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NOTES

Replicase Genes of Murine Coronavirus Strains A59 and JHM Are Interchangeable: Differences in Pathogenesis Map to the 3′ One-Third of the Genome[▽]

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The important roles of the spike protein and other structural proteins in murine coronavirus (MHV) pathogenesis have been demonstrated; however, the role of the replicase gene remains unexplored. We assessed the influence of the replicase genes of the highly neurovirulent MHV-JHM strain and the hepatotropic and mildly neurovirulent A59 strain in acute infection of the mouse. Analysis of chimeric A59/JHM recombinant viruses indicates that the replicase genes are interchangeable and that it is the 3′ end of the genome, encoding the structural proteins, rather than the replicase gene, that determines the pathogenic properties of these chimeras.

Coronaviruses form a group of pathogenic, enveloped, single-stranded, positive-sense RNA viruses. Coronaviruses induce acute self-limited and chronic persistent infections. Mouse hepatitis virus (MHV) is the prototype of group 2 coronaviruses. Various strains of MHV induce different patterns of pathogenesis. The JHM strain is a highly neurovirulent strain that causes severe acute encephalitis and chronic demyelination, but not hepatitis, while the A59 strain is dualtropic, causing moderate to severe hepatitis, mild to moderate acute meningoencephalitis, and chronic demyelination in C57BL/6 mice. Using a combination of targeted RNA recombination to precisely manipulate the coronavirus genome and in vivo approaches (the mouse model), we have previously reported that the coronavirus spike protein is a major determinant of pathogenesis (14, 15, 17, 18). Interestingly, we have also found that expression of the "hepatotropic" A59 spike glycoprotein within the background of the "neurotropic" JHM strain does not reproduce the A59 hepatotropic phenotype (14). Furthermore, expression of the JHM spike within the A59 background does not reproduce the rapid kinetics of mortality or the type of

immune response following JHM infection of the mouse (5, 11, 20). Thus, our studies demonstrated that genes other than the spike gene play a role in coronavirus tropism and virulence (14). These results prompted us to further investigate the roles of the structural and nonstructural genes in coronavirus pathogenesis.

The replicase of coronaviruses is encoded by gene 1, the 5′ two-thirds of the genome (21 kb out of 32 kb for murine coronavirus), and is comprised of a protein complex of up to 16 viral subunits that together with a number of cellular proteins forms the replicase complex (22). The roles of the replicase proteins in coronavirus pathogenesis remain poorly understood. In the present study, we sought to determine the contribution of the replicase gene to neurovirulence and to the outcome of hepatitis. Using a reverse genetics system (8), we generated isogenic recombinant MHV-A59 and -JHM viruses that differ only in the replicase gene. We generated chimeric recombinant A59 viruses that express the replicase gene of the nonhepatotropic JHM strain (repJHM-RA59) and chimeric recombinant JHM viruses that express the replicase gene of the hepatotropic A59 strain (repA59-RJHM) (Fig. 1). These chimeric recombinant viruses were compared to wild-type A59 and JHM recombinant viruses (RA59 and RJHM). (The so-called wild-type recombinant viruses [RA59 and RJHM] encode the same genes as and exhibit phenotypes indistinguishable from those of the corresponding nonrecombinant wild-type viruses, as we have described previously [13, 14].) Our data demonstrate that the differences between A59 and JHM replication kinetics in vitro, pathogenesis, and viral load in vivo are not determined by their replicase gene. Rather, the 3′ one-third of the murine coronavirus genome (spike gene

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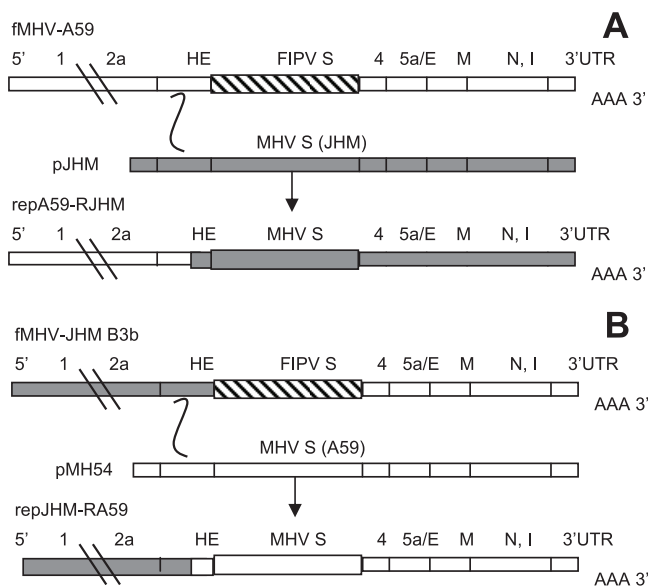▽ Published ahead of print on 1 November 2006.

