MHV-A59 ORF1A REPLICASE PROTEIN NSP7-NSP10 PROCESSING IN REPLICATION

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1. INTRODUCTION

The highly conserved region at the carboxy-terminus of the MHV replicase ORF1a polyprotein is processed by the main protease (M^{pro}) into mature products including nsp7-nsp10, which are associated with replication complexes and presumably involved with RNA synthesis.[1-4] The exact function of these proteins, in either their pre- or postcleaved forms, is unknown. However, disruption of M^{pro} cleavage during any stage of the infection cycle blocks replication, suggesting that constitutive proteolytic processing of the nonstructural polyprotein is a requirement for efficient transcription.[5] In this report, we describe preliminary data defining the requirement for the proteolytic processing of the nsp7-nsp10 proteins in MHV-A59 replication. Through use of an efficient MHV-A59 reverse genetics system,[6] we ablated each of the M^{pro} cleavage sites associated with the nsp7-nsp10 cassette, and evaluated whether the mutated genome was capable of supporting a viable virus, and if so, characterized the M^{pro} processing of the mutated protein, transcription function, and *in vitro* growth fitness.

2. RESULTS AND DISCUSSION

The M^{pro} targets amino acid sequences Q-S/N/A, cleaving after the essential Glu at position 1 (P1).[7] To evaluate the importance of cleavage on virus replication, the cleavage sites flanking nsp7, nsp8, nsp9, and nsp10 were individually disrupted by mutating the P1 Glu to an Ala (Table 1). However, there are two potential M^{pro} cleavage sites at the nsp7/8 interface, an LQA (present at positions P5-P3) and LQS (P2-P1'). Although the LQS has been shown to be cleaved during M^{pro} processing,[3] it is possible that the upstream LQA site is also functional in the presence or absence of the LQS site. To address this possibility, both sites were mutated, either individually (nsp7*8.A and

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Table 1. Amino-acid sequences for the nsp7-nsp10 Mpro cleavage sites for MHV-A59 (top) and mutant viruses (bottom).

	MHV-A59 M^{pro} Cleavage Sites									
	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
nsp6/7	V	S	Q	I	**Q**	S	R	L	T	D
nsp7/8[a]	L	**Q**	A	L	**Q**	S	E	F	V	N
nsp8/9	T	V	V	L	**Q**	N	N	E	L	M
nsp9/10	T	V	R	L	**Q**	A	G	T	A	T
nsp10/11	G	S	Q	F	**Q**	S	K	D	T	N
	Mutant M^{pro} Cleavage Sites									
nsp6*7	V	S	Q	I	**A**	S	R	L	T	D
nsp7*8.A[b]	L	**A**	A	L	Q	S	E	F	V	N
nsp7*8.B[c]	L	Q	A	L	**A**	S	E	F	V	N
nsp7*8.A+B[d]	L	A	A	L	**A**	S	E	F	V	N
nsp8*9	T	V	V	L	**A**	N	N	E	L	M
nsp9*10	T	V	R	L	**A**	A	G	T	A	T
nsp10*11	G	S	Q	F	**A**	S	K	D	T	N

[a] There are two potential M^{pro} cleavage sites, an LQA and LQS.
[b] The upstream LQA site is mutated.
[c] The downstream LQS site is mutated.
[d] Both sites are mutated.

nsp7*8.B) or in combination (nsp7*8.A+B). The viability of the seven MHV-A59 mutants were tested by transferring the mutations (or combination of mutations) to the MHV-A59 infectious clone, driving full-length transcripts, and electroporating the RNA into cells. Viability was confirmed by the formation of CPE and detection of leader-containing transcripts by RT-PCR.

