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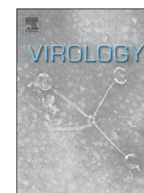

A key role for the carboxy-terminal tail of the murine coronavirus nucleocapsid protein in coordination of genome packaging

Lili Kuo, Cheri A. Koetzner, Paul S. Masters *

Wadsworth Center, New York State Department of Health, Albany, NY 12201, United States

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ABSTRACT

The prototype coronavirus mouse hepatitis virus (MHV) exhibits highly selective packaging of its genomic positive-stranded RNA into assembled virions, despite the presence in infected cells of a large excess of subgenomic viral mRNAs. One component of this selectivity is the MHV packaging signal (PS), an RNA structure found only in genomic RNA and not in subgenomic RNAs. It was previously shown that a major determinant of PS recognition is the second of the two RNA-binding domains of the viral nucleocapsid (N) protein. We have now found that PS recognition additionally depends upon a segment of the carboxy-terminal tail (domain N3) of the N protein. Since domain N3 is also the region of N protein that interacts with the membrane (M) protein, this finding suggests a mechanism by which selective genome packaging is accomplished, through the coupling of genome encapsidation to virion assembly.
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1. Introduction

The selective packaging of genomic RNA (gRNA) into assembling virions is a fundamental problem faced by many RNA viruses. Coronaviruses occupy a unique niche among positive-strand RNA viruses in two respects. First, they have helically symmetric nucleocapsids and, consequently, are not subject to the architectural constraints that govern packaging for viruses with icosahedral capsids (Prevelige, 2016). Second, although they synthesize a large molar excess of multiple subgenomic RNA (sgRNA) species during infection, coronaviruses very selectively incorporate gRNA into virions (Makino et al., 1990; Escors et al., 2003).

Coronavirus gRNA packaging has been most intensively studied in two betacoronaviruses, mouse hepatitis virus (MHV) and bovine coronavirus (BCoV), and in the alphacoronavirus transmissible gastroenteritis virus (TGEV). For MHV, a genomic packaging signal (PS) was originally identified through analyses of packaged defective-interfering (DI) RNAs, which are extensively deleted genomic remnants that replicate by appropriating the RNA synthesis machinery of a helper virus (Makino et al., 1990; van der Most et al., 1991). The MHV PS is situated roughly 20.3 kb from the 5′ end of the genome, embedded in the segment of gene 1 that encodes the nonstructural protein 15 (nsp15) subunit of the replicase-transcriptase (Fosmire et al., 1992; Cologna and Hogue, 2000; Narayanan and Makino, 2001). This localization places the PS solely in gRNA and not in any of the six species forming the 3′-nested set of sgRNAs of MHV.

A structure recently proposed for the MHV PS (Chen et al., 2007b) models it as a 95-nt bulged stem-loop containing four repeat units, each with an AA (or GA) bulge; an internal loop separates the PS into quasi-symmetric upper and lower halves (Fig. 1A). This structure is consistent with chemical and enzymatic probing profiles and is highly conserved among lineage A betacoronaviruses (such as MHV and BCoV). We previously manipulated the MHV PS in the intact virus, rather than in DI RNAs, with the goal of obtaining insights into the mechanism of packaging (Kuo and Masters, 2013). Extensive disruption of the PS structure with 20 coding-silent mutations (in a mutant designated silPS) or outright deletion of the PS resulted in the packaging of abundant amounts of sgRNA in addition to gRNA in highly purified virions. We found that the PS was not essential for MHV viability, but it conferred a distinct selective advantage to genomes that harbored it. Additionally, the PS remained functional when transposed to an ectopic genomic site, a noncoding region created downstream of the replicase-transcriptase gene. This work showed that the PS indeed governs the selective incorporation of gRNA into virions.

To begin to identify interacting partners of the PS, we modified the nucleocapsid (N) protein, the molecule that coats the gRNA and winds it into a helically symmetric filament within the virion (Masters, 2006). N is a mostly basic phosphoprotein containing two structurally separate RNA-binding domains, the amino-

* Corresponding author.
E-mail address: Paul.Masters@health.ny.gov (P.S. Masters).