FIG. 1. Scheme of targeted RNA recombination. Feline cells (FCWF) were infected with fMHV-A59, a chimeric recombinant MHV-A59 virus expressing the feline infectious peritonitis virus (FIPV) spike (A), or with fMHV-JHM B3b, a chimeric recombinant MHV-JHM virus expressing the FIPV spike (B), and then electroporated with pJHM-derived (A) or pMH54-derived (B) in vitro-transcribed RNA to generate recombinant JHM viruses expressing the replicase gene of A59 (repA59-RJHM) (A) and A59 viruses expressing the replicase gene of JHM (repJHM-RA59) (B). Infected and electroporated FCWF cells were overlaid onto murine L2 cells, and recombinant viruses were selected for their ability to infect murine cells as previously described (15). Four independent chimeric viruses and two independent wild-type recombinant viruses per construct were generated and characterized in vitro and in vivo. The positions of open reading frames (ORF1, ORF2a, ORF4, and ORF5a), genes, plasmids, and 3′ untranslated region (3′UTR) are indicated.

through the 3′ end [Fig. 1]) determines replication kinetics, virulence, and pathogenesis.

Selection of isogenic chimeric recombinant murine coronaviruses that differ in the replicase gene. Figure 1 shows a scheme of targeted RNA recombination. Targeted RNA recombination allows the manipulation of all open reading frames (ORFs) downstream of the hemagglutinin esterase (HE) gene (spike, ORF4, ORF5a, small membrane [E], membrane [M], nucleocapsid [N], and the internal I protein encoded within N). In order to generate A59 and JHM chimeric viruses differing only in the replicase (gene 1), targeted recombination was carried out between interspecies chimeric helper viruses in the A59 (fMHV-A59) and JHM (fMHV-JHM B3b) genetic backgrounds and in vitro-transcribed RNA derived from transcription vectors pMH54 (A59) and pJHM (JHM) as previously described (14, 15). Thus, the A59 and JHM replicase genes and ORF2a were provided by the helper viruses fMHV-A59 and fMHV-JHM B3b, respectively, and genes downstream of HE were provided by the transcription vectors pMH54 (A59) and pJHM (JHM). For each recombinant type virus, four chimeric (repJHM-RA59 and repA59-RJHM), and two wild-type (RA59 and RJHM) independent recombinant viruses were evaluated in order to minimize the possible interference of spurious mutations. Note that independent recom-

binant viruses of the same genotype are not distinguished by name. In each experiment, data for one representative of each genotype are shown.

The entire spike and portions of the M and N genes of the chimeric viruses were sequenced to verify the correct genetic composition of each chimeric recombinant. Portions of the HE gene of each chimeric recombinant were also sequenced. Due to the composition of the transcription vectors and selection against the feline coronavirus spike (Fig. 1), the crossovers in the recombinant genomes must occur within the HE gene. All the viruses in the A59rep-RJHM group have the 5′ end of the HE gene derived from A59 and the 3′ end from JHM, with crossovers between nucleotides 1000 and 1100 (from the 5′ end of the HE gene) (data not shown). Conversely, all the viruses in the JHMrep-RA59 group have the 5′ end of the HE gene derived from JHM and the 3′ end from A59, with crossovers before nucleotide 1000 (data not shown).

