Structural aspects of encapsidation signals in RNA viruses

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Chapter II

Encapsidation signals in positive-strand RNA viruses - a literature review

Introduction

In the life cycle of a positive-strand RNA virus, genomic RNA is translated for expression of viral proteins, replicated for propagation of the virus, and encapsidated for assembly of virions. Assembly of an RNA virus is initiated with the binding of nucleocapsid proteins or core protein complexes to the encapsidation signal(s) located in the viral genomic RNA(s). The presence of an encapsidation signal in the genomic RNA promotes the oligomerization of nucleocapsid proteins and/or the interactions with other viral proteins, so that the genomic RNA is specifically packaged thereby forming virus particles. The encapsidation signals in RNA viruses are usually *cis*-acting elements exhibiting specific sequence and/or particular secondary structures that interact with (nucleo-)capsid proteins. To date, many positive-strand RNA viruses have been studied for their encapsidation signals. In this review, the structural aspects of these encapsidation signals in the positive-strand RNA viruses are highlighted.

The packaging origin of tobacco mosaic virus

In tobacco mosaic virus (TMV), the type member of the genus *Tobamovirus*, a particular fragment of genomic RNA had been originally found tightly associated with the capsid protein disks and resistant to nuclease digestion (Zimmern & Butler, 1977). The fragment is a 500-nt region located in the 3'-half of the TMV RNA (Guilley *et al*., 1979; Ohno *et al*., 1977; Zimmern & Wilson, 1976). The fragment containing the packaging origin was further narrowed down to the sequence between the position of nt 5420 and 5546 in TMV genomic RNA, which contains the core sequence of packaging signal (PS), 5'-AAGAAGUC(U)G-3' (Goelet *et al*., 1982; Jonard *et al*., 1977; Zimmern, 1977). The

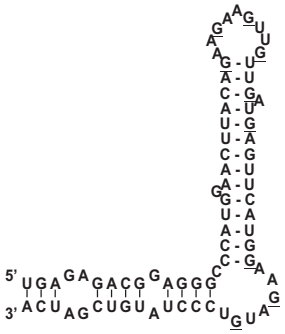

Figure 1. The packaging origin of the Tobacco mosaic virus. The critical G residues for capsid protein binding are underlined.

secondary structure for the packaging region was then proposed (Fig. 1) (Zimmern, 1983).

Structure probing of the main stem of TMV packaging origin was in agreement with the stem-loop feature, and alterations of the secondary structure were reported to interfere with the initiation of packaging (Turner *et al*., 1988). The core sequence located in the loop region was found critical for efficient packaging; sequence deletions or substitutions resulted in reduced packaging efficiency (Turner & Butler, 1986), while heterogeneous RNA containing the packaging origin could be encapsidated into virions (Sleat *et al*., 1986; Sleat *et al*., 1988a, b).

Sequence comparisons further suggested that packaging origins in all known tobamoviruses exhibit stable hairpin structures with the looped-out common target sequence, GANGUUG (Okada, 1986). It was then suggested that the G residues which appear with 3-nt intervals in the origin-of-assembly sequence initiate RNA encapsidation bi-directionally (Wilson & McNicol, 1995). Thus, the $(GNN)_n$ motifs were suggested to be specifically recognized by the disks made of TMV capsid proteins *in vitro*. On the other hand, since the genomic RNA of tobamoviruses has a statistically significant bias for G at the first position every third (or 3n) nucleotides in the region, the high frequency of GNN codons results in high Val, Ala, Gly, Asp, or Glu content in the corresponding proteins (Okada, 1999). However, the functional relevance of this observation has yet to be determined.

Although the mechanism of TMV assembly has been studied intensively *in vitro*, the nature of the *in vivo* elongation process remained unclear. It is still not known how TMV is packaged in plant cells, though self-assembled pseudovirus particles which contain heterogeneous RNAs have been reported in *E. coli* (Hwang *et al*., 1994).

