Analysis of Nonessential Gene Function in Recombinant MHV-JHM

Gene 4 knockout recombinant virus

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1. INTRODUCTION

The large size of the coronavirus genome has made reverse genetics difficult. Targeted recombination, a technique developed by Masters and colleagues, has facilitated the introduction of mutations into the coronavirus genome (Kuo et al., 2000). Previous work by Skinner and Siddell showed that MHV-JHM ORF 4 encodes a 15 kDa protein composed of 139 amino acids. This protein is relatively rich in threonines and includes a hydrophobic region. The N-terminus contains a potential membrane-anchoring region and the C-terminus has a possible RNA binding region (Skinner and Siddell, 1985). MHV-S, a natural variant, does not encode a functional ORF 4 suggesting that the ORF 4 product was not necessary for growth in tissue culture cells or animals. Additionally this strain contained a deletion within ORF 5a (Yokomori and Lai, 1991). Lack of mRNA 4 synthesis most likely resulted from a point mutation in the intergenic sequence (UCUAAAC to UUUAAAC). In this study, targeted recombination was used to genetically disrupt ORF 4. This recombinant virus was then analyzed in a murine model of encephalitis.

The Nidoviruses (Coronaviruses and Arteriviruses).
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2. MATERIALS AND METHODS

2.1 Recombinant Virus

Initially, targeted recombination (Kuo et al., 2000) was used to construct a chimeric virus containing MHV-JHM genes 1 and 2, the surface (S) glycoprotein of feline coronavirus (FCoV) and MHV-A59 genes 4-7 (designated FCV-MHV-JHM clone B3b) (Figure 1). Subsequently, mutations were introduced into this virus using targeted recombination.

2.2 Construction of plasmids

A plasmid containing gene 3 from MHV-JHM and genes 4-7 from MHV-A59 (pMH54-4) was kindly provided by Dr. Susan Weiss, University of Pennsylvania. PCR was used to replace genes 4-7 in this plasmid with homologous sequences from the JHM strain (Figure 1). JHM-specific DNA fragments were prepared either from plasmids encoding JHM proteins (a gift from Dr. S. Siddell, University of Wurzburg) or from JHM-specific RNA harvested from infected tissue culture cells (Pewe et al., 1996). The fidelity of all PCR products was confirmed by sequencing.

2.3 Measurement of LD_{50}

Pathogen-free C57Bl/6 mice were purchased from the National Cancer Institute (NCI). Dilutions of each virus were prepared in phosphate buffered saline and each was delivered in 30 μl under anesthesia to 3-5 mice. Virus was inoculated into the left cerebrum. LD_{50} values were calculated by the Reed-Muench method (Reed and Muench, 1938).

2.4 Viral RNA analysis

Virus from infected brains was amplified by passage through 17Cl-1 cells as described previously. RNA was harvested from 17Cl-1 cells infected with this amplified virus (m.o.i. 0.5-1.0) and analyzed by Northern blot analysis as described previously (Perlman et al, 1990, Pewe et al, 1996). Processed blots were exposed to film or analyzed using a phosphoimager.

3. RESULTS AND DISCUSSION

3.1 Construction of recombinant MHV-JHM

In all previous cases, targeted recombination was used to introduce mutations into the MHV-A59 background. MHV-JHM is useful for studies of neuropathogenesis. Consequently we adapted targeted recombination to MHV-JHM (Figure 1). Initially, a chimeric MHV-JHM containing the FCoV surface glycoprotein was developed using standard techniques (Kuo et al., 2000). Next a donor plasmid was constructed using an MHV-A59 based plasmid (pMH54-4). This plasmid contained the JHM S gene sequences with MHV-A59 genes 4-7. MHV-JHM genes 4-7 were cloned using PCR products amplified from plasmids or cDNA derived from MHV-JHM and inserted into the MHV-A59-based plasmid.