Viral kinetics in vitro. Cells were infected at a multiplicity of infection of 1 PFU/cell, and the time course of released and cell-associated virus in murine fibroblast cells (L2) was determined by plaque assay (Fig. 2) as previously described (15). MHV-A59 and -JHM viruses exhibit very different replication kinetics in vitro. A59 replicates to higher titer, whereas JHM replicates with slower kinetics and to a lower final titer and displays higher levels of cell-to-cell fusion and cytotoxicity (3). We have previously reported that RA59 and RJHM recombinant viruses generated by targeted RNA recombination mimic in vitro phenotypes of wild-type A59 and JHM viruses (14, 17). In this study, we found that the replication kinetics of chimeric recombinant A59 viruses expressing the replicase gene of JHM (repJHM-RA59) were very similar to those of RA59 (Fig. 2). In contrast, chimeric recombinant JHM viruses expressing the A59 replicase (repA59-RJHM) replicated with delayed kinetics and to a lower final titer compared to RJHM (Fig. 2). These results demonstrate that, at least in the cell type used here, structural genes, rather than the replicase gene, have a major role in A59 and JHM replication kinetics. We have previously shown that expression of the A59 spike in the JHM background is not sufficient to reproduce the efficient replication of A59 (14).

Neurovirulence. MHV-A59 is dualtropic, causing mild to moderate hepatitis, acute meningoencephalitis, and chronic demyelination in C57BL/6 mice. In contrast, MHV-JHM is a highly neurovirulent strain that causes severe acute and fatal encephalitis, but not hepatitis. We have previously generated recombinant wild-type A59 and JHM viruses (RA59 and RJHM) and demonstrated that these recombinant wild-type viruses mimic the pathogenesis induced by the nonrecombinant A59 and JHM strains (14, 18). To determine the virulence of chimeric recombinant A59 and JHM viruses expressing the replicase gene of JHM (repJHM-RA59) and A59 (repA59-RJHM), 4-week-old C57BL/6 mice were infected intracranially with 10-fold serial dilutions of virus, five mice per dilution. Fifty percent lethal dose (LD_{50}) values were calculated as previously described (19). Chimeric repJHM-RA59 (LD_{50}s of 3.6 to 3.8 $\log_{10}$ PFU), and repA59-RJHM (LD_{50}s of 1.0 to 1.2 $\log_{10}$ PFU) recombinant viruses were as virulent as wild-type recombinant viruses RA59 (LD_{50}s of 3.6 to 3.8 $\log_{10}$ PFU) and RJHM (LD_{50} of 1.0 $\log_{10}$ PFU), respectively. These data suggest that the murine coronavirus 3′ genes, rather than the
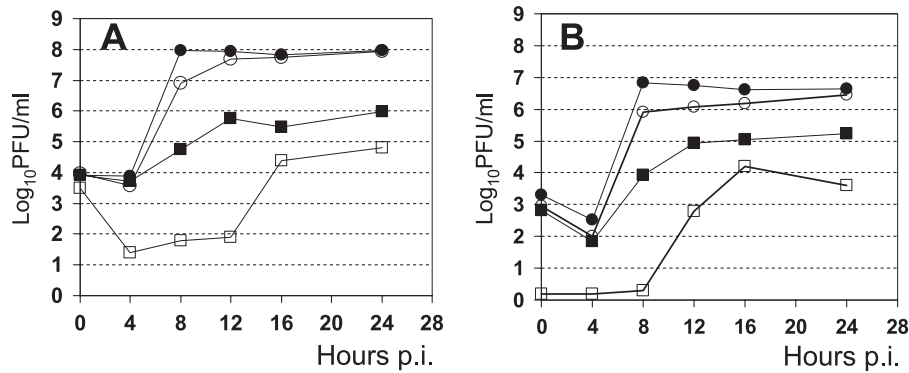
FIG. 2. Viral growth kinetics of recombinant viruses in murine L2 cells. Released (A) and cell-associated virus (B) of RA59 (●), RJHM (■), repJHM-RA59 (○), and repA59-RJHM (□). No significant differences were observed between RA59 and repJHM-RA59 viruses, demonstrating that a chimeric recombinant A59 virus expressing the replicase gene of JHM replicates similarly to the wild-type recombinant A59. In contrast, a chimeric recombinant JHM virus expressing the A59 replicase gene (repA59-RJHM) replicated to lower levels than RJHM did. At the indicated times, virus titers were determined in cells and culture supernatants by plaque assay in L2 cells. The data shown represent the mean titers of triplicate samples. Four independent chimeric recombinant viruses were analyzed. Data from one independent recombinant virus per construct are shown.