The encapsidation signal in turnip yellow mosaic virus

The knowledge of encapsidation signals in the genus *Tymovirus* was mostly obtained from studies on turnip yellow mosaic virus (TYMV). Virions of TYMV were found unstable and leading to decapsidation at high pH *in vitro* (Kaper, 1971). It had been further shown that TYMV RNA specifically binds to empty protein capsid at low pH though the RNA might not be fully encapsidated (Briand *et al.*, 1975).

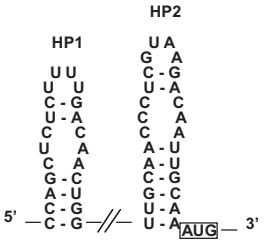

Figure 2. Turnip yellow mosaic virus packaging signal. The two mismatch containing hairpins, HP1 and 2, are shown with the boxed AUG start codon of the TYMV *gp1* gene.

Regions for initiating coat protein (CP) binding are located in the 5'-UTR of TYMV genomic RNA, which folds into two stem-loop structures, assigned as hairpin 1 (HP1) and HP2, with characteristic C:C and

C:A mismatches (Hellendoorn *et al*., 1996) (Fig. 2). These mismatches were shown protonatable at low pH *in vitro* facilitating infectious virion assembly (Bink *et al*., 2002, 2003; Hellendoorn *et al*., 1996, 1997; van Roon *et al*., 2004).

An *in vivo* evolutionary assay also suggested that mutants with Watson-Crick base-pairs tend to revert to having C:C and/or C:A mismatches in HP2 (Bink *et al*., 2003; Hellendoorn *et al*., 1997). Specific interactions between the two 5′ proximal hairpins and CPs were further proposed to regulate the pH-dependent translation and encapsidation of TYMV RNAs (Bink *et al*., 2003).

More recently, virion assemblies have been observed *in vivo* when TYMV RNA and CP were transiently expressed in agro-infiltrated non-host plants, *N. benthamiana* leaves, and both empty capsids and virions containing (sub-)genomic RNAs were found (Cho & Dreher, 2006). However, applying a similar method to study the TYMV encapsidation in Chinese cabbage, it was found that mutations in HP1 and HP2 seriously interfere with viral replication but not encapsidation (Shin *et al*., 2008; 2009). Moreover, it was also demonstrated that TYMV can efficiently replicate in plants held in the dark showing sufficient encapsidation of the propagating RNAs (Rohozinski & Hancock, 1996). These observations could be contradictory to the previous model for TYMV encapsidation, which stated that the packaging occurs at low pH generated by proton gradients produced by photosynthetic activity in the light (Shin *et al*., 2009). Thus, how the protonation of TYMV HP1 and HP2 hairpins is achieved remains unclear.

The PS in brome mosaic virus and alfalfa mosaic viruses

The tRNA-like structure (TLS) at the 3′ terminus of genomic RNAs has been suggested to function as a PS in brome mosaic virus (BMV), the type member of the genus *Bromovirus.* The presence of TLSs, both *in cis* and *in trans,* has been shown to initiate BMV encapsidation *in vitro*, while disruption of the TLS resulted in no packaging (Choi *et al*., 2002). However, the necessity of 3′ TLS for BMV encapsidation was uncertain. The encapsidation of mutant RNA1 and RNA2 lacking 3′ TLS was proposed to be promoted by host tRNA (Rao, 2006). It was further reported that only RNA3, but not RNA1 or RNA2, requires the 3′ TLS *in cis* for BMV encapsidation *in vivo* (Annamalai & Rao, 2007), suggesting that the packaging of TLS-less RNAs *in vivo* can be promoted by *trans* complementation of cellular tRNAs or that the packaging is independent to TLS *in vivo* (Annamalai & Rao, 2007).

UV cross-linking and band-shift assays demonstrated that BMV CP binds to the BMV TLS (Choi *et al*., 2002; Damayanti *et al*., 2002). Nevertheless, CP binding affinity of certain RNA elements does not necessarily imply their functioning as PS. For instance, the interaction between the CP and the BMV 3′ and 5′ UTR was reported for regulating the translation inhibition and the

minus-strand RNA synthesis, rather than directing genomic RNA packaging (Yi *et al*., 2009; Zhu *et al*., 2007). Moreover, in the case of the closely related cowpea chlorotic mottle virus (CCMV), encapsidation of genomic RNA *in vitro* requires no TLS (Annamalai & Rao, 2005). On the other hand, deletion of the 3′-TLS from the TYMV genomic RNA did not alter viral RNA encapsidation in *N. benthamiana* (Cho & Dreher, 2006), the specificity of the packaging was reported to be coupled with replication (Annamalai & Rao, 2006).

