madison), anti-E protein rabbit polyclonal antibody (25), anti-eIF2 α (total) rabbit polyclonal antibody (Cell Signaling Technology), anti-eIF2 α (phosphoserine-52 specific) rabbit polyclonal antibody (Invitrogen), and anti-PKR (mouse) rabbit polyclonal antibody (Santa Cruz Biotechnology).

For Northern blotting analysis, purified cytoplasmic RNA was fractionated in an agarose gel containing formaldehyde. RNA was blotted and then UV cross-linked onto a Nytran Supercharge membrane (Schleicher and Schuell). A DNA probe corresponding to the 5' end of the murine 18S rRNA (nucleotides [nt] 30 through 529) was generated by PCR and labeled with an AlkPhos Direct kit (Amersham). Hybridization was carried out at 56°C for 18 h, and the blot was washed and developed according to the manufacturer's instructions.

Nucleotide sequence accession number. The complete MHV-S genomic sequence has been deposited in GenBank under accession number GU593319.

RESULTS

IFN sensitivity of MHV-S. Two early studies on the *in vitro* effects of IFN on MHV infection showed significant differences among various strains of MHV, with respect to sensitivity to this antiviral agent (13, 50). Although their results were somewhat discrepant, both reports indicated that MHV-S was more highly sensitive to IFN than were other strains, suggesting to us that MHV-S lacks one or more activities used by MHV to counter the IFN-induced antiviral state. To seek confirmation of those prior results, we adopted a standard protocol in which mouse L2 cell monolayers were pretreated with successively higher doses of mouse IFN- α for 24 h and then infected with MHV at a multiplicity of 2.0 PFU per cell. At 18 h postinfection, released virus was harvested and titers were determined by plaque assay on L2 cells. As shown in Fig. 1, and consistent with other reports (37, 58, 61), we observed MHV-A59 to be relatively resistant to IFN treatment, typically showing an inhibition of 10-fold or less at the highest dose tested (1,000 U/ml). In contrast, MHV-S was markedly inhibited by as little as 10 U/ml IFN and showed a drop of infectious titer on the order of 1,000-fold at the highest dose of IFN. Identical results were obtained when the same experiment was carried out with mouse L929 cells (data not shown). Even in the absence of IFN treatment, MHV-S titers were 5-fold lower than those of MHV-A59. A number of factors could account for this differ-

FIG. 1. Differing IFN sensitivities of MHV-A59 and MHV-S. Mouse L2 cell monolayers were treated with the indicated doses of IFN- α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells. Open and shaded symbols represent results from two separate experiments. In the absence of IFN, the infectious titers were 6.3×10^7 and 6.7×10^7 PFU/ml for MHV-A59 and 1.5×10^7 and 1.0×10^7 PFU/ml for MHV-S. At a dose of 1,000 U/ml IFN, the infectious titers were 7.2×10^6 and 7.3×10^6 PFU/ml for MHV-A59 and 1.9×10^4 and 6.4×10^3 PFU/ml for MHV-S.

ence, but it was possibly due to inhibition of MHV-S by basal levels of ISG expression.

Mapping of the phenotypic difference between MHV-S and MHV-A59. The dramatic divergence in IFN response between these two MHV strains raised the possibility that a genetic approach could be used to localize the basis for that difference. Because the amount of previously available sequence data for MHV-S was limited (26, 31, 59), we determined the entire genomic sequence of this virus. The MHV-S genome was found to have a total length of 31,147 nt (exclusive of polyadenylate), compared with 31,335 nt for MHV-A59, and it is highly homologous to all other strains of MHV whose sequences are available (Table 1). The greatest pairwise identity

of encoded open reading frames (ORFs) between MHV-S and MHV-A59 occurs in the gene 1 replicase polypeptide. Downstream of the replicase gene, sequence identity remains very high but a number of differences can be noted (Fig. 2). The HE gene of MHV-A59 is a pseudogene lacking both an independent TRS and a start codon (27), whereas the MHV-S HE ORF is intact and functional (26). The greatest sequence divergence among the canonical structural genes lies in the spike (S) protein ORF, consistent with the different pathogenicities of MHV-S, which is enterotropic (39), and MHV-A59, which is hepatotropic and neurotropic (56). On the other hand, there are only a minimal number of variant amino acids between the membrane (M) and N proteins of the two viruses, and the somewhat higher divergence of the envelope (E) proteins reflects the fact that the MHV-A59 E protein is five amino acids shorter than that of all other strains of MHV. In both viruses, gene 4 is a pseudogene. For MHV-A59, this status results from an early frameshift which truncates ORF 4 (57); in MHV-S, TRS4 is nonfunctional (59). The most salient dissimilarity between the two viral genomes is the absence in MHV-S of ORF 5a (59) (Fig. 2); this ORF is intact in MHV-A59 and in every other known strain of MHV.