replicase gene (gene 1), determine A59 and JHM virulence. Furthermore, it is not the spike gene alone that determines virulence, as expression of the JHM spike from the A59 background does not reproduce the extremely high neurovirulence of JHM (5, 11). HE protein has been shown to enhance the neurovirulence of a virus expressing the JHM spike in the A59 background (7). However, HE cannot be a factor that contributes to the high virulence of A59rep-RJHM, as the 5′ portion of the HE gene of these viruses is derived from A59 and thus cannot be either transcribed or translated (7).

Quantification of infectious virus in liver and brain. Plaque assays were performed to measure infectious virus in the brains and livers of C57BL/6 mice over 1 week postinfection (p.i.) (1, 3, 5, and 7 days p.i.). Viral load was determined as PFU per gram of tissue, as described previously (15). First, we sought to determine whether repJHM-RA59 and repA59-RJHM exhibited differences in viral loads in liver compared to RA59 and RJHM (Fig. 3A and B). Mice were inoculated intrahepatically with low (500 PFU/mouse) and high (10^5 PFU/mouse) doses of virus as previously described (15). Chimeric repJHM-RA59 and repA59-RJHM viruses replicated to a similar extent and with similar kinetics compared to RA59 and RJHM, respectively. RJHM replicated poorly, whereas RA59 viral load was similar to the range that we have previously observed for A59 strain (13). These findings demonstrated that the proteins encoded downstream of gene 2 (spike through nucleocapsid), rather than replicase, determine the viral load in the liver.

Next, we assessed viral load in brain and liver after intracranial inoculation with LD_{50} viral doses (Fig. 4A and B). No significant differences in viral load were observed in the brains of mice inoculated with RA59, RJHM, repJHM-RA59, and repA59-RJHM viruses (Fig. 4A). This is consistent with previous observations that murine coronavirus pathogenesis in the central nervous system (CNS) does not correlate with viral load but rather results from a combination of the direct effects of infection and immune-mediated processes (17, 18). Interestingly, repA59-RJHM kills mice even more quickly than RJHM, as evidenced by the absence of any animals still alive at day 7 (Fig. 4). In contrast, viral load in the liver following

intracranial inoculation, like that following intrahepatic inoculation (Fig. 3), mapped to the 3′ genes, rather than the replicase gene (gene 1) (Fig. 4B). In the liver, chimeric recombinant viruses repJHM-RA59 and repA59-RJHM replicated to
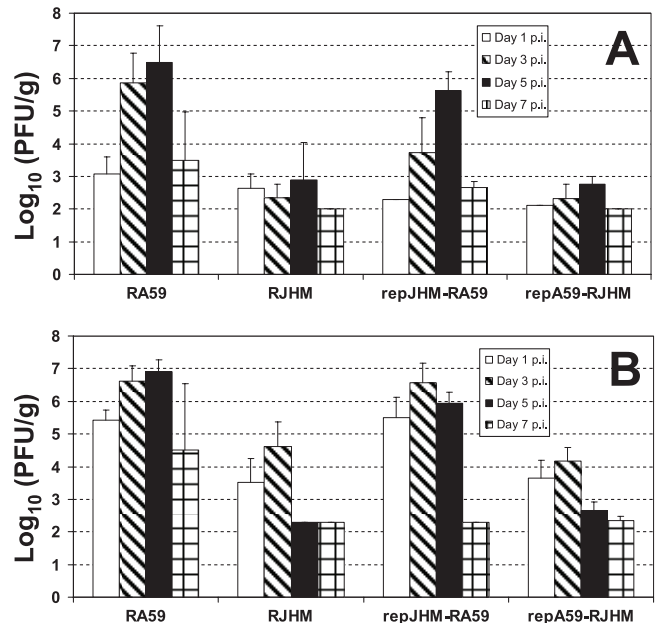