In alfalfa mosaic virus (AMV), the presence of the 3′ UTR was reported to promote encapsidation (Gougas *et al*., 2004) though it is the stem-loop conformer, rather than the tRNA-like conformer, that interacts with CP and initiates the encapsidation. The stem-loop conformer, of which the multiple CP-binding AUGC motifs are spatially orientated, was shown to interact strongly with AMV CP (Gougas *et al*., 2004; Laforest *et al*., 2004), but the stabilized TLS conformer was shown to decrease binding affinity with CP (Olsthoorn *et al*., 1999; Chen & Olsthoorn, unpublished data). Thus, it is unlikely that encapsidation of BMV requires the 3′ TLS, *per se*, although the 3′-UTR may function cooperatively with internal PS located within the coding region of BMV RNA1 (Duggal *et al*., 1993) and RNA3 (Choi & Rao, 2003; Damayanti *et al*., 2003) (*vide infra*).

Alternatively, two non-overlapping regions in BMV RNA3 were proposed to contain the internal PS (Fig. 3) in two independent studies (Damayanti *et al*., 2003; Choi & Rao, 2003). Damayanti *et al*. (2003) demonstrated that a 69-nt sequence which folds into two distinct stem-loops (SL), the SL1 and SL2 (Fig. 3A), is crucial for BMV RNA3 packaging in barley protoplasts. Disruptions of the SL1 and/or SL2 secondary structure resulted in poor packaging. However, Choi and Rao (2003) proposed another region in the BMV RNA3, from the position of nt 602 to 817, which folds into three structural elements A, B, and C (Fig. 3B) as the encapsidation signal. Deletion of either one or both of the elements resulted in less or no packaging of BMV. Thus, both the two non-overlapping regions and their secondary structures are crucial for BMV encapsidation. In addition, the specificity of

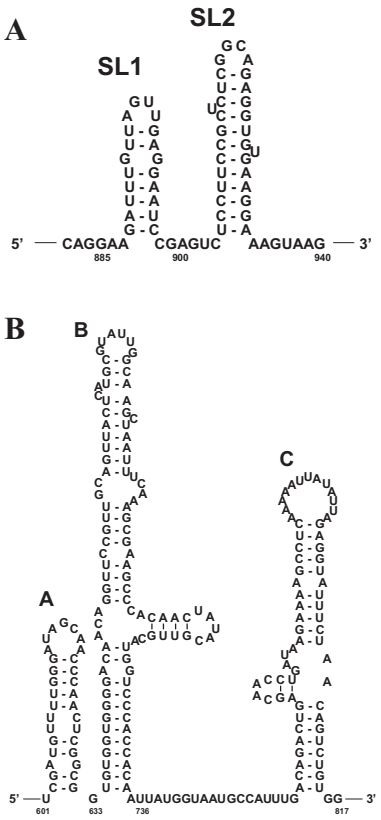

Figure 3. Brome mosaic virus packaging signals. The secondary structures of the packaging signals proposed by (A) Damayanti *et al*., 2003, *J Virol*. 77:9979-9986 and (B) Choi & Rao 2003. *J Virol*. 77:9750-9757.

packaging BMV genomic RNAs, and possibly that of other bromoviruses as well, is dependent on coupling the replication-dependent transcription and the translation of CP (Annamalai & Rao, 2006), which may complicate the identification of bromovirus encapsidation signals *in vivo*.

