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Tomato spotted wilt virus transcriptase in vitro displays a preference for cap donors with multiple base complementarity to the viral template

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Abstract

Transcription of segmented negative-strand RNA viruses is initiated by cap snatching: a host mRNA is cleaved generally at 10–20 nt from its 5′ capped end and the resulting capped leader used to prime viral transcription. For *Tomato spotted wilt virus* (TSWV), type species of the plant-infecting *Tospovirus* genus within the *Bunyaviridae*, cap donors were previously shown to require a single base complementarity to the ultimate or penultimate viral template sequence. More recently, the occurrence in vitro of "re-snatching" of viral mRNAs, i.e., the use of viral mRNAs as cap donors, has been demonstrated for TSWV. To estimate the relative occurrence of re-snatching compared to snatching of host mRNAs, the use of cap donors with either single, double, or multiple complementarity to the viral template was analyzed in pair-wise competition in TSWV in vitro transcription assays. A strong preference was observed for multiple-basepairing donors.

Keywords: *Bunyaviridae*; *Tospovirus*; TSWV; Transcription; Cap snatching; Endonuclease-cleavage

Introduction

Cap snatching is the mechanism used by all segmented negative-strand RNA viruses to initiate transcription of the viral genome. It was first described for *Influenza A virus* (FLUAV) (Bouloy et al., 1978; Plotch et al., 1979) and since then has been reported for viruses infecting hosts from both the animal and plant kingdoms (Bellocq et al., 1987; Huiet et al., 1993; Kormelink et al., 1992b; Raju et al., 1990; Simons and Pettersson, 1991). During cap snatching, host mRNAs are recruited by the viral polymerase complex and subsequently cleaved by the virally encoded endonuclease, generally at ~10–20 nt from the ^{7m}G-capped 5′-end. The resulting capped leaders are then used to prime transcription of the viral genome.

Knowledge of the sequence specificity and the requirements for leader length of a suitable donor RNA is still limited. For a number of bunyaviruses, a viral endonuclease cleavage specificity has been suggested based on the last nucleotide in the leader preceding the viral sequence in the mRNA (Bishop et al., 1983; Bouloy et al., 1990; Jin and Elliot, 1993a, 1993b). However, the host mRNAs from which the capped leader sequences had been snatched, and therefore the actual cleavage site, remained unknown. For FLUAV, recent investigations have shown that only capped RNA fragments with a CA 3′-terminus are effectively used as primers in vitro and that the A residue of the CA-terminated cap donor may basepair to the ultimate U-residue at the 3′-end of the viral template (Rao et al., 2003). To explain data from transcription initiation studies for *Hantaan virus* (genus *Hantavirus*, family *Bunyaviridae*), an additional "prime-and-realign" mechanism has been proposed (Garcin et al., 1995) in which transcription is primed with a capped leader RNA that is extended for a few nucleotides and then released. Progressive elongation takes place only after backward re-alignment by virtue of the viral 3′ terminal sequence repeats. This mechanism, which depends on

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basepairing of an extended capped-RNA leader to the viral template, would also explain the presence of repetitive sequences within the 5′-leader of viral mRNAs from several orthobunyaviruses (Jin and Elliot, 1993a; Vialat and Bouloy, 1992).

Tomato spotted wilt virus (TSWV) is the type species of the plant-infecting viruses of the *Tospovirus* genus within the family *Bunyaviridae*. The TSWV genome is tripartite and consists of ambisense S and M RNA segments, and a negative sense L RNA (Fig. 1A). The S RNA (2.9 kb) codes for the nucleoprotein (N) in viral complementary (vc) sense and a non-structural protein (NSs), that acts as a suppressor of silencing (Bucher et al., 2003; Takeda et al., 2002), in viral (v) sense (De Haan et al., 1990). The M RNA (4.8 kb) codes for the glycoprotein precursor (GP) in vc-sense and the cell-to-cell movement protein (NSm) in v-sense (Kormelink et al., 1992a, 1994; Storms et al., 1995). The L RNA (8.9 kb) codes in vc-sense for the viral RNA-dependent RNA polymerase (RdRp) or L protein (De Haan et al., 1991), which is also assumed to encompass the endonuclease activity required for cap snatching.