FIG. 3. Viral load in the livers of C57BL/6 mice at 1, 3, 5, and 7 days p.i. after intrahepatic inoculation with 500 PFU (A) and 10^5 PFU (B) of recombinant wild-type viruses RA59 and RJHM and chimeric recombinants repJHM-RA59 and repA59-RJHM. Viral titers were determined by plaque assay and presented as log_{10} PFU/gram of liver. Error bars represent logarithmic standard deviations (SDs). The limit of detection was 200 PFU/g of tissue. RA59 and repJHM-RA59 replicated to higher viral titers than RJHM and repA59-RJHM did ($P < 0.05$). No significant differences in viral titers were observed among viruses differing only in the replicase gene (RA59 versus repJHM-RA59 and RJHM versus repA59-RJHM). Data represent the averages plus SDs of four independent chimeric and two wild-type recombinant viruses. Ten to 20 mice were evaluated for each point.
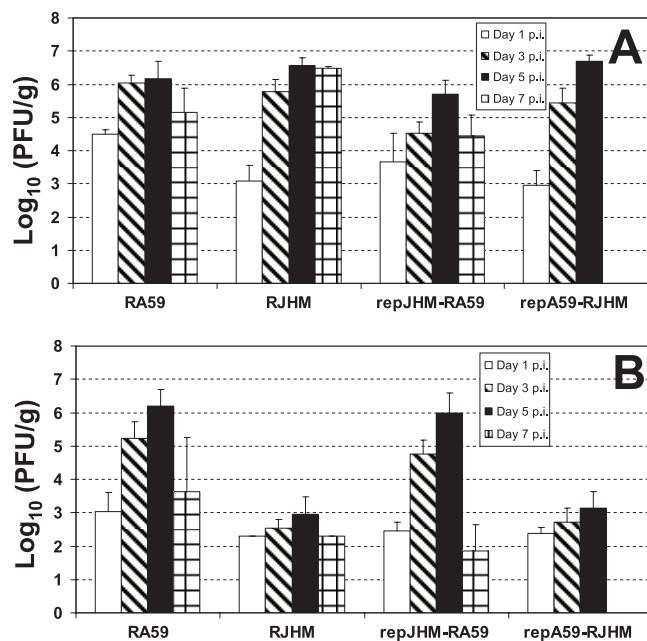
FIG. 4. Viral load in the brains (A) and livers (B) of C57BL/6 mice at 1, 3, 5, and 7 days p.i. after intracranial inoculation. Mice were inoculated intracranially with LD_{50}s of RA59, RHJM, repJHM-RA59, and repA59-RJHM recombinant viruses. No significant differences in viral titers were observed for viruses in the brain. In the liver, RA59 and repJHM-RA59 replicated to higher titers than RJHM and repA59-RJHM did ($P < 0.05$). Data represent the averages plus standard deviations (error bars) of four independent chimeric and two wild-type recombinant viruses. Ten to 20 mice were evaluated for each point.

the same levels as wild-type RA59 and RJHM did, respectively. These results suggested that the replicase gene does not determine the highly neurovirulent phenotype of JHM or the dualtropic phenotype of A59 (mildly neurovirulent and hepatotropic). The data obtained in this study in combination with previous data (5, 14) demonstrate that one or more 3′ genes other than the spike gene contribute to the pathogenic phenotypes of chimeric viruses.

Viral antigen localization and spread in liver and brain. We next examined whether there were differences in the localization of viral antigen in liver and brain that could be associated with the replicase gene. Immunohistochemistry was performed in liver and brain at day 5 p.i., which is the peak of viral replication. Viral antigen was detected using anti-N monoclonal antibody 1.16.1 (provided by J. Leibowitz, Texas A&M University) as previously described (15). RJHM induced no to minimal changes in the liver (Table 1), with scattered foci of virus-stained hepatocytes (data not shown). RA59 caused moderate hepatitis (Table 1), with multiple foci of inflammation and necrosis that colocalized with viral antigen (data not shown), as expected (13). Viral antigen spread was significantly more extensive in the CNSs of mice infected with RJHM than in those infected with RA59, as previously reported (18); similarly, inflammation in the CNS was similar to that previously observed (data not shown) (18). Interestingly, no significant differences were observed in the pathology induced by chimeric recombinant repJHM-RA59 and repA59-RJHM viruses

compared to RA59 and RJHM wild-type viruses, respectively. These findings indicate that the abilities of murine coronaviruses to spread and induce pathology in the liver as well in the brain depends on the proteins encoded in the 3′ end of the genome, rather than the replicase gene.

