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Mutagenesis of the murine hepatitis virus nsp1-coding region identifies residues important for protein processing, viral RNA synthesis, and viral replication

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Abstract

Despite ongoing research investigating mechanisms of coronavirus replication, functions of many viral nonstructural proteins (nsps) remain unknown. In the current study, a reverse genetic approach was used to define the role of the 28-kDa amino-terminal product (nsp1) of the gene 1 polyprotein during replication of the coronavirus murine hepatitis virus (MHV) in cell culture. To determine whether nsp1 is required for MHV replication and to identify residues critical for protein function, mutant viruses that contained deletions or point mutations within the nsp1-coding region were generated and assayed for defects in viral replication, viral protein expression, protein localization, and RNA synthesis. The results demonstrated that the carboxy-terminal half of nsp1 (residues K_{124} through L_{241}) was dispensable for virus replication in culture but was required for efficient proteolytic cleavage of nsp1 from the gene 1 polyprotein and for optimal viral replication. Furthermore, whereas deletion of nsp1 residues amino-terminal to K_{124} failed to produce infectious virus, point mutagenesis of the nsp1 amino-terminus allowed recovery of several mutants with altered replication and RNA synthesis. This study identifies nsp1 residues important for protein processing, viral RNA synthesis, and viral replication.

Keywords: Coronavirus; Replication; Reverse genetics; Nonstructural protein; Protein processing; RNA synthesis

Introduction

Coronaviruses belong to a family of enveloped positive-strand RNA viruses that are responsible for devastating illnesses in livestock and domestic animals. The identification of a novel human coronavirus as the etiological agent of Severe Acute Respiratory Syndrome (SARS) in 2003 highlighted the potential of this virus family to also cause severe human disease (Kuiken et al., 2003). Even with continuing research addressing how coronaviruses replicate and cause disease, the functions of many viral proteins remain to be elucidated. For example, 14–16 nonstructural proteins (nsps) are expressed from coronavirus gene 1 polyproteins, but at least seven of these nsps have no known roles in viral replication. In the past, gene 1 has been referred to as the "replicase gene" and gene 1 nsps as "replicase proteins" named by their molecular weight in kilodaltons. More recently, nsps have been named based on their order in gene 1 and are numbered consecutively beginning at the amino-terminus of the polyprotein (i.e., nsp1 through nsp16) (Harcourt et al., 2004; Prentice et al., 2004b; Snijder et al., 2003). The gene 1 nsps of the coronavirus murine hepatitis virus (MHV) are similar in number, size, and organization to those of SARS coronavirus (SARS-CoV) (Marra et al., 2003; Snijder et al., 2003). This resemblance suggests that MHV is an excellent model for studies of coronavirus nsp function and may

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increase our understanding of SARS-CoV replication and pathogenesis.

Following entry of MHV into a host cell, the first event in the virus life cycle is translation of two polyproteins from gene 1 of the input RNA genome. Gene 1 is comprised of two overlapping open reading frames (ORF1a and ORF1b) that are connected by a −1 ribosomal frameshift (Fig. 1A) (Bonilla et al., 1994; Bredenbeek et al., 1990; Brierley et al., 1989; Lee et al., 1991; Pachuk et al., 1989). Translation of either ORF1a or the ORF1ab fusion results in possible 495-kDa or 803-kDa polyproteins, respectively. These co-amino-terminal polyproteins are proteolytically processed by three virus-encoded proteinases, including two papain-like proteinases (PLP1 and PLP2 within nsp3) and a picornavirus 3C-like proteinase (nsp5), to yield at least 16 mature gene 1 nsps as well as intermediate precursors. To date, all MHV gene 1 nsps tested co-localize with sites of active viral RNA synthesis at cytoplasmic viral replication complexes on intracellular double-membrane vesicles (Bost et al., 2000, 2001; Brockway et al., 2003, 2004; Gosert et al., 2002; Prentice et al., 2004a; Shi et al., 1999; van der Meer et al., 1999). Nsps are thought to mediate replication of the MHV RNA genome and subgenomic RNA synthesis at these membrane-bound complexes. However, the viral proteinases are the only MHV nsps with experimentally confirmed functions (Baker et al., 1989; Bonilla et al., 1995; Lu et al., 1996; Lu et al., 1995). Based on homology to proteins with known functions, roles in viral RNA synthesis have been predicted for several other MHV gene 1 nsps. These proteins include two *trans*-membrane scaffolding proteins (nsp4 and nsp6) (Gosert et al., 2002), an RNA-dependent RNA polymerase (nsp12) (Cheng et al., 2005; Gorbalenya et al., 1989; Lee et al., 1991), an RNA helicase (nsp13) (Seybert and Ziebuhr, 2001; Seybert et al., 2000), and several RNA processing enzymes (nsp14, nsp15, and nsp16) (Bhardwaj et al., 2004; Ivanov et al., 2004; Thiel et al., 2003; Ziebuhr, 2005). Currently, there are no known or envisaged functions in replication for at least seven MHV gene 1 proteins (nsp1, nsp2, and nsp7–11).