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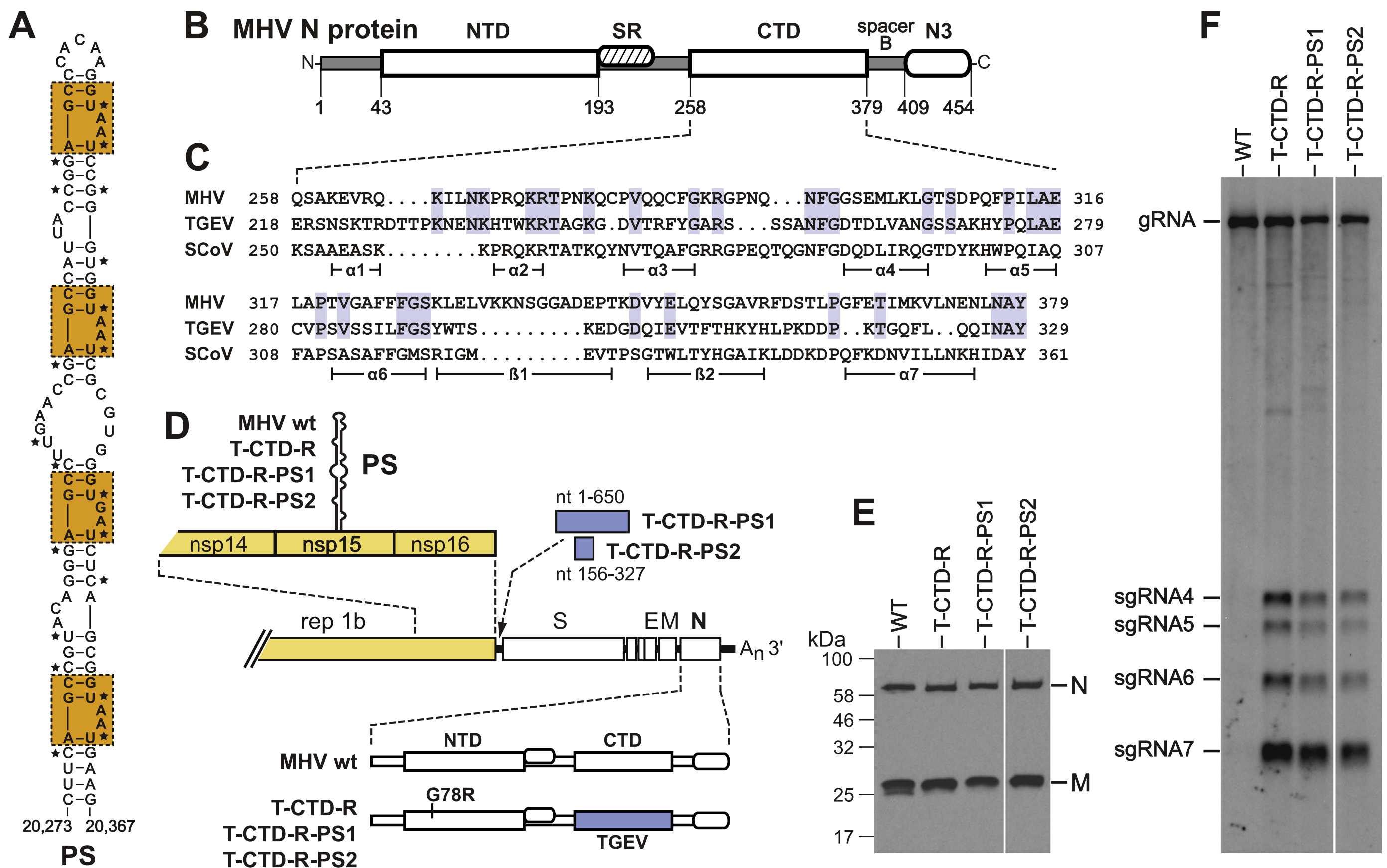