After the initial finding of 5′ non templated sequences on TSWV mRNAs (Fig. 1A; Kormelink et al., 1992b; Van Poelwijk et al., 1996), indicative of cap snatching, this mechanism of initiation was studied further using co-infection assays with *Alfalfa mosaic virus* (AMV) (Duijsings et al., 1999, 2001). These studies led to a model for cap snatching, in which the cap donor is required to have a single base complementarity to the ultimate or penultimate 3′ residue of the viral template (Duijsings et al., 2001). More recently, an in vitro assay based on purified TSWV virus particles was established, in which viral transcription was demonstrated to require the presence of rabbit reticulocyte lysate (RRL; Van Knippenberg et al., 2002). The α- and β-globin mRNAs present in this lysate, which have a dinucleotide sequence (AG and GA, respectively) at 14 nt from the 5′ capped

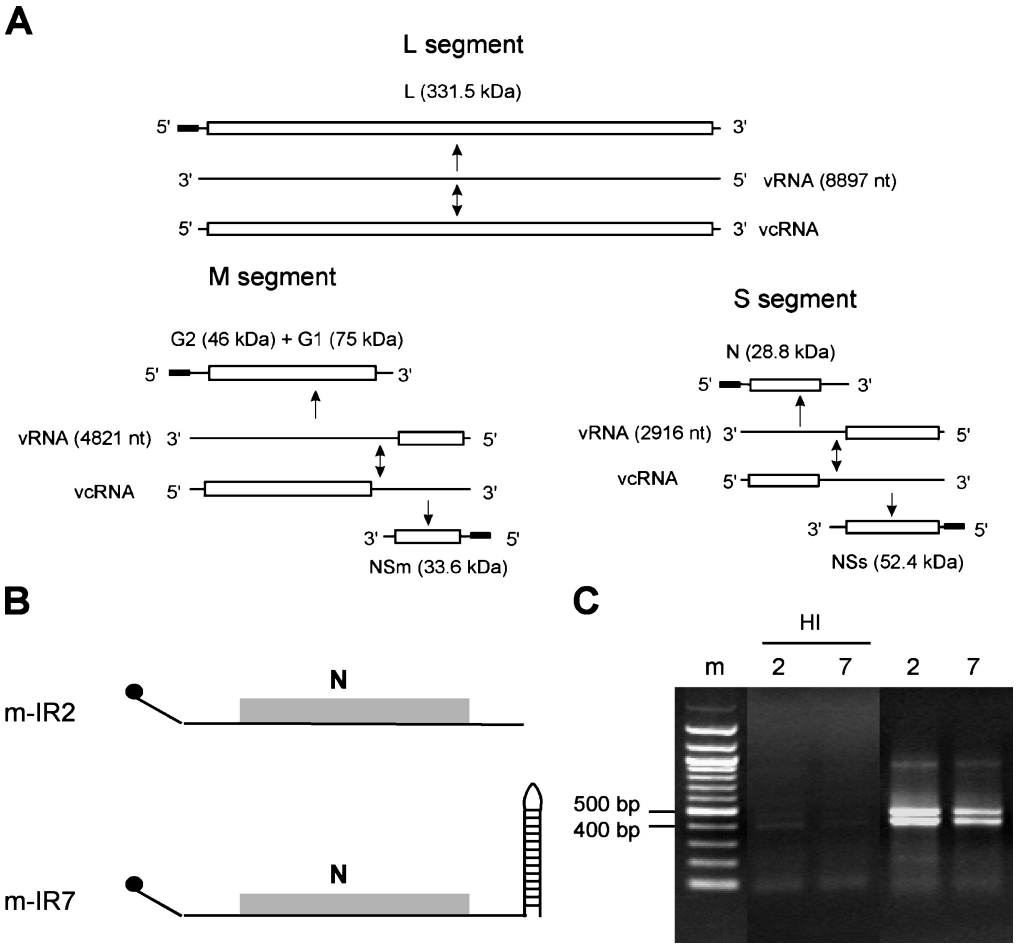

Fig. 1. Re-snatching of viral transcripts containing or lacking a 3′-terminal hairpin. (Panel A) Diagram of TSWV genome organization. mRNA 5′ leaders derived from host mRNAs are indicated by black boxes. (Panel B) Schematic representation of full-length N mRNA transcripts m-IR2 and m-IR7 containing a capped AMV RNA3-derived leader sequence and with (m-IR7) or without (m-IR2) the 3′-end hairpin structure. (Panel C) RT-PCR analysis of cap snatching of m-IR2 and m-IR7 in a TSWV in vitro transcription assay. HI: negative controls using heat inactivated virus; m: 100-bp molecular weight marker; 2: m-IR2 as cap donor; 7: m-IR7 as cap donor. The double bands correspond to the expected sizes of the RT (upper band) and nested PCR (lower band) products, respectively, and are attributed to an excess of RT primer in the reaction.

end that may be involved in basepairing to the 3′-end of the viral template, were both used as cap donor to prime transcription in vitro. Moreover, an exogenously added synthetic capped N-gene transcript containing an AMV RNA3-derived leader, i.e., representing a viral mRNA, was used as cap donor in vitro (Van Knippenberg et al., 2002). These findings implied that apparently there is no limit to the extent of complementarity between cap donor and viral template and, furthermore, that the mere presence of viral sequences within the cap donor is not sufficient to protect a viral transcript from being "re-snatched" by the viral endonuclease.