Based on the observed differences in genes other than the replicase gene, we determined whether the relative IFN resistance of MHV-A59 maps to the downstream end of the viral genome. To accomplish this, we used targeted RNA recombination, a method in which transfected synthetic donor RNA is allowed to recombine with coronavirus genomic RNA in infected cells and recombinants are then isolated through screening or by counterselection of some phenotypic property of the recipient virus (28). Initially, we established that IFN pretreatment had a much more pronounced effect on the size of plaques of MHV-S than on those of MHV-A59 (Fig. 3A). Cells infected with MHV-S were thus transfected with RNA transcribed from vector pMH54 (24), which contains the 3'-most 8.6 kb of the MHV-A59 genome, and progeny able to form large plaques were obtained through screening on cells that had been treated with 10 or 100 U/ml IFN- α (Fig. 3B). For eight isolated candidate recombinants (four from either IFN concentration), genomic compositions were analyzed by RT-PCR using primers in gene 4 and in the E gene that bounded

TABLE 1. Percent amino acid identity between MHV-S proteins and those of other strains of MHV^a

Strain	% Amino acid identity MHV-S protein:								
	1a	1b	2a	HE	S	4 ^b	E	M	N
MHV-A59	97.2	98.6	91.3	93.6 ^b	80.8	92.4 ^b	88.6	97.4	96.0
MHV-JHM	95.8	98.5	94.3	94.5	82.3	91.8	94.3	96.9	94.9
MHV-1	95.6	98.2	91.3	92.4 ^b	91.3	90.2	93.2	98.2	94.5
MHV-3	93.7	97.7	90.9	86.0 ^b	83.2	90.2	94.3	97.8	95.2
MHV-2	92.8	97.9	92.5	73.9 ^b	93.2	91.0 ^b	94.3	96.1	94.1
MHV-DVIM	NA ^c	NA	91.7	72.1	94.9	91.0	96.6	99.6	94.4
SDAV	NA	NA	90.2	91.6	92.4	90.2	94.3	96.1	91.9
MHV-RI	NA	NA	NA	73.3	83.0	NA	NA	95.2	96.3
MHV-TY	NA	NA	NA	NA	95.2	NA	NA	97.4	94.5
MHV-Y	NA	NA	NA	NA	95.9	95.1	98.9	97.4	95.2

^a GenBank accession numbers: MHV-S, GU593319; MHV-A59, AY700211; MHV-JHM, FJ647226; MHV-1, FJ647223; MHV-3, FJ647224; MHV-2, AF201929; MHV-DVIM, AY771998; SDAV, AF207551; MHV-RI, AF091735, U14645, L37757, and L37758; MHV-TY, AF190406, AF190407, and AF190408; MHV-Y, U14646, AY525270, L37756, and L37759.

^b Pseudogene.

^c NA, sequence not available.

FIG. 2. Comparison of the genomic compositions of MHV-A59 and MHV-S. For each strain, the region downstream of replicase gene 1 is shown. Rectangles represent ORFs for structural and accessory proteins; dotted lines indicate pseudogenes. In each MHV-S ORF, vertical lines indicate amino acid residues that are nonidentical to those of MHV-A59; note that this representation accentuates differences between the ORFs of the two strains (see Table 1). Open circles represent TRSs; the filled circle marks the nonfunctional mutant TRS upstream of MHV-S gene 4. The solid triangle highlights the deletion of gene 5a in MHV-S.

the gene 5a region (Fig. 3C). All eight candidates gave rise to the same 413-bp product as MHV-A59, rather than the 108-bp product obtained with MHV-S. This indicated that each recombinant had acquired at least the genes downstream of gene 4 from the MHV-A59 parent. Further analysis, through sequencing of RT-PCR products obtained from the HE gene region, revealed that the eight MHV-S/A59 chimeric recombinants had been produced by five independent crossover events, all of which occurred between nucleotides 136 and 598 of the HE gene of MHV-S. The IFN dose responses of two independent recombinants, 4 and 8, were compared to those of the two parent viruses, and both recombinants were found to have the same relative IFN resistance as MHV-A59 (Fig. 3D). These results showed that one or more MHV-A59 genes downstream of gene 2a suffice to completely counteract the IFN sensitivity of MHV-S.

