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Murine Coronavirus Packaging Signal Confers Packaging to Nonviral RNA

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Studies of defective interfering (DI) RNAs of the murine coronavirus mouse hepatitis virus (MHV) suggest that a 69-nucleotide-long packaging signal is necessary for MHV genomic RNA packaging into MHV particles. In this study we showed that when RNA transcripts that consisted of a non-MHV sequence and the packaging signal were expressed in MHV-infected cells, they were packaged into MHV particles. Those RNA transcripts that lacked the packaging signal or those containing a mutated packaging signal did not package efficiently. Thus, the presence of the packaging signal was sufficient for RNA packaging into MHV particles.

Packaging of viral genomic RNA into virus particles involves recognition and interaction of various molecules and is an essential step in the multiplication of any RNA virus. RNA packaging signals necessary for virus RNA packaging are described for several RNA viruses and hepadnaviruses (1, 5, 7, 11, 20, 27, 36, 37, 41, 42). Among these viruses, there are several examples in which the identified packaging signal is necessary and sufficient for viral RNA packaging (1, 7, 11, 42). The packaging signal(s) of some RNA viruses is mapped within more than one viral genomic region (20, 27, 36); for many viruses, it is not known whether a combination of multiple genomic regions or only one region is necessary for viral RNA packaging. Nor is it known if the packaging signal alone can suffice for viral RNA packaging.

Coronaviruses are large enveloped viruses containing a long, single-stranded, positive-sense genomic RNA that cause a variety of diseases in humans and animals (35). A prototype coronavirus, mouse hepatitis virus (MHV), contains a 31 kb-long MHV genomic RNA (17, 26). Other coronaviruses, such as infectious bronchitis virus (40), transmissible gastroenteritis virus (TGEV) (29), and bovine coronavirus (9), package virus-specific subgenomic RNAs as well as nonviral cellular RNA. Coronavirus contains four proteins, S, M, sM, and N protein; all except N protein are found in the virus envelope. S protein binds to host-cell receptors (38) and induces cell fusion (4). M protein, a triple-spanning transmembrane protein (2), is the most abundant glycoprotein. A minute amount of sM protein associates with MHV envelope (39). N protein and the genomic RNA form a helical nucleocapsid (21). Recently Risco et al. demonstrated that TGEV particles contain a spherical internal core, which seems to consist of mostly M protein and a lesser amount of N protein (28). They also showed that the helical nucleocapsid locates inside of the internal core (28). M protein and sM protein are necessary for packaging of viral nucleocapsid (12) and MHV envelope formation (3, 12, 34), while S protein is dispensable for both these functions (3, 12, 34). The MHV nonstructural proteins, including RNA polymerase and RNA proteinases, are translated from MHV-specific genomic-sized mRNA 1. Other MHV structural and nonstructural proteins are synthesized from six to seven smaller species of subgenomic mRNAs, mRNA 2 to mRNA 7. These mRNAs form a nested set with a shared 3′-coterminal structure (15, 18). Characteristically, all MHV mRNAs have an identical 5′-end leader sequence of approximately 72 to 77 nucleotides (nt) (14, 30). Among the mRNAs, only mRNA 1 is packaged into MHV particles (16), indicating that there is a mechanism which allows specific packaging of mRNA 1 into MHV.

Studies of cloned defective interfering (DI) RNAs of the JHM strain of MHV (MHV-JHM) identified a 69-nt-long MHV RNA signal (packaging signal) (Fig. 1) that is necessary for MHV DI RNA packaging into MHV particles (5). This packaging signal resides at about 20 kb from the 5′-end of the MHV genome and presumably is necessary for packaging of MHV mRNA 1 into MHV particles. The packaging signal forms a stable stem-loop structure (Fig. 1) that is important for its biological function. Although a 61-nt-long sequence within the 69-nt-long packaging signal also has a packaging function, sometimes the packaging efficiency of DI RNAs containing the 61-nt-long packaging signal is lower than those containing the 69-nt-long packaging signal (5). Analysis of a DI RNA from the A59 strain of MHV (MHV-A59) narrowed down a sequence containing the packaging signal to within 650 nt, which includes the 69-nt-long packaging signal (33).