The PS in turnip crinkle virus

The *in vitro* dissociation and reassembly of turnip crinkle virus (TCV), the type member of the genus *Carmovirus*, have shown that neutral pH promotes formation of ribonucleoprotein (RNP) complexes which consist of viral RNA tightly bound to CP subunits (Sorger *et al*., 1986; Golden & Harrison, 1982). It was reported that the presence of RNA is required for specific virion assembly initiated by TCV CP. The heterogeneous TMV and the closely related tomato bushy stunt virus (TBSV) genomic RNAs are shown to be packaged less efficiently than the TCV RNAs, although the 18S non-viral rRNA could also be packaged with high efficiency by TCV CP (Sorger *et al*., 1986).

The specificity and the high efficiency of TCV encpasidation were hypothesized to be initiated by formation of RNP complexes at a specific site in the TCV genome (Sorger *et al*., 1986; Skuzeski & Morris, 1995). The CP interacting RNA sequences have been identified using nuclease protection assays. Five protected RNA fragments which are clustered into two regions of the TCV genome were discovered (Wei *et al*., 1990). These two clusters of sequences were subsequently reported to direct encapsidation by independent researches.

One of the two clusters, consisting of fragments a, f, and c, is situated within a region of approximately 400 nts that includes the leaky stop codon in the TCV polymerase gene. The other cluster consists of fragment d and e and is located within the CP coding region near the 3'-end of the genome. Notably, these two fragments are also present in the subgenomic RNAs which are corresponding to the 3'-terminal 1700 and 1500 nts of the TCV genomic RNA (Carrington *et al*., 1987; 1989).

The TCV CP was suggested to contain both specific and non-specific RNA binding activities, and the specific interacting sites of the TCV RNA were first proposed to be located in the 386-nt sequences corresponding to fragments a, f, and c. (Wei & Morris, 1991). However, wobble mutations applied to the 386-nt sequence, which altered the secondary structure of the region while keeping the amino acid sequence of encoded polymerase protein identical, disabled the replication of TCV (Wei *et al.*, 1992). On the other hand, binding affinity between TCV CP and the 386-nt transcripts was shown no better than

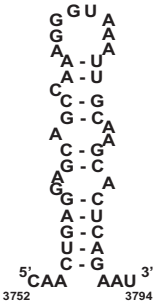

Figure 4. The packaging signal of Turnip crinkle virus. The secondary structure of the TCV PS is a stem-loop with a bulge and internal loops.

that between TCV CP and heterogeneous RNA (Skuzeski & Morris, 1995). Thus, studies of the 386-nt in fragments a, f, and c as PS was abandoned because of the difficulties in constructing viable mutants and studying the specificity of CP binding (Wei & Morris, 1991; Wei *et al*., 1992; Skuzeski & Morris, 1995).

A 186-nt region consisting of the fragments d and e at the 3' end of the TCV CP-coding region was subsequently shown to be crucial for specific encapsidation in protoplasts (Qu & Morris, 1997). Furthermore, it was shown that the size of the viral RNA to be packaged is another critical factor for stable virion assembly. Eventually, it was shown that a 28-nt bulged hairpin within the 186-nt region (Fig. 4) is indispensable for packaging and directs encapsidation (Qu & Morris, 1997).

The PS in Sindbis virus

Large amounts of genomic and subgenomic RNAs are replicated and/or transcribed in alphaviruses infected cells, but usually only the genomic RNAs are packaged into virions. This implies the existence of an encapsidation signal which is responsible for the selective packaging of genomic RNA (Frolova *et al*., 1997). In Sindbis virus, the type member of the genus *Alphavirus*, a 600-nt fragment from the position of nts 721 to 1306 in the viral methyltransferase nsP1 gene was reported to contain the specific signal for CP binding, which directs encapsidation of defective interfering (DI) RNAs (Geigenmüller-Gnirke *et al.*, 1993; Weiss *et al*., 1989). Sindbis virus DI RNA which has one copy of the CP binding site was shown to strongly interact with CP *in vitro* while the DI RNA lacking the CP binding site does not bind to CP (Weiss *et al*., 1989). Such difference in CP binding on the other hand resulted in efficient packaging of signal containing DI RNA and no packaging of signal-less DI RNA, respectively (Weiss *et al*., 1989). The CP binding site in the 600-nt fragment was further specified to the 132-nt span from the position of nts 945 to 1076 in the Sindbis virus genomic RNA (Fig. 5), which specifically binds to CP or the 68-amino-acid peptide derivative (Weiss *et al*., 1994). Insertion of the PS in the subgenomic RNA of the Sindbis virus replicon, particularly to the 5'-end, led to significant incorporation of the subgenomic RNA into extracellular particles (Bredenbeek *et al*., 1993; Frolova *et al*., 1997).