To further localize the gene(s) responsible, we created a reciprocal chimera (MHV-A59/S) composed of MHV-A59 containing the MHV-S counterparts of all of the genes downstream of the S gene, including the mutant TRS4 of MHV-S (Fig. 4A). This and all subsequent mutants were generated by standard targeted RNA recombination using host range-based selection via the interspecies coronavirus fMHV, which was transduced with donor RNA synthesized from constructed derivatives of the pMH54 transcription vector (14, 24, 28). The IFN dose response of the MHV-A59/S chimera was nearly indistinguishable from that of MHV-S (Fig. 4B). Identical results were obtained with a second, independent isolate of the same chimeric mutant (data not shown). These results showed that all, or almost all, of the IFN sensitivity of MHV-S is due to one or more genes downstream of the S gene. In particular, the possibility that the S protein plays a significant role in IFN antagonism could be ruled out.

Testing of the role of gene 5a. Since the absence of gene 5a stood out as the most notable difference in MHV-S with respect to MHV-A59, we next constructed a mutant of MHV-A59 containing a knockout of gene 5a (Fig. 5A). Disruption of this gene was carried out through replacement of the ORF 5a start codon with CUA, which is not known to be used as an alternative eukaryotic translation initiation codon (reference 7

and references therein). It should be noted that ORF 5a does not contain any internal AUG codons. Additionally, we replaced the fourth codon of ORF 5a with a stop codon. Because of the small overlap between ORFs 4 and 5a, coding changes were concomitantly generated in the final sense codon and the stop codon of ORF 4, but these were silent (Fig. 5A). The resulting MHV-A59 5a-KO mutant was found to be much more sensitive to the action of IFN than was MHV-A59; however, it was not as severely inhibited by IFN as was MHV-S. On a logarithmic scale, the IFN dose response of the 5a-KO mutant fell roughly midway between those of the two parent strains (Fig. 5B). This magnitude of response indicated that accessory protein 5a is a major IFN antagonist for MHV, but there may be additional factors that contribute to the high IFN sensitivity of MHV-S.

The MHV E gene, which was originally designated gene 5b, is the second ORF in subgenomic RNA5 (1, 2, 46). Evidence has been presented that translation of the E protein is initiated by means of an internal ribosome entry site (IRES) that encompasses part of gene 5a (17, 52). Although the 5a-KO mutations fell 140 nt upstream of the 5' boundary of the IRES, we wished to determine whether synthesis of E protein had been inadvertently altered as a consequence of the 5a-KO mutations. Western blot analysis of lysates from cells infected with the 5a-KO mutant or with wild-type MHV-A59, at two different multiplicities of infection, revealed that no such alteration had occurred. The mutant and the wild type produced equivalent levels of E protein relative to N protein (Fig. 5C). Thus, E protein expression was not significantly affected in the 5a-KO mutant, and the IFN sensitivity phenotype of this virus could be attributed solely to the disruption of 5a protein expression. Consistent with that conclusion, we found that MHV-S could not be rescued from the inhibitory action of IFN by growth in a line of 17Cl1 cells that constitutively expresses the MHV E protein (25; data not shown).

To examine whether differences between the N proteins of MHV-A59 and MHV-S could account for the remainder of the greater IFN sensitivity of the latter strain, we generated mutants of MHV-A59 containing the N gene of MHV-S, in the presence or absence of the 5a-KO mutations (Fig. 6A). The

FIG. 3. Mapping of the IFN resistance of MHV-A59. (A) Differential effect of IFN on the sizes of plaques formed by MHV-A59 and MHV-S. L2 cells were treated with 0, 10, or 100 U/ml IFN- α for 24 h prior to the performance of standard plaque assays. Monolayers were stained with neutral red at 48 h postinfection and photographed 4 h later. (B) Targeted recombination between MHV-S genomic RNA and synthetic donor RNA transcribed from pMH54, which contains all of the MHV-A59 genes downstream of the HE gene (24). Progeny obtained from infected and transfected cells were screened on L2 cells that had been pretreated with 10 or 100 U/ml IFN- α , and candidate recombinants were isolated as viruses that formed large plaques. (C) Analysis of eight candidate recombinants. In the schematic, vertical lines in each MHV-S ORF indicate amino acid residues that are nonidentical to those of MHV-A59. Note that primers (arrows) are not drawn to scale; the two primer hybridization sites are identical between MHV-A59 and MHV-S. RT-PCR products crossing the gene 5a region were analyzed by agarose gel electrophoresis; the sizes (base pairs) of markers in the first lane are indicated to the left of the gel. Denoted beneath the gel are the five independent groups (a to e) into which the recombinants were found to fall, based on sequencing of the crossover sites in the HE gene. (D) IFN dose responses of independent recombinants 4 and 8, compared to MHV-A59 and MHV-S. Mouse L2 cell monolayers were treated with IFN- α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells.