These observations led to the question whether the virus would have a preference for either single or multiple basepairing cap donors, since a preference for single basepairing donors would diminish the odds of viral transcripts being re-snatched in vivo. To investigate the likelihood of re-snatching occurring in vivo, the preference of TSWV for donors with either single, double, or multiple base complementarity to the viral template was examined by pair-wise competition in TSWV in vitro transcription assays. It is demonstrated that, in vitro, cap donors with longer stretches of complementarity to the viral template are used preferentially to cap donors with shorter stretches of complementarity.

Results

Competition between single and double basepairing cap donors

As a first step to investigate whether TSWV transcriptase has a preference for cap donors with longer stretches of base complementarity to the viral RNA template, a competition experiment was performed in which a mixture of single and double basepairing (capped) AMV RNA3 molecules was added to the TSWV in vitro transcription assay (Van Knippenberg et al., 2002) and tested as cap donors. These cap donors will be referred to as wt-AMV3 (single basepairing) and mut-AMV3 (double basepairing) (Table 1). Mut-AMV3 (AMV RNA3 mutant A18G19C20), harboring a double base complementarity to the viral RNA template, was designed by changing residues A19A20 into G19C20. To discriminate leaders generated from this mutant from leaders derived from wt-AMV3, an additional marker nucleotide was introduced by changing A12 into C12. Prior to the competition experiment, both wt-AMV3 and mut-AMV3 were added individually to in vitro TSWV transcription reactions and tested as cap donor. After extraction, the RNA was analyzed for de novo synthesized N gene transcripts by RT-PCR using a set of nested primers for the N gene in combination with a primer corresponding to the first 11 nt of the AMV RNA3 leader. Subsequent cloning and sequence analyses confirmed that both wt-AMV3 and mut-AMV3 were used as cap donor (Table 2). Furthermore, while the initial assays contained 100 ng/μl of AMV RNA3 as cap donor (Van Knippenberg et al., 2002), titration experiments demonstrated that in fact 8 ng/μl was already sufficient (results not shown).

In the competition experiment, wt-AMV3 and mut-AMV3 were added in equimolar amounts to in vitro TSWV transcription assays. When high equimolar amounts (32 ng/μl each, both transcripts are of equal size) of the cap donors were used, RT-PCR cloning and sequencing of de novo synthesized N gene transcripts revealed that 19 out of the 23 clones analyzed contained the marker nucleotide at position 12 (Table 2), indicative of the presence of the mut-AMV3 leader sequence. Only 4 clones contained the wt-AMV3 leader (Table 2). When low equimolar amounts (8 ng/μl) of both cap donors were provided, 14 out of 21 clones contained the leader derived from mut-AMV3 (Table 2).

To further substantiate the preference for mut-AMV3 as cap donor, two additional competition experiments were performed in which the wt-AMV3 and mut-AMV3 cap donors were added in a 5:1 (40 ng/μl wt-AMV3 vs. 8 ng/μl mut-AMV3) and a 10:1 ratio (80 ng/μl wt-AMV3 vs. 8 ng/μl mut-AMV3). RT-PCR cloning of de novo synthesized N gene transcripts showed that 5 out of 6 clones represented mRNAs with a leader derived from mut-AMV3 for the 5:1 ratio and 11 out of 18 clones in the case of the 10:1 ratio (Table 2). Remarkably, the sequence data also showed that, occasionally, basepairing and subsequent cleavage took place at residues A19, A20, or A21 of wt-AMV3. Taken together, these competition experiments demonstrated a strong preference for AMV cap donors with double base complementarity to the viral RNA template compared to those with only a single base complementarity.

Table 1
Cap donors used in competition assays

Name of cap donor	5' end sequence of cap donor
wt-AMV3	5'-GUAUUAAUAC CAUUUUCAAA AUAUUCCAAU
mut-AMV3	5'-GUAUUAAUAC CCUUUUCAGC AUAUUCCAAU
AMV3-N	5'-GUAUUAAUAC CAUUUUCAGA GCAAUUGUGU

The nucleotide residues potentially basepairing to the viral template (3′-UCUCG…) are underlined; the marker nucleotide used to discriminate mut-AMV3 leaders from wt-AMV and AMV3-N-derived leaders is shaded.