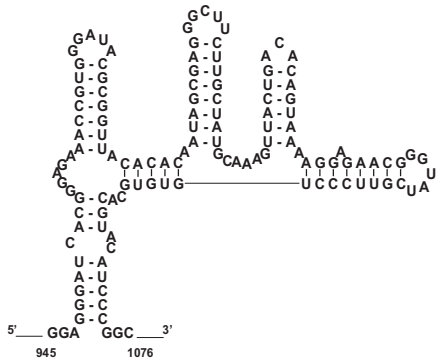

Figure 5. The packaging signal of Sindbis virus. The secondary structure of the Sinbis virus PS is shown to consist of several stem-loop structurs.

In contrast to what has been reported for Sindbis virus, the PS of the related Ross River virus (RRV) was

found at multiple positions in the genomic RNA. Another difference considering between the encapsidation signals of RRV and Sindbis virus is that the Sindbis virus RNA could not be recognized by RRV CP, neither *vice versa* (Frolova *et al*., 1997).

Among all the potential encapsidation signals identified in Sindbis virus, the most promising one lies between the positions of nts 2761 and 3062 in the nsP2 gene, of which the corresponding sequence was proposed to contain homologues of the Semliki Forest virus (SFV) PS, another alphavirus (Frolova *et al*., 1997). It has been found that not only the homology of the PS but also that of the CPs is high between RRV and SFV (Frolova *et al*., 1997). It was further suggested that the location(s) of the encapsidation signals may be correlated to the conservation of the CPs in alphaviruses (Frolova *et al*., 1997; Strauss & Strauss, 1994). The specificity of encapsidation is determined by the interaction between the PS and the binding CP. Nevertheless, it was reported that a mutant lacking residues 97 to 106 in the CP resulted in non-specific encapsidation of Sindbis virus subgenomic RNA (Owen & Kuhn, 1996). This suggested that the specificity of encapsidation *in vivo* may not only be determined by the presence of the PS in the genomic RNA but also by the properties of the CP in Sindbis virus.

More recently, a model of the 132-nt RNA PS which can promote the dimerization of CPs was described for Sindbis virus (Linger *et al*., 2004). Mobility shift assays showed that a 32-amino acid peptide is capable of recognizing the Sindbis virus encapsidation signal, and that the bound RNA molecule undergoes a conformational change. The multiple purine-rich stem-loops (Fig. 5) within the encapsidation signal was shown to be crucial for efficient packaging, which directs early events of the nucleocapsid assembly (Linger *et al*., 2004).

The PS in Mouse hepatitis virus

The PS in mouse hepatitis virus, the type member of the genus *Coronavirus*, was indentified near the 3' end of the open reading frame (ORF) 1b in its genomic RNA (Makino *et al*., 1990; van der Most *et al*., 1991). Sequence deletion analysis suggested that a 190-nt region corresponding to 20.2-20.4kb of the genomic RNA was necessary for packaging of MHV DI RNA. A 69-nt core sequence within the region was further proposed as the PS (Fig. 6A) which was computationally predicted to consist of a stem-loop structure (Fosmire *et al*., 1992) and found to direct packaging of heterogeneous RNA (Lin & Lai, 1993; Woo *et al*., 1997). However, the 69-nt element showed only one-fifth efficiency in directing genome packaging compared to what has been shown for the 190-nt sequence (Fosmire *et al*., 1992), while the 69-nt PS was on the other hand shown to bind nucleocapsid (N) protein much weaker than the 190-nt