We know that the MHV packaging signal is necessary for MHV DI RNA packaging, but we did not know whether this packaging signal was sufficient for RNA packaging. Replication of MHV-JHM DI RNAs requires three discontinuous regions; deletion of a part of these *cis*-acting replication signals abolishes DI RNA replication (13, 19). If MHV RNA packaging requires not only the packaging signal but also another MHV region(s) located within the *cis*-acting replication signal, then deletion analysis of MHV DI RNAs would not identify a putative secondary packaging signal; DI RNAs that have a deletion within the *cis*-acting replication signals do not replicate. Consequently, development of another RNA packaging system was needed to examine whether the packaging signal is sufficient for MHV RNA packaging.

To examine whether the packaging signal was necessary and sufficient for MHV RNA packaging into MHV particles, we studied whether expressed non-MHV RNA transcripts carry-

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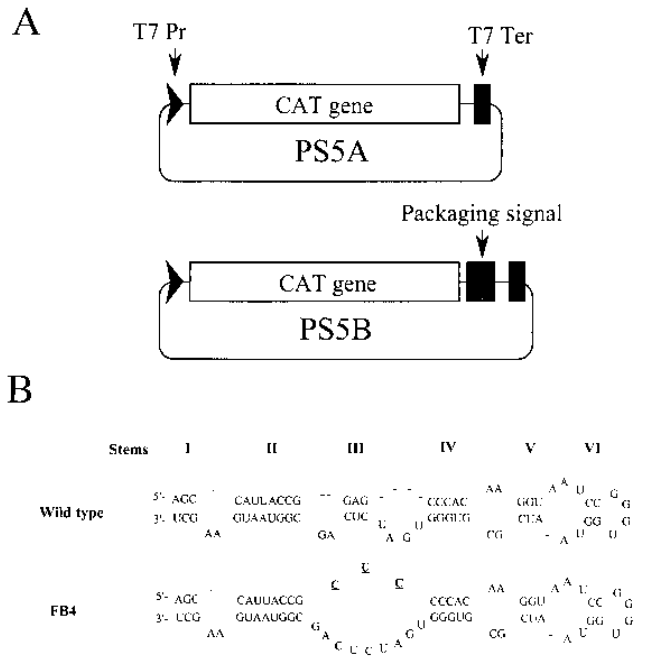

FIG. 1. Schematic representation of the transcription plasmids (A) and secondary structure of the 69-nt-long packaging signal (B). (A) Both PS5A and PS5B have a CAT gene between the T7 promoter (T7 Pr) and the T7 terminator (T7 Ter). PS5B contains the 69-nt-long packaging signal between the CAT gene and the T7 terminator. (B) Wild type and FB4 represent the 69-nt-long packaging signal present in PS5B and PS5BM, respectively. FB4 packaging signal was derived from MHV DI cDNA, FB4 (5). Underlined sequence represent mutated nucleotides in FB4.

ing the chloramphenicol acetyltransferase (CAT) gene plus the packaging signal were packaged into MHV particles. For this analysis, PS5A was constructed by inserting a CAT gene into the polylinker site of pT7ET-1 vector, which consisted of a pT7-3 (10)-derived sequence including the T7 promoter, a polylinker site, and pET-17b vector (Novagen)-derived T7 terminator (Fig. 1). DNA fragments that corresponded to the 69-nt-long packaging signal (Fig. 1), to a mutated packaging signal (Fig. 1), and to the 190-nt-long MHV sequence including the 69-nt-long packaging signal (5) were created by PCR and inserted downstream of the CAT gene in PS5A to create PS5B, PS5BM, and PS5B190, respectively. PS5B contained the 69-nt-long packaging signal between the CAT gene and the T7 terminator (Fig. 1). PS5BM contained a mutated 69-nt-long packaging signal that was derived from FB4 MHV DI RNA (Fig. 1); the mutated packaging signal in FB4 lacks packaging activity (5). PS5B190 had a structure similar to PS5B, except that it contained a 190-nt-long MHV sequence (nt 20353 to 20421 from the 5′ end of MHV-JHM genomic RNA) that included the 69-nt-long packaging signal and its flanking sequences (5). MHV genomic RNA and naturally occurring MHV DI RNAs that package efficiently contain this 190-nt-long region (5).

