Structural aspects of encapsidation signals in RNA viruses

Chen, S.C.

Chapter VIII

General discussion

General features of the packaging signals in positive-strand RNA viruses

Specific interaction between the (nucleo-)capsid proteins and their binding site(s) in the genomic (g) RNA is generally believed to dominate the selectivity of virion packaging. Historically, such interactions were used for the discovery of the so-called packaging signal (PS) *in vitro*, which is the critical RNA sequence that directs selective packaging of gRNA. Nuclease protection assays have allowed researchers to identify viral RNA sequences that tightly bind to (nucleo-)capsid proteins, and this was how the very first RNA sequence of the packaging origin was identified in the *Tobacco mosaic virus* (TMV) (Zimmern & Butler, 1977). Specific interactions between the PSs and the (nucleo-)capsid proteins have also been shown for alphaviruses (Owen & Kuhn, 1996; Weiss *et al*., 1989), retroviruses (Berkowitz *et al*., 1995; Zhang & Barklis, 1995), and bacteriophages *in vitro* (Witherell *et al*., 1991).

The presence of repeating (structural) motifs which are specific for (nucleo-)capsid protein binding is a general feature for PSs. For instance, the $(GNN)_n$ motifs found in TMV (Wilson & McNicol, 1995), the C:C and C:A mismatches in the two adjacent hairpins in *Turnip yellow mosaic virus* (TYMV) (Hellendoorn *et al*., 1997; Bink *et al*., 2003), and the AUGC motifs in *Alfalfa mosaic virus* (AMV) (Gougas *et al*., 2004; Laforest *et al*., 2004). The repetition of these (nucleo-)capsid protein binding motifs in PSs may enhance the binding efficiency, while on the other hand the specific orientation of these motifs can promote the oligomerization of (nucleo-)capsid proteins.

The group IIa coronavirus PS: the structural motifs and the interaction with nucleocapsid proteins

In the *Mouse hepatitis virus* (MHV), a type member of coronavirus (CoVs), the PS is considered to interact with the nucleocapsid (N) protein for genome encapsidation and virion assembly, which was defined within nucleotides (nts) 20,208 to 20,398 of the MHV gRNA comprising the 190-nt element of MHV PS (Makino *et al*., 1990; van der Most *et al*., 1991; Fosmire *et al*., 1992). It has been shown that the region is indispensable for virion packaging and able to direct packaging of heterogeneous RNA containing this region (Lin & Lai, 1993; Woo *et al*., 1997). Complexes of N protein and PS were identified in the lysate of MHV-infected cells, and the interaction between the PS-containing RNA and the N protein showed selectivity against tRNA and cytoplasmic RNA (Molenkamp & Spaan, 1997). However, specificity of the interaction between PS and N-protein is poor *in vitro* since the MHV N-protein binds to tRNA as well as to the 96-nt MHV PS transcripts (Chen & Olsthoorn, unpublished data). This is probably due to the fact that the N proteins exhibit both PS-specific and non-specific activity in RNA binding (Cologna *et al*., 2000; Wei & Morris, 1991).

Difficulties in studying the specificity of the PS-N protein interaction have hampered the understanding of the actual N-protein binding sites in MHV PS. However, as demonstrated in Chapter III of this thesis, structural characterization of the group IIa PS has led to a better model for the 96-nt MHV PS, *i.e.* a stem-loop (SL) structure which consists of repeating substructural motifs, the AA-bulges (Chen *et al*., 2007). Our new model fits the mutagenesis results much better than the 69-nt stem-loop model reported by Fosmire *et al.* (1992). These bulges are the potential binding sites for the N proteins and presumably promote oligomerization of N proteins that are orientated alongside the PS, triggering the subsequent encapsidation of the entire gRNA.

The RNA-N protein interaction is rather non-specific in CoVs as not only the gRNA was found to form intracellular ribonucleoprotein (RNP) complexes in infected cells but also subgenomic RNAs and non-CoV RNA (Baric *et al*., 1988; Cologna & Hogue, 2000; Cologna *et al*., 2000; Masters, 1992; Narayanan *et al*., 2000; Narayanan & Makino, 2001). Thus, the selectivity of the gRNA packaging may not be solely determined by the RNA-N protein interaction. Narayanan *et al*. (2000; 2001) have reported a specific interaction that occurs between the viral membrane (M) protein and the gRNA-RNP complex at the budding site in infected cells. The 190-nt PS was shown to mediate the specific interaction between the M protein and the RNP complex. They have further demonstrated that the interaction between the M protein and the PS-containing RNA directs a specific packaging in the absence of N protein, although whether the M protein directly interacts with the PS was unclear (Narayanan *et al*., 2003). This is consistent with a previous observation that for CoVs the formation of virus-like particle (VLPs) can occur in the absence of N-protein (Vennema *et al*., 1996).