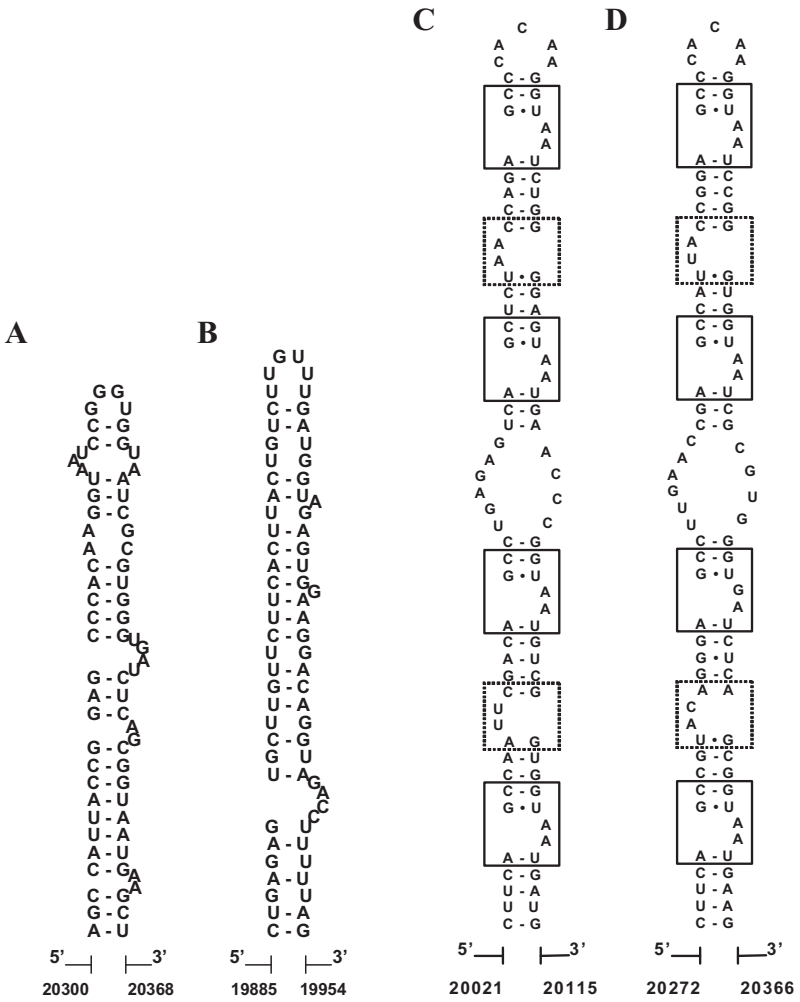

Figure 6. The packaging signals of coronaviruses. (A) The 69-nt MHV packaging signal proposed by Fosmire *et al*., (1992, J Virol. 66:3522-30) and (B) the homologue in SARS-CoV proposed by Hsieh *et al*., (2005, J Virol. 79:13848-55). (C, D) The 96-nt packaging signal of the group IIa coronaviruses BCoV and MHV, respectively (Chen et al., 2007, J Virol. 81:6771-4).

sequence does (Molenkamp & Spaan, 1997). It has led to the assumption that the flanking sequences are necessary for promoting the 69-nt PS to fold into specific secondary structure which is selectively recognized by N protein (Molenkamp & Spaan, 1997; Narayanan & Makino, 2001). Nevertheless, mutagenesis on the 69-nt element based on the originally proposed secondary structure led to obscure results (Fosmire *et al*., 1992). In addition, secondary structure of the MHV 69-nt PS unexpectedly exhibited poor homology to the predicted secondary structure of the closely related bovine coronavirus (BCoV) PS discovered later on (Cologna & Hogue, 2000). The structural homology between MHV and BCoV PS was not discovered until a phylogenetic analysis

was done on sequences corresponding to PS in all group IIa CoVs and an intensive structure probing study was applied to the MHV PS (Chen *et al*., 2007).