To express the RNA transcripts from these plasmids in MHV-infected cells, we infected DBT cells (8) with recombinant vaccinia virus vTF7-3 that expresses T7 DNA-dependent RNA polymerase (6) at a multiplicity of infection of 5. After 1 h of virus adsorption, cells were independently transfected with each plasmid using a lipofection procedure (10). At about 4 h postinfection (p.i.) of vTF7-3, the cells were superinfected with MHV at a multiplicity of infection of 5. Twelve hours after MHV superinfection, the supernatant was collected and the cell debris were removed by low-speed centrifugation. Clarified supernatant was placed on a discontinuous sucrose gradient consisting of 20% (wt/wt) sucrose and 60% sucrose in NTE buffer (0.1 M NaCl, 0.01 M Tris-HCl [pH 7.5], 0.001 M EDTA) and centrifuged at 26,000 rpm for 3 h at 4°C in a Beckman SW28 rotor. Partially purified MHV at the interface between 60 and 20% sucrose was collected and pelleted by centrifugation at 38,000 rpm for 1.5 h at 4°C in a Beckman SW41 rotor. Virus pellets were suspended in proteinase K solution containing 0.1 M Tris-HCl (pH 7.5), 12.5 mM EDTA, 0.15 M NaCl, 1% sodium dodecyl sulfate, and 200 μg of proteinase K per ml, and incubated at 37°C for 30 min. Virus particle-associated RNAs were extracted twice with phenol-chloroform followed by ethanol precipitation. Intracellular RNAs were also extracted at 12 h p.i. of MHV infection as described previously (22). To remove putative contamination of plasmid DNA in virus particle-associated RNAs and intracellular RNA preparations, RNA samples were incubated with 3 U of RNase-free DNase (Promega) in 30 μl of a buffer (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 40 U of RNasin [Promega]) for 6 h at 37°C. After incubation, samples were adjusted to pH 5.5 using 0.3 M sodium acetate and RNA was extracted with acid phenol-chloroform (Ambion) and precipitated with ethanol.

Northern blot analysis using a CAT gene-specific probe revealed that RNA transcripts from all of the transfected plasmids were expressed at a similar level in vTF7-3-infected, MHV-infected cells (Fig. 2A). Analyses of virus particle-associated RNAs revealed that only PS5B transcripts and PS5B190 transcripts, but not PS5A transcripts, were reproducibly detected in the pelleted virus particles (Fig. 2A). The amounts of virus particle-associated PS5B transcripts and PS5B190 transcripts were similar. In most cases, PS5BM transcripts were not detected in the pelleted virus particles, while in some experiments we detected a minute amount of PS5BM transcripts in virus particle-associated RNAs; the amount of PS5BM was less than 20% of that of PS5B. A band that migrated slightly faster than PS5B transcripts and PS5B190 transcripts (shown by an asterisk in Fig. 2A, lanes 13 to 16) was not seen in other experiments (data not shown) and was probably an experimental artifact. These results demonstrated that RNA transcripts containing an intact 69-nt-long packaging signal were packaged into MHV particles, whereas RNA transcripts lacking the packaging signal or those containing the mutated packaging signal were not packaged.

To know the buoyant density of MHV particles containing PS5B transcripts, MHV particles released from the PS5B transcript-expressing cells were centrifuged either overnight or for 3 h on a 20 to 60% continuous sucrose gradient. After centrifugation, fractions were collected and virus particle-associated RNAs were extracted. Northern blot analysis using a CAT probe and MHV-specific probe showed that MHV particles containing PS5B transcripts and those containing MHV genomic RNA were not separable under these centrifugation conditions (data not shown), indicating that the PS5B transcript-containing MHV particles and the infectious MHV had similar buoyant densities of approximately 1.19 g/cm^3.

We examined RNase A susceptibility of the virus particle-associated PS5B transcripts to clarify that the PS5B transcripts were indeed packaged inside MHV particles. If PS5B transcripts were packaged inside MHV particles, then the PS5B transcripts should be resistant to RNase A treatment of virus particles. If the PS5B transcripts were associated with MHV particles outside the particles, then the PS5B transcripts should be digested with RNase A. Clarified culture fluid from PS5B