Interestingly, in contrast to MHV the N protein of SARS-CoV was found to be required for the formation of VLPs, containing non-viral RNA fused to the putative SARS-CoV PS, nt 19715 to 20294 of the SARS-CoV gRNA (Hsieh *et al*., 2005). Thus, the requirement for the N protein for the initiation of specific packaging may be different in MHV and SARS-CoVs. However, the region proposed to contain the SARS-CoV PS (Hsieh *et al*. (2005) does not exhibit the typical conserved structural motifs present in the group IIa CoV PS, suggesting that the region may not strongly direct gRNA packaging. This may further indicate that the presence of the N protein improves the efficiency and/or the selectivity of packaging, particularly when a weak PS is used to package heterogeneous RNA. We hypothesize that, in group IIa CoVs, the N proteins strongly bind to the AA bulges orientated in the PS to initiate encapsidation of the entire gRNA, while the M protein may specifically interact with the CACAA loop sequence. These two interactions may take place sequentially in different compartments. Subsequently, the membrane bound M protein interacts with the RNP complex by the specific protein-protein interaction between the N and M proteins to incorporate the RNP into enveloped vesicles (Hurst *et al*., 2005;

2009). A similar mechanism may apply to other CoVs, *e.g.* the SARS-CoV, though the PS which consists of the N protein binding motifs is probably different from the one reported by Hsieh *et al*. (2005). Chapter IV of this thesis describes why the conserved UUCYGY motifs present in the 5'UTR of SARS-CoV gRNA may be the potential N-protein binding sites (*vide infra*).

Interpretation of the 5' stem-loop 5 as the PS in group I, IIb, IIc and IId CoVs

To date, the PS is still unknown in group I, IIb, IIc, and IId CoVs. Nevertheless, the PS of transmissible gastroenteritis virus (TGEV), a group I CoV, has been pinpointed to the 5' 649 nts of the gRNA (Escors *et al*., 2003). Such a location is different from that of the group IIa CoVs, *e.g.* MHV and BCoV, of which the PSs are located in the 3' end of open reading frame (ORF) 1ab (Makino *et al*, 1990; Cologna & Hogue, 2000; Hsieh *et al*., 2005)**.** Recently, Hsieh *et al*. (2005) have identified an element similar to MHV PS in the corresponding ORF1ab in SARS-CoVs, which directs the packaging of non-viral RNA and forms VLPs. However, the secondary structure of this element does not feature the typical AA-bulges that are a hallmark of the PS of group IIa coronaviruses (Chapter III). In fact, sequence alignment of all group I and II CoVs ORF1abs suggested that the sequence corresponding to the MHV PS is exclusively present in group IIa CoVs (Chapter IV). On the other hand, we have shown that the conserved substructural hairpins present in stem-loop 5 (SL5) of group I and II CoVs are exclusively absent in the group IIa CoVs. The mutually exclusive presence of the PS and these substructural hairpins has led to the assumption that the inserted SL5 substructures in group I, IIa, IIb, and IIc CoVs are the counterpart of the PS present in the 3' proximal region of the ORF1ab in group IIa CoVs, and thus may function as a PS.

One of the general features for a typical PS, which is to consist of repetitive motifs, further suggests that the SL5 substructural hairpins are the potential PS. We have shown that the PS of group IIa CoVs exhibits repeating conserved AA-bulge motifs which are likely to be the N protein binding sites (Chapter III). Repetitive motifs were also found in the loops of the SL5 substructural hairpins present in group I, IIb, IIc, and IId CoVs, which are the 5'-UUCYGY-3' sequences (Chapter IV). Thus, the nature of the structural motifs that we have found in these SL5s strongly suggests that this region is responsible for N protein binding.