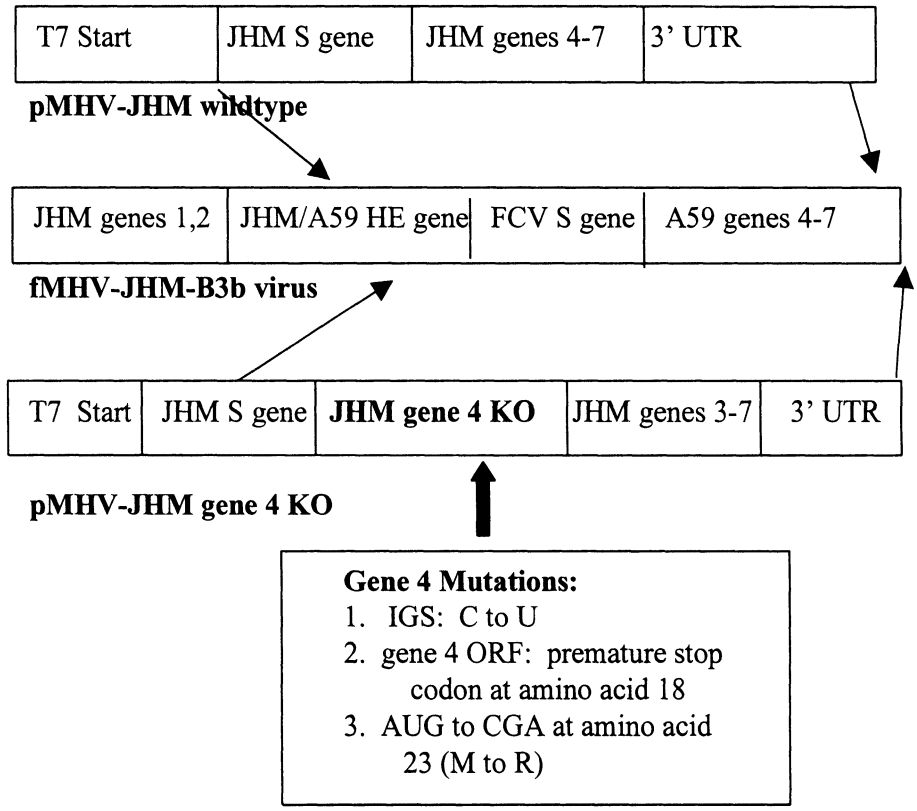

Figure 1. Strategy for construction of wildtype recombinant MHV-JHM(upper portion) and MHV-JHM gene 4 knockout recombinant(lower portion). Recipient virus and donor plasmid were constructed as described in Materials and Methods. Gene 4 was genetically disrupted by insertion of three mutations as shown above.

The strategy for knocking out expression of gene 4 involved introducing three separate mutations (Figure 1). The same point mutation which had been identified in the intergenic sequences of MHV-S, the natural variant lacking gene 4 mRNA, was introduced into the IGS of our MHV-JHM gene 4 (Yu and Leibowitz, 1994). This change should eliminate transcription initiating at gene 4. In addition, a premature stop codon at amino acid 18 was introduced. This mutation should result in premature termination of any translation occurring from residual gene 4 RNA. Finally, an AUG downstream from this termination codon was also mutated. Though not used by MHV-JHM, this AUG may be functional in MHV-A59 infected cells.

The MHV-JHM wildtype and MHV-JHM gene 4 knockout plasmids were transcribed into an 8 KB RNA product. This RNA was electroporated into feline FCWF cells (American Type Culture Collection) previously infected with fMHV-JHM-B3b. Recombinants were selected and the three mutations in gene 4 were confirmed.

3.2 Mouse model of neuropathogenesis

C57Bl/6 infected intracranially or intranasally with wild type MHV-JHM develop an invariably fatal acute encephalitis. In initial studies, the MHV-JHM wildtype and gene 4 knockout recombinants were used to infect 6 week old C57Bl/6 mice. Intranasal inoculation of mice with undiluted stocks of virus demonstrated that both the recombinant wildtype MHV-JHM and the MHV-JHM-gene 4 KO recombinant were capable of causing acute encephalitis with similar kinetics.

These results show that there was not a high level of attenuation after genetic disruption of gene 4. In order to define more precisely any attenuation of the gene 4 KO recombinant, LD_{50} after intracranial inoculation was determined. Serial dilutions of virus were prepared and inoculated into 3-4 mice per dilution. The recombinant wildtype MHV-JHM had an LD_{50} of 9 pfu, similar to what we observed for nonrecombinant wildtype MHV-JHM (3 pfu). The LD_{50} of MHV-JHM gene 4 KO recombinant was 73, higher than the wild type recombinant.