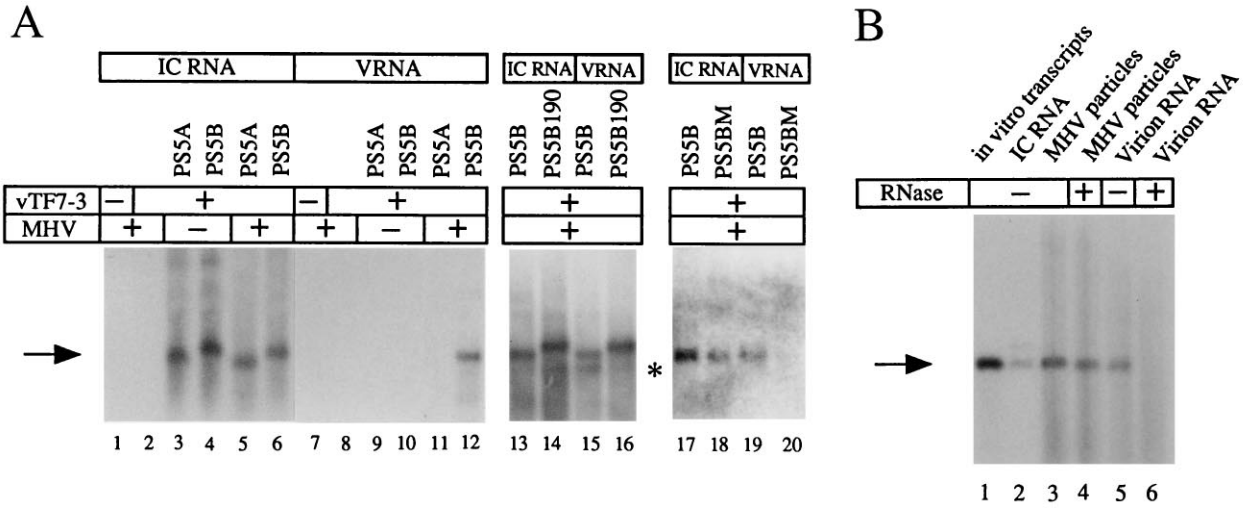

FIG. 2. Northern blot analysis of expressed RNA transcripts which were packaged into MHV particles (A) and virus particle-associated RNA samples after RNase A treatment (B). (A) Plasmid DNAs were transfected into vTF7-3-infected cells (lanes 2 to 6, 8 to 12, and 13 to 20) or mock-infected cells (lanes 1 and 7). At about 4 h p.i. of vTF7-3, cells were infected with MHV-A59 (lanes 1, 2, 5 to 8, and 11 to 20) or mock infected (lanes 3, 4, 9, and 10) and culture supernatant was harvested at 12 h p.i. with MHV. Intracellular RNAs were also extracted at 12 h p.i. with MHV. MHV particles were partially purified and virus particle-associated RNA was extracted. One-tenth of the intracellular RNA from the DNA-transfected cells (approximately 1.2×10^6 cells) and virus particle-associated RNA extracted from virus samples released into supernatant of approximately 2.4×10^6 cells were separated in 1% agarose gel containing formaldehyde, transferred to nylon membrane, and analyzed using Northern blot. A CAT gene-specific probe consisting of the entire length of the CAT sequence was prepared by a random priming labeling procedure (22) and used as a probe. IC RNA and VRNA represent intracellular RNA and virus particle-associated RNA, respectively. (B) Virus particle-associated RNAs suspended in 500 μl of sucrose solution (lanes 5 and 6) and 500 μl of partially purified virus sample in the same sucrose solution (lanes 3 and 4) from PS5B-expressing cells were incubated at 37°C for 30 min in the presence of RNase A (lanes 4 and 6) or in the absence of RNase A (lanes 1 to 3 and 5). After incubation, RNAs were extracted and analyzed by Northern blot analysis using a probe corresponding to the entire CAT sequence. The arrow shows PS5B transcripts. IC RNA, intracellular RNA extracted at 12 h p.i. with MHV. Lane 1, in vitro-synthesized PS5B transcripts.