actual N chimeric construct contained a substitution encompassing 17 out of the 18 N protein amino acid residues that are different in MHV-S, and it also included the final residue of the MHV-S M protein (I228) in place of T228 of the MHV-

A59 M protein. The N chimera mutant alone exhibited an IFN dose response indistinguishable from that of its MHV-A59 parent (Fig. 6B). This suggested that sequence differences between the MHV-A59 and MHV-S N proteins do not make a

FIG. 4. Mapping of the IFN sensitivity of MHV-S. (A) Schematic comparison of the genomes of MHV-A59, MHV-S, and the chimera MHV-A59/S; the latter was constructed by targeted RNA recombination as described in Materials and Methods. MHV-A59/S contains, in an otherwise MHV-A59 background, all of the MHV-S sequence downstream of the S gene (including nonfunctional TRS4 of MHV-S). The 3' untranslated region of the chimeric virus corresponds to that of MHV-A59 but differs from the MHV-S 3' untranslated region by only 1 nt. (B) IFN dose response of MHV-A59/S, compared to those of MHV-A59 and MHV-S. Mouse L2 cell monolayers were treated with IFN- α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells. Open and shaded symbols represent results from two separate experiments.

substantial contribution to the IFN sensitivity of MHV-S. Nevertheless, there was a small enhancement of the IFN sensitivity of the 5a-KO mutant when the gene 5a knockout mutations were paired with the MHV-S N gene. However, such an effect was less apparent in an independent replicate of the experiment that is shown in Fig. 6B. Thus, although the MHV N protein may act as an IFN antagonist (58), differences between the N proteins of MHV-A59 and MHV-S do not make a strong contribution to the phenotypic difference between these two strains.

As a direct test of whether the 5a protein can mitigate the inhibition of MHV-S by IFN, we introduced gene 5a at an ectopic position in the genome of the MHV-A59/S chimera. This insertion was accomplished through replacement of the 2a-HE transcription unit with a rebuilt transcription unit containing the MHV-A59 gene 5a (Fig. 7A). We found that such a reconstitution produced a virus [MHV-A59/S chimera+5a(MHV)] that was refractory to the action of IFN to the same extent as was the wild-type MHV-A59 parent (Fig. 7C). In contrast, as we had observed before (Fig. 4B), the MHV-A59/S chimera exhibited a sensitivity to IFN as severe as that of MHV-S. To further assess the function of accessory protein 5a, we constructed additional derivatives of the MHV-A59/S chimera harboring the gene 5a homolog from BCoV or from HCoV-

HKU1. These related accessory proteins have 51% and 49% sequence identity, respectively, with their MHV-A59 counterpart (Fig. 7B). The three gene 5a substitution mutants were assessed for their sensitivity to an intermediate dose of IFN (100 U/ml) in comparison to wild-type MHV-A59 and the MHV-A59/S chimera (Fig. 7D). As an additional control, we examined the IFN sensitivity of an MHV-A59 Δ 2a-HE mutant (Fig. 7A). The latter virus was somewhat more sensitive to IFN than was wild-type MHV-A59, a finding that could indicate that accessory protein 2a plays some role in IFN antagonism. (The HE protein is not expressed in MHV-A59.) The 2a gene has previously been shown to be nonessential for replication in tissue culture (44), but its deletion or mutation does affect pathogenesis in the mouse host (8, 38). Despite the lack of gene 2a, each of the constructed recombinants that contained a 5a protein homolog was able to overcome most of the inhibition of the MHV-A59/S chimera caused by IFN (Fig. 7D). This result demonstrated that MHV accessory protein 5a is a member of a family of related coronavirus proteins that counteract one or more of the antiviral activities induced by IFN.

Noninvolvement of the protein kinase R (PKR)/eukaryotic initiation factor 2 α (eIF2 α) system or the oligoadenylate synthetase (OAS)/RNase L system in the differing IFN sensitivities of MHV-S and MHV-A59. IFN treatment of cells leads to