The 3′ end of the genome encodes several structural proteins other than the spike; since spike alone does not determine pathogenic outcome, the data here imply that one or more of these determine the ability of murine coronavirus A59 to replicate in the liver and induce hepatitis and/or contribute to the very minimal hepatitis characteristic of JHM infection (14). Previous studies using viruses that were not isogenic also mapped pathogenic properties to the 3′ end of the genome (4, 10). Indeed, there are data implicating both membrane and nucleocapsid proteins in MHV pathogenesis (1, 9). The M protein of porcine coronavirus transmissible gastroenteritis virus has been shown to have interferogenic activity, and mutations in the M protein that impair N glycosylation decrease this activity (9). For MHV, while the glycosylation state of M protein does alter the ability to replicate in vitro, it may affect the ability to induce alpha interferon in vitro and also the ability to replicate in the liver in vivo (1). The N protein of MHV has been implicated in fulminant hepatitis (16) via its role in the upregulation of transcription of the immune procoagulant molecule, fibrinogen-like protein 2 (fgl2) (2, 16). In addition, we have recently observed that a chimeric virus in which the N protein of JHM is expressed within the A59 background is significantly more neurovirulent than wild-type A59 is (unpublished data).

We have demonstrated that, in the context of chimeric A59/JHM recombinant viruses, the abilities of murine coronaviruses to replicate in the brain and induce high neurovirulence or to replicate in the liver and induce hepatitis are not determined by the replicase gene. However, there are some examples of how coronavirus replicase proteins may affect virulence. A single amino acid substitution (Tyr6398His) in the MHV-A59 replicase ORF1b p59-nsp14 protein, an exoribonuclease (ExoN) (12) does not affect replication in vitro, but it does results in attenuation of virulence for a recombinant MHV-A59 following intracranial infection of C57BL/6 mice (21). In a recent study, it was shown that the most amino-terminal replicase protein, nsp1, of another group two coronavirus, se-

TABLE 1. Virus-induced histopathology in the liver

Inoculation dose and virus	% of mice with hepatitis[a]				
	None	Minimal	Mild	Moderate	Severe
500 PFU					
RA59			20	60	20
RJHM	80	10	10		
repA59-RJHM	40	60			
repJHM-RA59			40	60	
10^5 PFU					
RA59				10	90
RJHM	20	70	10		
repA59-RJHM	10	80	10		
repJHM-RA59			10	80	10

[a] The percentages of mice with minimal, mild, moderate, and severe hepatitis at day 5 after inoculation with 500 PFU or 10^5 PFU directly into the liver. Ten to 20 mice were examined per viral group and dose.

vere acute respiratory syndrome coronavirus, promotes host mRNA degradation and thus inhibits host protein synthesis; these authors suggested that this activity may play an important role in pathogenesis by inhibiting host innate immune response genes (6). There are potentially, as yet undiscovered, roles in pathogenesis for other components of the replicase, which include several enzymatic activities, as well as for the nonstructural proteins encoded by ORF2a, ORF4, and ORF5a.

In summary, we have generated two types of recombinant chimeric JHM-A59 viruses, (i) viruses in which the entire JHM replicase gene was introduced into the A59 background (JHMrep-RA59) and (ii) viruses in which the entire A59 replicase gene was introduced into the JHM background (repA59-RJHM). We have performed in vitro replication kinetics analysis and in vivo studies in order to compare the pathogenesis induced by chimeric repJHM-RA59 and repA59-RJHM compared to RJHM and RA59 (recombinant wild-type viruses). In vitro studies demonstrate that the replication kinetics of JHMrep-RA59 virus are similar to those of RA59. In vivo studies demonstrate that the replicase gene of JHM strain does not account for the nonhepatotropic phenotype of JHM. Taken together, our results suggest that in the context of A59/JHM chimeric viruses, replicase genes may be exchanged without detectable change in phenotype in vitro or pathogenesis in vivo; thus, the 3′ one-third of the genome encoding spike through nucleocapsid, rather than the replicase gene, determines the murine coronavirus pathogenic phenotype.

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