transcript-expressing cells was placed on a discontinuous sucrose gradient consisting of 20% sucrose and 60% sucrose in NTE buffer and centrifuged at 26,000 rpm for 3 h at 4°C in a Beckman SW28 rotor. A total of 2 ml of partially purified virus sample was collected from the interface between 20 and 60% sucrose. Half the partially purified virus sample was mixed with an equal amount of 2× proteinase K solution, and the mixture was incubated at 37°C for 30 min. Virus particle-associated RNAs were extracted twice with phenol-chloroform followed by ethanol precipitation and then resuspended in 1 ml of 40% sucrose in NTE buffer. The other half of the partially purified virus sample (1 ml) was kept at 0°C during the preparation of virus particle-associated RNAs. Half of the partially purified virus sample and that of the virus particle-associated RNA sample were incubated at 37°C for 30 min in the presence of 0.1 ng of RNase A per ml. Another half of the virus sample and that of virus particle-associated RNA sample were incubated in the absence of RNase A. After incubation, 50 μg of tRNA and 500 μl of 2× proteinase K solution was added to each sample and the samples were further incubated for 30 min at 37°C. RNA was extracted with phenol-chloroform and precipitated with ethanol. Northern blot analysis showed that PS5B transcripts in the partially purified MHV was resistant to RNase A treatment, whereas naked PS5B transcripts present in the partially purified virus particles were completely digested by RNase A (Fig. 2B). These data clearly indicated that PS5B transcripts were packaged inside MHV particles. Thus the 69-nt-long packaging signal was sufficient for packaging of the expressed non-MHV RNA transcripts into MHV particles.

The non-MHV RNA transcripts containing the 69-nt-long packaging signal were packaged into MHV particles, yet how does the packaging signal facilitate packaging of non-MHV RNA transcripts? One possibility is that expressed non-MHV RNA transcripts interact with N protein to form helical nucleocapsid. M protein may recognize helical nucleocapsid formed by N protein and the expressed transcripts, because M protein is one of the packaged components of the internal core and, in vitro, M protein interacts with nucleocapsid but not with free N protein (32). If this is the case, the packaging signal may be a nucleation site for N protein, although MHV N protein tends to bind to RNA nonspecifically in vitro (24). One possibility for a specific recognition of packaging signal by N protein is that an unidentified cellular protein or an MHV-specific protein may specifically bind the packaging signal to form a protein-packaging signal complex. N protein may not specifically bind to the packaging signal directly, rather it may specifically bind to this protein-packaging signal complex. Once N protein binds to the protein-packaging signal complex, the N protein may undergo structural alteration which enables it to bind other N proteins along the RNA chain to form helical nucleocapsid. Another possibility for how the packaging signal facilitates packaging of non-MHV RNA transcripts is that the packaging signal may directly interact with a component of the spherical internal core during assembly of the spherical internal core.

The PS5B-containing MHV particles, MHV DI particles, and infectious MHV particles had a similar buoyant density (5, 23). Consequently, we do not know whether PS5B transcripts were copackaged with MHV genomic RNA to form virus particles or whether they were separately packaged. DI particles of TGEV are separable from infectious helper virus by sucrose gradient centrifugation (25), whereas using similar conditions we could not separate MHV DI particles from infectious MHV particles (5, 12, 23). It is tempting to speculate that MHV particles are efficiently formed only if the total mass of packaged RNAs is similar to that of MHV genomic RNA. Yet, TGEV DI particles are separable from infectious TGEV by sucrose gradient centrifugation (25), raising the question of

whether the total mass of DI RNAs in the DI particles is different from that of the genomic RNA.

Although MHV N protein tends to bind RNA nonspecifically (24), N protein is reported to specifically bind to the leader sequence of MHV mRNAs (31). The biological significance of N protein binding to the leader sequence during MHV replication is not clear. Our present data indicated that the binding of N protein to the leader sequence is not essential for MHV RNA packaging, because PS5B and PS5B190 do not contain leader sequence. However, it is still possible that binding of N protein to the leader sequence may have some effect on RNA packaging, e.g., the N protein-leader RNA interaction may increase the efficiency of RNA packaging.

The implication that the MHV particles containing non-MHV RNAs may be developed as an expression vector is exciting. Because MHV is a large virus that carries a 31-kb-long genomic RNA, an MHV expression vector should be capable of delivering large RNA molecules of non-MHV origin that are attached to the MHV packaging signal. Furthermore, the system established in this study will be useful to study the disassembly processes of coronavirus nucleocapsid, a process which must occur after infection, possibly simultaneously with the start of coronavirus genomic RNA translation. Characterization of nucleocapsid disassembly also will pertain to the development of a coronavirus-based expression vector system.

The first two authors contributed equally to this study.

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