A new secondary structure model for MHV and other group IIa CoVs PS is a 96-nt element with two stem-bulge structures separated by internal loops, which consist of repeating 2-nt (preferably AA) bulges (Fig. 7A). The model fits the mutagenesis data originally obtained by Fosmire *et al*. (1992) much more evidently, and the 96-nt PS clearly explained why the flanking sequences of the 69-nt element is required for N protein binding and how the 190-nt element directs packaging much more efficiently (Lin & Lai, 1993; Molenkamp & Spaan, 1997; Narayanan & Makino, 2001; Woo *et al*., 1997), i.e. the 190-nt region includes the entire 96-nt MHV PS while the 69-nt element possesses only a truncated 96-nt PS and is possibly unable to fold into specific secondary structures required for encapsidation (Chen *et al*., 2007).

The putative PS in other coronaviruses has also been located. In another group II CoV, the severe acute respiratory syndrome (SARS) CoV, homologues of the MHV 69-nt PS were proposed in the corresponding region of SARS-CoV ORF1ab (Hsieh *et al*., 2005) (Fig. 7B). The sequence is able to incorporate non-viral RNA into virus-like particles. In transmissible gastroenteritis virus (TGEV), a group I coronavirus, the PS was located in the 5' 649 nts, though the specific sequence and the (secondary) structure of the signal have not yet been investigated (Escors *et al.*, 2003).

The PS in Aichi virus and Poliovirus

Poliovirus and Aichi virus are members of the *Picornaviridae*. Poliovirus contains a 3'-polyadenylated 7450-nt positive-sense genomic RNA which consists of three coding regions, P1, P2, and P3. It has been shown that only the positive-sense viral genomic RNA is packaged into virions, which is covalently bound to a small viral protein, the VPg, at its 5'-terminus (Lee *et al*., 1977; Nomoto *et al*., 1977; Novak & Kirkegaard, 1991). Although the VPg is associated with the genomic RNA, the specificity and the efficiency of the encapsidation are not likely to be dependent on the interaction between VPg and genomic RNA because the VPg-bound viral RNA was found not to be packaged in cells (Harber *et al*., 1991) while encapsidation has been shown in mutants of which the amino acid sequence in VPg

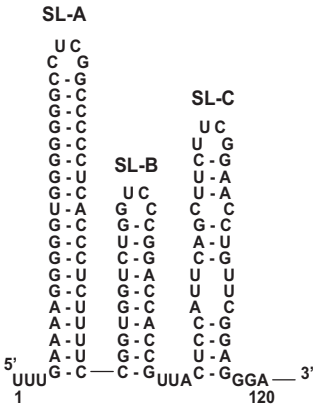

Figure 8. The packaging signals of Aichi virus. The secondary structure of the Aichi virus packaging signal is shown, exhibiting three stem-loop structures.

was seriously mutated (Kuhn *et al*., 1988). The specificity of the encapsidation was subsequently suggested to be coupled with the replication of poliovirus (Nugent *et al*., 1999).

Although no encapsidation signals have been identified yet in the poliovirus, studies on packaging of the poliovirus DI RNA have indicated some clues. First, the signal may not be located in the coding region of the structural proteins (P1) since that the encapsidation was observed in mutant viruses or DI RNA with this region truncated and/or substituted by non-poliovirus sequences when the P1 proteins were expressed *in trans* (Ansardi *et al*., 1993; Barclay *et al*., 1998; Kajigaya *et al*., 1985; Porter *et al*., 1995). Secondly, the encapsidation signal may not be located in the 5'UTR or in the coding region of the non-structural 2A protease because substituting the 5'UTR and the coding region of the 2A protease for the corresponding sequence of other viruses still leads to encapsidation of poliovirus genomic RNA (Lu *et al*., 1995; Xiang *et al*., 1995).

In another picornavirus, the Aichi virus which is associated with acute gastroenteritis in humans (Yamashita *et al*., 1998), a potential PS has been proposed to be located in the 5'-proximal 120 nts, where three stem-loop structures (SL-A, SL-B, and SL-C) have been predicted (Fig. 8). The SL-A was shown to be required for viral RNA replication and encapsidation (Sasaki *et al*., 2001; Sasaki & Taniguchi, 2003). However, the role of secondary structure in directing encapsidation was still unclear because sequence substitutions in the stem of SL-A seriously interfered with the encapsidation although the secondary structure was maintained (Sasaki *et al*., 2001).

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