The ability to interact with (nucleo-)capsid protein is another general property of PSs (Weiss *et al*., 1989; Owen & Kuhn, 1996). The interaction between the PS and the N protein has been shown in MHV (Molenkamp & Spaan, 1997). We have found that the SL5 containing transcripts of SARS-CoV and HCoV-229E can strongly interact with SARS-CoV and HCoV-229E N proteins *in*

vitro, respectively (Chen & Olsthoorn, unpublished data), indicating that the SL5 is the potential PS in these two viruses.

The 96-nt MHV PS transcripts were found not to significantly interfere with the binding between the SARS-CoV SL5-containing transcripts and the SARS-CoV N protein, or the binding between the SARS-CoV SL5-containing transcripts and the HCoV-229E N protein. This observation indicated that the SARS-CoV and the HCoV-229E N proteins preferably recognize the N protein binding sites present in the SARS-CoV SL5, which presumably are the 5'-UUCYGY-3' motifs, but not the other type of the CoV N protein binding sites, the AA-bulges located in MHV PS and other group IIa CoVs.

Since the number and the sequence of the conserved motifs in SL5 are slightly different in SARS-CoV and HCoV-229E, we were interested in the specificity of each N protein-SL5 interaction. We have found that the interaction between SARS-CoV SL5 transcript and the N protein was not disrupted by the HCoV-229E SL5 transcripts. On the other hand, transcripts of the SARS-CoV SL5, rather than that of the HCoV-229E SL5, were better competitors to disrupt the heterogeneous binding between the HCoV-229E N protein and the SARS-CoV SL5 transcripts. This implies that the binding interfaces between the homogenous and the heterogeneous interactions may be different and once one of the interacting modes has been induced-fit, it may be hard to compete with.

To sum up, the SL5 and the structural motifs in this region match to the general features of PSs, i.e. the (oriented) repetition and the N-protein binding affinity. We hypothesized that the SL5 and the sub-structural SL5a, b, and c in the 5'UTR of the group I, IIb, IIc, and IId CoVs are the functional counterparts of the PS present in the ORF1ab of the group IIa CoVs.

Roles of the interaction between the coat protein and the AUGC repetition in Alfalfa mosaic virus 3'-terminus

The AUGC repeats in the gRNA 3'-terminus, which are characteristic of the alfalfa mosaic virus (AMV) and ilarviruses, were found to be important for coat protein (CP) binding (Gougas *et al*., 2004; Houser-Scott *et al*., 1994; Houser-Scott *et al*., 1997; Laforest *et al*., 2004; Reusken *et al*., 1994; Rocheleau *et al*., 2004). The CP binding site 1 (CPB1) which consists of two AUGC repeats is a likely candidate for the origin of assembly of AMV virus particles (Bol, 2005). It was found that the intermolecular base pairing between the AUGC motifs is promoted in the presence of CP, resulting in a stable and compact co-folded complex (Gougas *et al*., 2004). However, deletion of the 3' UTRs of AMV RNAs 1 and 2 did not abrogate their encapsidation (Vlot *et al*., 2001). This indicates that the 3' AUGC repetition in AMV is dispensable for encapsidation because the assembly of virions may be initiated from internal CP

binding sites, which also consist of AUG(C) repeats (Zuidema & Jaspars, 1984). Thus, the 3' AUGC repeats are not that critical for encapsidation but the strong interaction with CP regulates the conformational switch in the AMV 3'-termini of the gRNAs.

The conformational switch between the tRNA-like (TL) and the coat protein binding (CPB) conformers in the gRNAs is crucial for the AMV lifecycle, and it has been proposed that the CP regulates the switch between translation and replication of AMV (Bol *et al*., 2005; Olsthoorn *et al*., 1999; Chapter VI). In the initial stage of the AMV infection, the semi-uncoated gRNAs may maintain the CPB conformation in the 3' terminus, thereby enhancing translation of viral replicase (Bol, 2005; Krab *et al*., 2005; Neeleman *et al*., 2004). Subsequently, the synthesized replicase protein binds to the minus-strand promoter, the hairpin E near the gRNA 3'-terminus (Olsthoorn *et al*., 1999), for the synthesis of the minus-strand RNA, and the 3' TLS facilitates the synthesis to be initiated from the very 3'-end of the template RNA (Olsthoorn *et al*., 2002). The synthesis of minus-strand RNA requires the dissociation of the CP from the 3' end of the parental RNAs, which is possibly mediated by the replicase proteins, to switch the CPB conformation to the TL conformation (Bol, 2005). The CP dissociation not only switches off the translation but also clears the template plus-strand RNA from ribosomes for synthesizing the minus-strand RNA (Bol, 2005). Once the minus-strand RNA 3 is synthesized, the synthesis of the subgenomic RNA 4 which encodes the CP can be initiated by the subgenomic promoter that is internally located in the minus-strand RNA 3 (Haasnoot *et al*., 2000, 2003). The presence of subgenomic RNA 4 results in the massive expression of CP subsequently. Although the mechanism of the switch from minus-strand to plus-strand RNA synthesis is largely unknown, it is believed that the strong binding affinity between the AMV CP and the gRNA 3' termini may eventually stop all the RNA synthesis at the high level of CP during the late stage of the AMV lifecycle, followed by the encapsidation of the replicated gRNAs. At last, the encapsidated gRNAs are no longer accessible to ribosomes for translation and subsequently are packaged into virions.