FIG. 5. IFN sensitivity of the MHV-A59 gene 5a knockout mutant. (A) Construction of the 5a-KO mutant. Shown above the schematic of the MHV-A59 genome is the wild-type sequence of the gene 4-gene 5a junction. TRS5 is located within ORF 4, 32 nt upstream of the sequence segment shown. Indicated below the genome are four point mutations made to eliminate the start codon of ORF 5a and to replace the fourth codon with a stop codon. Mutations were constructed in donor RNA transcription vector pMH54 (24) and incorporated into MHV-A59 via targeted RNA recombination as described in Materials and Methods. (B) IFN dose response of the 5a-KO mutant, compared to those of MHV-A59 and MHV-S. Mouse L2 cell monolayers were treated with IFN- α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells. Open and shaded symbols represent results from two separate experiments. (C) Western blot assays of lysates from 17C1 cells that were either mock infected or infected with wild-type MHV or the 5a-KO mutant at a multiplicity of 1.0 or 0.2 PFU per cell. Blots were probed with polyclonal anti-MHV E antiserum (25) or with monoclonal anti-N antibody J.3.3.

the induction of more than 200 ISGs, the products of a number of which have been shown to have antiviral activity (19, 33, 40, 43). The two earliest-discovered and most extensively studied among these IFN-induced antiviral proteins are PKR and OAS. PKR becomes activated through autophosphorylation, which is triggered by its binding to double-stranded RNA (dsRNA). Activation enables PKR to phosphorylate its substrates, which include the α subunit of the pivotal translation initiation factor eIF2. Phosphorylation causes eIF2 to bind essentially irreversibly to the nucleotide exchange factor eIF2B, thereby preventing recycling of eIF2 and shutting down host cell protein synthesis. To assess the potential role of PKR during MHV infection, we examined PKR and eIF2 α by Western blot analysis of lysates prepared from cells treated under conditions identical to those used in our IFN dose-response experiments. In both mock-infected and infected cells, the latter at both 6 and 10 h postinfection, there occurred a

marked induction of PKR by IFN (Fig. 8A). However, in IFN-treated cells, we observed no differences between PKR in mock-infected cells and PKR in cells that were infected with MHV-A59, MHV-S, or the MHV-A59/S chimeric virus. In particular, we did not detect a more slowly migrating band corresponding to the phosphorylated form of PKR, even though samples were resolved by SDS-PAGE on long (20-cm) gels. Consistent with this finding, there were no significant differences in the level of phosphorylated eIF2 α between uninfected cells and cells infected with either MHV-A59 or MHV-S, irrespective of IFN treatment or time postinfection (Fig. 8B). In contrast, a high level of phosphorylated eIF2 α was seen in uninfected cells that had been treated with arsenite, which effects eIF2 α phosphorylation by a stress pathway independent of PKR (20).

The second well-characterized IFN-induced antiviral activity, OAS, also becomes activated by its binding to dsRNA.

FIG. 6. IFN sensitivities of chimeras of MHV-A59 containing the MHV-S N gene in the presence or absence of the gene 5a knockout. (A) Schematics of the 3' ends of mutant genomes, compared to those of MHV-A59 and MHV-S. In each MHV-S ORF, vertical lines indicate amino acid residues that are nonidentical to those of MHV-A59; X denotes the 5a-KO mutations shown in detail in Fig. 5A. The N chimera mutant (and the 5a-KO+N chimera mutant) contain all of the amino acid residues of the MHV-S N protein that differ from those of the MHV-A59 N protein, except for V321A; additionally, the carboxy-terminal residue of the M protein (T228) is changed to its MHV-S counterpart (I228). Mutants were constructed by targeted RNA recombination as described in Materials and Methods. (B) IFN dose responses of chimeras, compared to those of MHV-A59, MHV-S, and the 5a-KO mutant. Mouse L2 cell monolayers were treated with IFN- α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells. Open and shaded symbols represent results from two separate experiments.

Activated OAS synthesizes a series of 2'-5'-linked adenylate oligomers, which then bind to and activate a constitutive enzyme, RNase L. This endoribonuclease targets both cellular and viral RNA and is commonly assayed through monitoring of rRNA decay (30, 41, 43, 45, 58). To assess the potential role of the OAS/RNase L system during MHV infection, we examined the integrity of total RNA isolated at 10 h postinfection from untreated and IFN-treated cells that had been infected with MHV-A59, MHV-S, or the MHV-A59/S chimeric virus (Fig. 8C). We observed no differences among any of these samples, as analyzed by agarose gel electrophoresis. In particular, there was no enhancement of degradation of rRNA in IFN-treated cells that were infected with MHV-S or with the MHV-A59/S chimera. To further test this result, in a separate experiment, IFN-treated cells were infected with the same set of viruses and also with encephalomyocarditis virus, reovirus, or mengovirus as controls. RNA was again isolated and analyzed at 10 h postinfection. For the latter three viruses, minor additional bands could be detected by agarose gel electrophoresis, relative to the mock-infected control (data not shown). To ascertain the origin of these extra species, we performed Northern blotting with a probe specific for murine 18S rRNA and found that RNA from MHV-infected cells did not differ markedly from RNA from mock-infected cells (Fig. 8D). In contrast, for cells infected with either of the control viruses, each of which is known to activate the OAS/RNase L system (30, 41, 45), distinct breakdown products of the 18S rRNA were noted.