Three constructs produced replicating viruses. Of the viable mutants, the MHV-nsp7*8.A and MHV-nsp7*8.B cleavage mutants replicated to wild-type titers while MHV-nsp9*10 replication was reduced by about two logs. The genetic stability of the attenuated MHV-nsp9*10 mutant virus was analyzed after 15 serial passages on DBT cells. Plaque purified passage 15 virus, MHV-nsp9*10p15, displayed improved fitness of *in vitro* growth, as demonstrated by the near wild-type titers. Surprisingly, the recovered virus did not revert to wild-type sequence at the nsp9/nsp10 M^{pro} cleavage site, indicating that an as of yet unidentified mutation(s) has compensated for the virus's inability to properly process the nsp9-nsp10 precursor protein.

Immunoprecipitation (i.p.) using either anti-nsp8 (for the nsp7*8 mutants) or anti-nsp10 antibody (for the nsp9*10 mutants)[1] and Western blot analysis was used to verify whether or not mutation of the P1 Glu prevented cleavage of the viable viruses (data not shown). Bands corresponding to nsp10 (15-kDa protein) were absent in lysates of MHV-nsp9*10 and MHV-nsp9*10p15. However, a band of approximately 37 kDa, which is the predicted size for uncleaved nsp9-nsp10, was present in the mutants, but absent in the wild-type control cells. Analysis of the i.p.-Western blot suggested that MHV-A59 was able to use either of the two potential M^{pro} cleavage sites located at the nsp7/nsp8 site, likely accounting for the wild-type growth kinetics. Bands of approximately 22 kDa were precipitated with anti-nsp8 antibody for the wild-type control, MHV-nsp7*8.A, and MHV-nsp7*8.B. Notably, the MHV-nsp7*8.B band was slightly larger than that of the