Fig. 1. Effect of substitution of the TGEV N protein CTD in the MHV N protein. (A) The MHV packaging signal as modeled by the Olsthoorn laboratory (Chen et al., 2007b). The four repeat units are boxed. Stars mark 20 nucleotides that were mutated in the silPS mutant to disrupt the PS structure without altering the encoded segment of nsp15 amino-acid sequence (Kuo and Masters, 2013). (B) Domain structure of the MHV N protein: *NTD*, amino-terminal RNA-binding domain; *SR*, serine- and arginine-rich region; *CTD*, carboxy-terminal RNA-binding domain; *spacer B*, variable spacer region; *N3*, carboxy-terminal M-interacting domain. Amino-acid residue numbers are shown beneath the schematic. (C) Alignment of the CTDs of the MHV, TGEV, and SARS-CoV N proteins. GenBank accession numbers for the sequences shown are: MHV-A59, AY700211; TGEV, AJ271965; SARS-CoV, AY278741. Residues that are identical in MHV and TGEV are highlighted. Shown below the alignment are α-helices and β-strands determined for the SARS-CoV CTD crystal structure (Chen et al., 2007a). (D) Compositions of the TGEV CTD chimeras. The N proteins of all three TGEV mutants contain a substitution of the TGEV CTD replacing that of MHV, plus the reverting mutation G78R in the NTD. All mutants (as well as the isogenic wild-type MHV) contain the wild-type MHV PS within the nsp15 coding region and a short linker segment replacing nonessential genes between gene 1 and the S gene, as described previously (Kuo and Masters, 2013). T-CTD-R-PS1 and T-CTD-R-PS2, additionally contain nt 1-650 or nt 156-327 of the TGEV genome, respectively, inserted into the linker between gene 1 and the S gene. (E) Western blots of purified virions probed with anti-N and anti-M monoclonal antibodies; note that the anti-N antibody recognizes an epitope in MHV domain N3. (F) Northern blots of RNA isolated from purified virions. MHV RNA was detected with a probe specific for the 5′ half of the N gene.

terminal domain (NTD) and the carboxy-terminal domain (CTD) (Fig. 1B). (For a comprehensive review of coronavirus N protein structure, see Chang et al., 2014.) The CTD additionally mediates N-N dimerization and higher-order interactions in the nucleocapsid (Chang et al., 2013). This nomenclature can obscure the point that the actual carboxy terminus of N protein consists of a linker (spacer B) joining the CTD to N3, an acidic domain that binds to the endodomain of the membrane (M) protein during virion assembly (Kuo and Masters, 2002; Hurst et al., 2005; Verma et al., 2006, 2007; Kuo et al., 2016). Another linker, between the NTD and CTD, encompasses a serine- and arginine-rich region that associates with replicase subunit nsp3 in a crucial early event of infection (Hurst et al., 2010).

Although the MHV PS is conserved among lineage A betacoronaviruses, it is clear that severe acute respiratory syndrome coronavirus (SARS-CoV), a lineage B betacoronavirus, does not contain a homolog of the MHV PS (Joseph et al., 2007; Chen and Olsthoorn, 2010). We therefore turned to SARS-CoV chimeras for evidence of interacting partners of this RNA element, constructing MHV N protein mutants in which either of the two RNA-binding domains of N was substituted by its SARS-CoV counterpart (Kuo et al., 2014). Strikingly, we found that the SARS-CoV CTD chimera recapitulated the defective packaging phenotype of the silPS mutant and the PS deletion mutant. Furthermore, the region affecting PS recognition was localized in a partial CTD chimera to a central segment of 30 amino acids, which corresponds to helices α4-α6 of the SARS-CoV CTD (Chen et al., 2007a). Conversely, gRNA packaging selectivity in the SARS-CoV NTD chimera was identical to that of wild-type MHV. This demonstrated that the second RNA-binding domain of N, the CTD, is the major protein determinant of MHV PS recognition. In the work reported here, we show that PS recognition additionally depends upon a segment of the carboxy-terminal tail of the N protein, domain N3. This finding suggests a mechanism by which selective genome packaging is carried out.

2. Results

2.1. Substitution of the TGEV CTD in MHV N abolishes recognition of the MHV PS

We found previously that selective packaging of MHV genomic RNA was abrogated by the substitution of the SARS-CoV CTD, but not the NTD, into the MHV N protein. To extend this result to a still more phylogenetically distant N protein, we chose TGEV, an alphacoronavirus for which selective genomic packaging has been

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