Taken together, these results showed that the PKR/eIF2 α and OAS/RNase L systems are not differentially affected in IFN-treated cells that have been infected with MHV-A59 or MHV-S. Specifically, this outcome suggests that the 5a protein does not mediate its antagonistic effect against IFN through interaction with either of these antiviral systems.

DISCUSSION

Coronaviruses, like many other viruses, have evolved mechanisms to circumvent, at multiple levels, the innate immunity of their hosts. Although an intact type I IFN system is essential for the clearance of MHV-A59 infection *in vivo* (3, 4, 16, 21, 36), this virus almost entirely sidesteps the IFN response *in vitro*. Many strains of MHV avoid inducing IFN- β in fibroblasts or dendritic cells *in vitro*, but the exact basis for this capability is unresolved and may vary in different cell types. During MHV infection, activation and nuclear translocation of the factors NF- κ B and IRF-3, as well as the resulting transcription of IFN- β , are either delayed (37) or completely suppressed (61, 62). These forestalling effects have been proposed to be largely passive, since MHV infection does not block IRF-3 nuclear translocation and IFN- β transcription induced by poly(I:C) treatment or by coinfection with either of the paramyxoviruses Newcastle disease virus or Sendai virus (37, 53, 62). Additionally, however, MHV infection obstructs expression of IFN- β at some posttranscriptional step, and this obstruction appears to arise from an active mechanism of inhibition, given that the induction of IFN- β protein by coinfecting Newcastle disease virus or Sendai virus is also decreased (37). Another group 2 coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV), exhibits much greater sensitivity to the antiviral action of IFN than does MHV (6, 48). However, as observed with MHV, SARS-CoV infection does not induce IFN- β . It is unclear whether this failure to induce is because IRF-3 is not translocated to the nucleus of SARS-CoV-infected cells (53) or because IRF-3, although translocated, is prevented from becoming activated (47).

A number of coronavirus gene products have been implicated in the disruption of signal transduction events required

FIG. 7. Effect of substitution of gene 5a in the gene 2 region of the MHV-A59/S chimera. (A) Schematic comparison of the genomes of MHV-A59, the MHV-A59Δ2a-HE mutant, the MHV-A59/S chimera, and mutants in which the 2a-HE region of the MHV-A59/S chimera was replaced with gene 5a of MHV-A59 or the homolog of gene 5a from BCoV or HCoV-HKU1. Deletion and substitution mutants were constructed by targeted RNA recombination as described in Materials and Methods. (B) Amino acid sequence alignment of the MHV-A59 5a protein and its homologs from BCoV (12.7-kDa protein) and HCoV-HKU1 (ORF 4 product). The GenBank accession numbers of the sequences shown are as follows: MHV-A59, AY700211; BCoV, U00735; HCoV-HKU1, AY597011. Circles denote residues that are conserved among all group 2a coronaviruses. (C and D) Relative IFN resistances of gene 5a substitution mutants. The graph (C) shows the IFN dose response of the MHV-A59/S+5a(MHV) substitution mutant, compared to MHV-A59 and the MHV-A59/S chimera. Mouse L2 cell monolayers were treated with IFN-α for 24 h and then infected at a multiplicity of 2.0 PFU per cell. Virus was harvested at 18 h postinfection, and infectious titers were determined by plaque assay on L2 cells. Open and shaded symbols represent results from two separate experiments. Histograms (D) show the relative infectious titers (compared to untreated samples) for all three of the 5a homolog substitution mutants, compared to those of the control viruses, at an IFN-α dose of 100 U/ml. Each value is the mean of three replicate samples ± the standard deviation.

for the IFN response. For both MHV and SARS-CoV, one such gene product is nsp1, the first mature polypeptide processed from the replicase polyprotein. Expressed nsp1 has been shown to antagonize the induction of IFN-β, as well as downstream events required for ISG induction, through inhibition of the expression and activation of multiple transcription factors (55, 64). Most of these blocking effects likely result from the widespread suppression of host protein synthesis that

nsp1 brings about via host mRNA degradation (18, 29). That this role pertains in the intact virus has been shown through the construction of nsp1 mutants that both induce and are inhibited by IFN (29, 55) and, in the case of MHV, are highly attenuated *in vivo* (64). In addition to nsp1, other SARS-CoV proteins act as IFN antagonists when transiently expressed. The N protein and the products of accessory genes 3b and 6 have all been found to prevent synthesis of IFN-β through