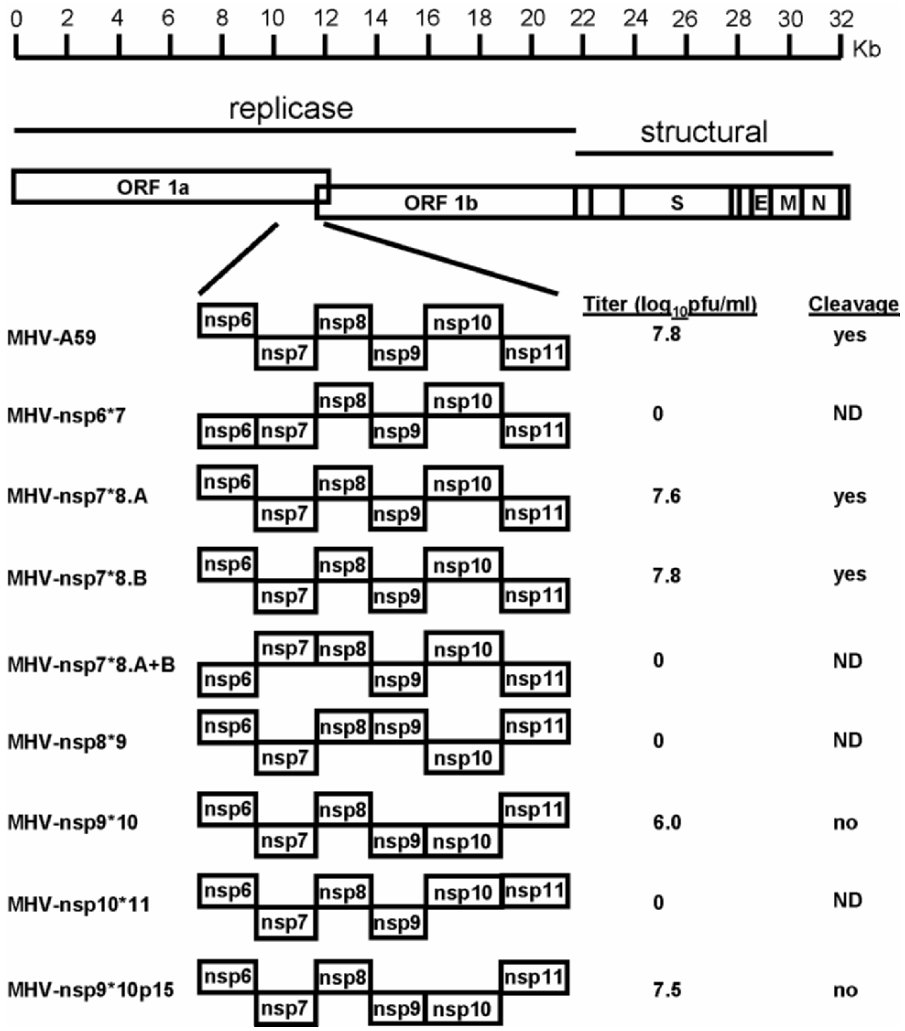

Figure 1. Summary of MHV-A59 and mutant M^{pro} processing of the nsp7-nsp10 cassette, viral titers, and verification of cleavage by immunoprecipitation and Western blot analysis of viable viruses. The picture of the nsp6-nsp11 proteins illustrates the predicted effects of mutating the P1 Glu M^{pro} cleavage sites, with uncleaved proteins forming a fusion protein. Peak log titers and the results of i.p.-Western blot analysis for M^{pro} cleavage at the site of mutation are also presented (yes = identification of proteolytically processed protein; no = identification of a fusion protein unprocessed by M^{pro}; ND = viral protein not detected due to lack of productive infection).

control or the first nsp7*8 mutant, consistent with the prediction that the LQA site was cleaved and yielded an nsp8 three amino acids larger than that of the LQS cleaved protein.

The cleavage site mutants were found to be similar to MHV-A59 in their transcriptional activity and cellular localization (results summarized in Figure 1). Northern blots hybridized with an RNA probe complementing the 5' end of the N-gene

showed no notable alteration in either the pattern or relative amounts of subgenomic to genomic RNA in mutant and control viruses. The distribution of the mutant proteins within cells was compared with their wild-type counterparts. The nsp7-nsp10 proteins are known to colocalize with sites of viral replication while being excluded from regions of virion assembly.[1,2] In order to determine if ablation of the M^{pro} processing interferes with the ability of the protein to traffic into the replication complex, an immunofluorescence (IFA) study was completed. Cells infected with either MHV-A59, mock, or mutant virus were dual-stained for either nsp8 or nsp10 (depending on the mutant, as above) and nucleocapsid (N), which co-localizes with sites of active viral replication, or membrane (M), which is targeted to regions of virus assembly. Regardless of the virus, the nsp8 and nsp10 colocalized with N and was separate from M (data not shown). We were unable to find a difference between the localization of the nsp8 or nsp10 proteins between the mutants or wild-type.

Cleavage site ablation resulted in lethal, debilitated, or near wild-type viability, and mutation of most of the M^{pro} cleavage sites was lethal (summarized in Figure 1). Lethality could be due to disruption of nsp7-10 proteolytic processing causing a failure of precursor, intermediate, or mature protein function within the replication complex. However, not all of the M^{pro} cleavage site mutants were nonviable. Based on the genetic analysis, MHV-A59 has two functional nsp7-nsp8 M^{pro} cleavage sites, LQA and LQS, and disruption of either of these potential sites fails to affect replication competence, M^{pro} cleavage pattern, or cellular localization *in vitro*. Possible *in vivo* effects need to be studied. In contrast, simultaneous mutation of both sites was lethal. The only other M^{pro} cleavage site harboring a viable mutation was that shared by the nsp9 and nsp10 proteins. In this case, the mutant virus was highly attenuated in its replication efficiency and was unable to proteolytically process the fused nsp9-nsp10 protein. Serial passage of this virus restored wild-type replication but did so without reverting the mutated cleavage site or the ability to process the nsp9-nsp10 protein. The data demonstrate that with the exception of cleavage between the nsp9 and nsp10 proteins, M^{pro} processing of the nsp7-nsp10 cassette is essential in coronavirus RNA transcription and replication.

3. REFERENCES

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