The mismatches in the 5' structural element of BaMV and its satellite RNAs

The functional aspects of the 5' structural element in BaMV are still poorly understood, although we have shown that the accumulation of the gRNA is reduced when the secondary structure of the 5' element was altered (Chapter VII). The structural homologue of the element in *Potato virus X* (PVX) was reported to interact to viral and cellular proteins (Kim *et al*., 2002; Miller *et al*., 1998). The C:C mismatch in the 5' terminal hairpin of PVX is crucial for CP binding, directing the encapsidation and the cell-to-cell movements. Kwon *et al*.

(2005) have further reported that strong CP binders selected from an RNA library by *in vitro* SELEX closely resemble the secondary structure of the wild-type PVX 5'-element, possessing the C:C mismatch. Interestingly, the presence of mismatch(es) is highly conserved in all structural homologues we have identified in potexviruses (Chapter VII). Previously, studies on the turnip yellow mosaic virus (TYMV) have shown that the protonatable C:C and C:A mismatches upstream to the start codon are crucial for CP mediated regulation of the encapsidation and or translation (Hellendoorn *et al*., 1997). Although we do not know if the C:A and C:C mismatches are protonated in the 5' element found in BaMV, it is likely that the two mismatches function as what has been reported in TYMV, which is to interact with CP. Interestingly, a TYMV mutant which has a perfect base-paired stem evolved mostly non-wild-type C:C and C:A mismatches after several passages in plants (Bink *et al*., 2002), indicating that the possession of the C:C and C:A mismatches may promote a better virus propagation. Since the C:C and C:A mismatches are also found in the 5' element of BaMV, which is upstream to the start codon, the mismatches could possibly function as a CP binding site as well, though the interaction should be further determined in the future.

The repetition of the mismatch-containing hairpins can be found in the 5'-proximal sequences of many RNA viruses. For instance, two mismatch-containing hairpins were found in TYMV and *Eggplant mosaic virus* (EMV) RNA while four such hairpins were located in the *Ononis yellow mosaic virus* (OYMV) RNA (Hellendoorn *et al*., 1996). Therefore, the binding affinity to the CP and the presence in repetition suggested that these mismatch-containing hairpins are PSs. Downstream of the 5' element of the BaMV reported in Chapter VII, we have identified another SL structure containing C:C and C:A mismatches (Chen and Olsthoorn, unpublished data). Thus, it is possible that one of the functions for the element, together with the downstream mismatch-containing hairpins, is to direct encapsidation.

Conclusions and perspectives

In this thesis, we have identified structural repetition in the known PS of group II CoVs and further shown that the sequence insertions in other CoVs can form characteristic repetitive structures that suggest their involvement in encapsidation. We also identified structural elements in the 5' UTR of the BaMV and other potexviruses, which consist of the potential CP binding mismatches. In addition, the role of the interaction between the AMV 3' terminus and the AMV CP was further studied for its regulation of the conformational switch.

In the exploration of novel PS in CoVs, we observed that sequence insertions that corresponded with the repeating motifs were seldom located in a region of the genome that encodes a functional domain of a viral (poly-) protein. Apparently, the constraints on viral RNA structure cannot always be

combined with its coding capacities. Thus, the PS and/or other large RNA elements present in coding regions are predominantly located in the "inter-domain" to moderate the interference caused by sequence insertions. This phenomenon may be exploited to search for unidentified cis-acting signals in the genomes of other RNA viruses.

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