FIG. 8. Status of the PKR/eIF2 α and OAS/RNase L antiviral systems in MHV-infected cells. Mouse L2 cell monolayers were left untreated or treated with 100 U/ml IFN- α for 24 h. Monolayers were then mock infected or infected with MHV-A59, MHV-S, or the MHV-A59/S chimera at a multiplicity of 2.0 PFU per cell. Cell lysates were prepared at 6 or 10 h postinfection. One untreated, mock-infected sample was treated with 0.5 mM sodium *m*-arsenite for 30 min prior to harvesting to provide a positive control for eIF2 α phosphorylation (10). Lysates were analyzed by Western blot assays probed with antibodies specific for PKR (A) or for total or phosphorylated eIF2 α (B). (C) For one set of samples, cells were harvested at 10 h postinfection and total cellular RNA was purified and analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide. The sizes (base pairs) of double-stranded DNA markers in the first lane are indicated to the left of the gel; the positions of the 28S and 18S rRNAs are shown at the right. (D) In a separate experiment, total cellular RNA was purified at 10 h postinfection from IFN-treated cells that had been mock infected or infected with MHV-A59, MHV-S, the MHV-A59/S chimera, encephalomyocarditis virus (EMCV), reovirus type 3, or mengovirus. RNA was separated by electrophoresis in a 1.2% agarose gel containing formaldehyde and analyzed by Northern blotting using a probe specific for the 18S rRNA.

inhibition of the function of IRF-3 (23). N protein additionally inhibits NF- κ B, and accessory proteins 3b and 6 inhibit signaling required for induction of ISGs. Finally, the papain-like proteinase (PLP) domain of SARS-CoV nsp3 is also reported to hinder the activation of IRF-3 and NF- κ B (9, 12, 60). It has not yet been established with viral mutants whether any of the

latter mechanisms are operative during SARS-CoV infection; for N and PLP, which are essential proteins, this will be difficult to demonstrate. In contrast to the attention that has been devoted to coronavirus antagonism of IFN induction and signal transduction leading to ISG expression, only one prior report has addressed

the means by which a coronavirus evades or counteracts the antiviral state that is generated by IFN. In that study, Ye and coworkers showed that incorporation of the MHV N gene into the vaccinia virus Δ E3L mutant was sufficient to render that mutant IFN resistant (58). The Δ E3L virus is highly sensitive to IFN because it lacks a dsRNA binding protein that inhibits PKR and OAS, in part by sequestering dsRNA (19, 33, 43). It was also shown that transient expression of N protein could partially rescue the abrogation of protein synthesis seen in cells infected with the Δ E3L mutant and could concomitantly prevent RNase L-mediated degradation of rRNA. These findings led to the proposal that N protein prevents activation of the OAS/RNase L system, and possibly also the PKR/eIF2 α system, by binding to dsRNA. It remains to be determined whether this potential mechanism of IFN antagonism operates during MHV infection.

In the current study, we have shown that gene 5a of MHV potently counteracts the antiviral action of IFN against MHV. This property of gene 5a was demonstrated through mapping of the essential differences between two strains, MHV-A59 and MHV-S, the latter of which exhibits orders-of-magnitude greater sensitivity to IFN than the former (Fig. 1). Selection of interstrain chimeric recombinants of MHV-S that had acquired relative resistance to IFN showed that one or more MHV-A59 genes downstream of gene 2a were sufficient to confer such resistance (Fig. 3). Conversely, transfer to MHV-A59 of the MHV-S genes downstream of gene 4 imparted an IFN-sensitive phenotype to MHV-A59 (Fig. 4), which further delimited the responsible genomic locus. Knockout of gene 5a of MHV-A59, the gene which represents the most prominent difference between the two strains, showed that the 5a protein accounts for a major part of the ability of MHV to counter the action of IFN (Fig. 5). Finally, restoration of gene 5a at an alternative genomic position was sufficient to rescue the MHV-A59/S chimera from inhibition by IFN (Fig. 7). To our knowledge, this is the first demonstration of a coronavirus gene product that can protect that same virus from antiviral activity(ies) induced by IFN. Our results provide a likely explanation for the earlier finding that a gene 4-5a deletion mutant of MHV-A59 was highly attenuated with respect to the wild type when inoculated intracranially into mice (8). Similarly, the absence of gene 5a correlates with the previously reported extremely low virulence of MHV-S in the mouse host (51).