Previous studies have provided intriguing evidence about the potential functions of the 28-kDa amino-terminal protein nsp1 (previously referred to as p28) in MHV replication. The kinetics of nsp1 expression suggest that it might have an early regulatory role during the viral life cycle. Nsp1 is the first mature protein processed from the gene 1 polyprotein and is likely cleaved quickly following trans-

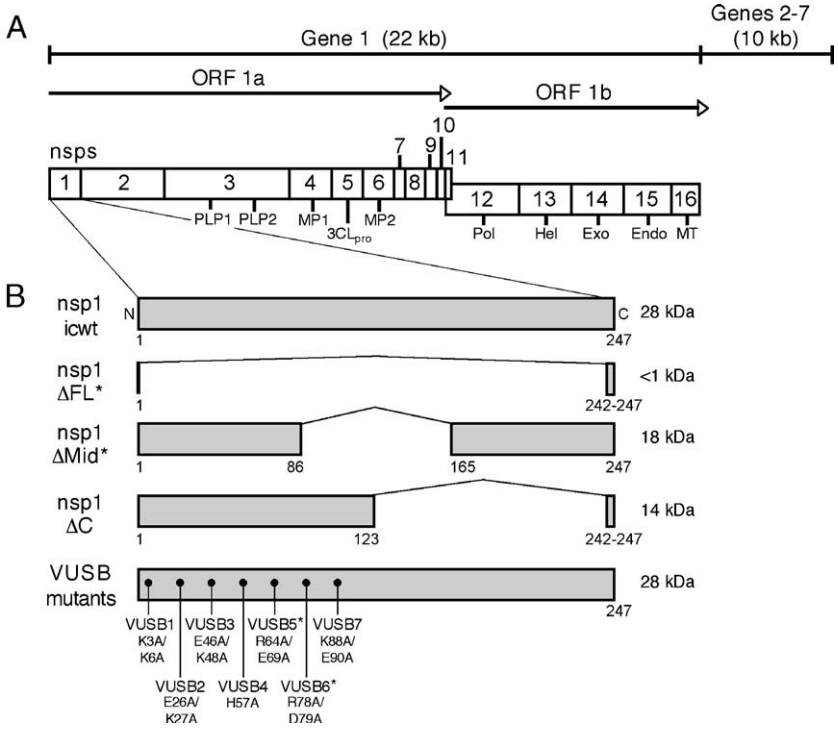

Fig. 1. Schematics of the MHV gene 1 polyproteins and nsp1 mutants. (A) Organization of the MHV gene 1 polyprotein. The 32-kb MHV genome is shown as a line, and the locations of gene 1 (22 kb) and genes 2–7 (10 kb) are indicated. Gene 1 is composed of two open reading frames (ORF1a and ORF1b). The ORF1a–ORF1b fusion polyprotein is illustrated with mature nonstructural protein (nsps) represented as numbered boxes. The gray box represents the amino-terminal cleavage product (nsp1). Nsps with confirmed or predicted functions include: two papain-like proteinases (PLP1 and PLP2 within nsp3), the 3C-like proteinase ($3CL_{pro}$; nsp5), two *trans*-membrane proteins (MP1 and MP2; nsp4 and nsp6, respectively), the RNA-dependent RNA polymerase (Pol; nsp12), the RNA helicase (Hel; nsp13), the 3′-to-5′ exonuclease (Exo; nsp14), the endoribonuclease (Endo; nsp15), and the RNA methyltransferase (MT; nsp16). (B) Nsp1 mutant proteins. The schematics illustrate the engineered deletions and point mutations within nsp1. Nsp1 amino acid numbers are listed below each protein, and the predicted protein size (in kilodaltons) is listed to the right of each. The amino-terminal charge-to-alanine mutations for each VUSB mutants are listed below the bottom nsp1 protein. The asterisks (*) indicate mutants that did not establish productive infections as determined by lack of recovered virus from electroporated cells.