Table 1. LD_{50} after intracranial inoculation.

	LD_{50}
JHM wildtype	3
JHM wildtype Recombinant	9
JHM-Gene 4 KO Recombinant	73

3.3 Analysis of recombinant viral RNA

Since the measurements of LD_{50} indicated that the gene 4 KO recombinant was only minimally attenuated, the next step was to confirm that the mutations into gene 4 were maintained after passage through mice. RNA, harvested from infected brains, was used to prepare cDNA (Pewe et al., 1996). Sequencing of this cDNA confirmed that the point mutations introduced into gene 4 were still present. In other experiments, virus from infected brains was harvested and passaged once through 17Cl-1 cells. RNA was harvested from cells infected with these amplified viruses and assessed by Northern blot analysis (Figure 2).

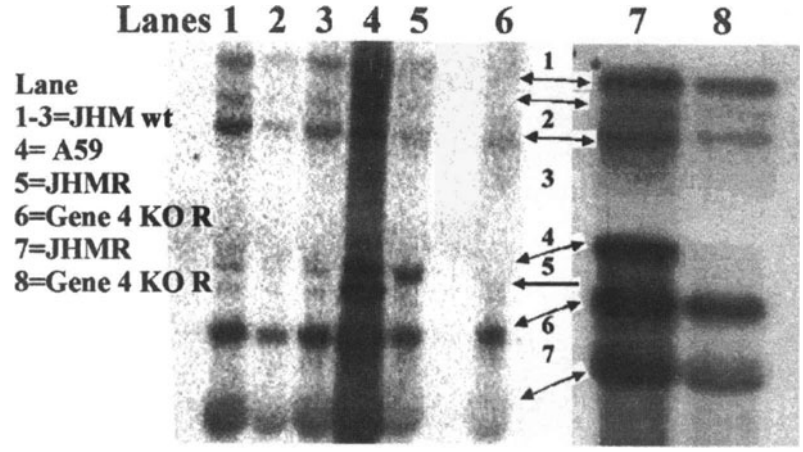

Figure 2. Northern blot analysis of RNA harvested from infected cells. RNA was prepared from cells infected with brain-derived virus as described in text. Bands corresponding to mRNA 4 and 5 were present in cells infected with MHV-JHM and MHV-A59. mRNA 4 was detected in cells infected with recombinant wildtype MHV-JHM but not in those infected with MHV-JHM gene 4 KO recombinant. mRNA 5 was not detected in either of these viruses but could be amplified by PCR. Levels of other mRNAs were not consistently changed by mutations introduced into gene 4.

As expected, two bands corresponding to mRNA 4 and 5 were easily visualized from MHV-A59 infected cells. Two bands, albeit fainter, are also visible in cells infected with wildtype MHV- JHM. Surprisingly only a band corresponding to mRNA 4 was detected in cells infected with recombinant wildtype MHV-JHM. The protein encoded by gene 5b is essential (Yu and

Leibowitz, 1994) making it unlikely the gene 5 transcript would be completely absent. Supporting this conclusion, a product corresponding to gene 5 could be amplified by PCR from viral RNA. In addition, we consistently observed that the mRNA 4 band is more intense than what we observe for that band in our wildtype virus. Introduction of the Sbf I site into the 3' end of the S gene alters the upstream context of the gene 4 intergenic sequence so that gene 4 transcription is enhanced (unpublished data). As shown in Figure 2, no gene 4 RNA is detected in cells infected with the MHV-JHM gene 4 KO recombinant. The pattern seen with the recombinant viruses is shown most clearly in Lanes 7-8 of Figure 2.

4. CONCLUSIONS

In summary, we have adapted targeted recombination to introduce mutations into the MHV-JHM strain. Our initial results show that genetic disruption of gene 4 results in mild attenuation in a murine model of acute encephalitis. We are in the process of confirming the lack of expression of gene 4 protein in cells infected with MHV-JHM gene 4 KO recombinants.

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