Although gene 5a plays a predominant role in the relative IFN resistance of most strains of MHV, knockout of this gene did not render MHV-A59 as fully susceptible to IFN as is MHV-S. Thus, other gene products that differ between the two strains cannot be ruled out as contributing to IFN antagonism. The N gene of MHV-S, when its presence was coupled with the 5a knockout mutations in an MHV-A59 background, appeared to augment IFN sensitivity (Fig. 6). However, this effect was not consistently observed and may depend on experimental variables that we cannot yet define. Moreover, an MHV-A59 mutant containing only the MHV-S N gene was as resistant to IFN as was wild-type MHV-A59. The latter finding does not argue against a role for N protein as an IFN antagonist (58), but it does show that differences in their respective N proteins do not make a major contribution to the contrasting IFN responses of the two MHV strains. It is conceivable that, in the absence of gene 5a, the role of N protein becomes more im-

portant in the sequestration or masking of viral RNA species that are capable of activating IFN-induced antiviral enzymes.

Synthesis of MHV 5a protein has previously been demonstrated *in vitro* (1, 2) but not unequivocally *in vivo*, owing to the unavailability of specific antisera (46). Our identification of gene 5a as an IFN antagonist raised the possibility that this gene acts indirectly, through its putative role as an IRES for translation of the E ORF (17, 52), rather than through its own protein product. However, we found that production of E protein was not significantly altered in the 5a-KO mutant (Fig. 5C), supporting the idea that disruption of 5a protein expression is indeed the cause of the IFN sensitivity of that mutant. An independent role for gene 5a, other than its IRES function, is also suggested by the observation that some group 2a coronaviruses (BCoV and HCoV-OC43) synthesize separate mRNAs for 5a and E. Consistent with this, it has been shown that MHV-S-infected cells contain ample amounts of subgenomic E mRNA relative to other viral mRNAs (5), thus arguing that the basis of the IFN sensitivity of MHV-S is not likely to be underexpression of E protein.

The most compelling evidence that gene 5a acts at the protein level, rather than at the RNA level, comes from our demonstration that insertion of gene 5a at a position some 4.6 kb upstream of the E gene confers IFN resistance on the otherwise highly sensitive MHV-A59/S chimera (Fig. 7A and C). This protection against IFN action must be independent of the putative *in situ* role of gene 5a as an IRES in MHV-A59. Additionally, we showed that the homologs of the 5a protein from BCoV and HCoV-HKU1, which are ~50% divergent from MHV 5a and from one another in amino acid sequence, also act as IFN antagonists. Collectively, all of the homologs of the 5a protein, which are found only in the group 2a coronaviruses, range from 107 to 112 amino acids in length and have a core of 30 invariant residues, including three cysteines (Fig. 7B). Because all coronaviruses have the same essential molecular and cellular biology, we would expect them to have the same inherent vulnerabilities with respect to the IFN system. Since no homologs of gene 5a are present in the group 2b coronaviruses (which include SARS-CoV and related viruses) or in the group 1 or group 3 coronaviruses, it therefore appears that some defenses against innate immunity are idiosyncratic across the different groups and subgroups of this family.

It is not yet clear which of the multiple ISG products can act against MHV, either *in vivo* or in tissue culture, and which of those is the target(s) of the 5a protein. In agreement with Ye and coworkers (58), we found that the PKR/eIF2 α and OAS/RNase L systems were not activated during MHV-A59 infection of IFN-treated cells. The same lack of activation has been reported for MHV-1-infected cells (63). Moreover, we observed that neither of the two antiviral systems was differentially affected between MHV-A59-infected cells and MHV-S-infected cells. These results may indicate that neither system has the capability of acting against MHV. It has been proposed that the dsRNA produced during coronavirus infections is shielded from host cell sensors (53, 62). Possibly, viral dsRNA is sequestered by N protein (58) or is masked within membranous replication compartments that are not accessible to PRRs or to dsRNA-activated antiviral enzymes. Alternatively, the PKR/eIF2 α and OAS/RNase L systems may be able to inhibit MHV, but their activation is blocked by one or more activities

that MHV-A59 and MHV-S possess in common. In either case, it appears that neither of these two most extensively studied antiviral systems is affected by the presence or absence of the 5a protein.

A number of additional ISG products with antiviral activity have been described and characterized to various extents, including ISG15, ISG20, MxA, ADAR1, viperin, and tetherin (19, 33, 40, 43); some of them have been found to be upregulated in MHV-infected mice (32). It remains to be determined which of these factors can exert significant control over MHV infections, either in immortalized cell lines or in primary cells (35), and whether all coronaviruses will turn out to be sensitive to the same subset of ISGs.

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