Competition between a double basepairing cap donor and a capped viral RNA transcript

The finding that a double basepairing cap donor is preferred over one with single basepairing raised the question whether this preference would extend to longer stretches of base complementarity to the template, especially in view of the earlier findings (Van Knippenberg et al., 2002) that viral mRNAs are re-snatched in vitro. To answer

Table 2
N and NS mRNA 5′-end sequences resulting from cap snatching of the indicated cap donors

Cap donor	Retrieved N mRNA 5' sequence	No. of clones
wt-AMV3	5'-GUAUUAAUACCAUUUUCAGAGCAAUUG	8
mut-AMV3	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	9
wt-AMV3 + mut-AMV3 (1:1; 32 ng/μl each)	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	18
	5'-GUAUUAAUACCCUUUUAGAGCAAUUG	1
	5'-GUAUUAAUACCAUUUUCAGAGCAAUUG	2
	5'-GUAUUAAUACCAUUUUCAAGAGCAAUUG	2
wt-AMV3 + mut-AMV3 (1:1; 8 ng/μl each)	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	13
	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	1
	5'-GUAUUAAUACCAUUUUCAGAGCAAUUG	6
	5'-GUAUUAAUACCAUUUUAAGAGCAAUUG	1
wt-AMV3 + mut-AMV3 (5:1; 40 resp 8 ng/μl)	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	4
	5'-GUAUUAAUACCCUUUUUAGAGAAUUG	1
	5'-GUAUUAAUACCAUUUUCAAGAGCAAUUG	1
wt-AMV3 : mut-AMV3 (10:1; 80 resp 8 ng/μl)	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	10
	5'-GUAUUAAUACCCUUUUUAGAGCAAUUG	1
	5'-GUAUUAAUACCAUUUUCAGAGCAAUUG	5
	5'-GUAUUAAUACCAUUUUCAAAGAGCAAUUG	1
	5'-GUAUUAAUACCAUUUUCAAAAGAGCAAUUG	1
mut-AMV3 + AMV3-N (1:1; 80 ng/μl each)	5'-GUAUUAAUACCCUUUUCAGAGCAAUUG	1
	5'-GUAUUAAUACCAUUUUCAGAGCAAUUG	13

TSWV viral sequence is underlined; the marker nucleotide C12 from mut-AMV3 cap donor is shaded; all sequences are N mRNA 5′-ends except for the assays using AMV3-N cap donor (last two sequences), which are NSs mRNA 5′-ends. In the cap donor column, "resp" refers to "respectively".

this question, a competition experiment was performed analogous to previous experiments, but now with mut-AMV3 in competition with a capped N gene transcript containing a wt AMV RNA3 leader. The latter, henceforth referred to as AMV3-N (Table 1), is capable of basepairing to the viral template (for transcription of NSs) over a stretch of 14 nucleotides (i.e., nucleotides 18–31 are complementary to template 3′ residues 1–14).

When equimolar amounts (80 ng/μl each) of both cap donors were added, RT-PCR cloning of de novo synthesized NSs gene transcripts revealed that 13 out of 14 clones contained an AMV3 leader lacking the marker nucleotide as present in mut-AMV3, thus representing NS mRNAs with a leader derived from re-snatching of AMV3-N (Table 2). In other words, the viral mRNA transcript, with a long stretch of complementarity to the viral RNA template, was preferentially used as a cap donor.

Re-snatching of full-length TSWV mRNAs in vitro

So far, the experiments that demonstrated the occurrence of re-snatching (Van Knippenberg et al., 2002) were performed using synthetic transcripts that represented only the 5′ half of the N mRNA. Recent mapping of the 3′-ends of TSWV S-segment mRNAs indicated they contained a predicted long stable stem-loop structure and conserved sequence motif (Van Knippenberg et al., 2005) which are located in the intergenic region of this genome segment (Figs. 1A and B; De Haan et al., 1990). To analyze whether the presence of the 3′ terminal hairpin may protect viral transcripts from being re-used as cap donor, full-length AMV3-N transcripts with (m-IR7) or without (m-IR2) the 3′ terminal hairpin (Fig. 1B) were tested as cap donors in vitro. For both m-IR2 and m-IR7 donors, RT-PCR cloning (Fig. 1C) and sequence analyses of de novo synthesized NS transcripts revealed the presence of the AMV3 leader, indicating that the presence of the 3′ terminal hairpin alone does not protect viral mRNAs from re-snatching.

Endonuclease cleavage site in multiple-basepairing cap donors

In the case of multiple basepairing cap donors, endonucleolytic cleavage might, in principle, occur anywhere along the stretch of basepairing residues. To investigate where precisely cleavage takes place in this case, the fate of a capped AMV3-N transcript in a TSWV in vitro transcription assay was analyzed by primer extension (Fig. 2). To this end, primer ML01, identical to S vRNA sequence 2869–2887, was extended on AMV3-N transcripts before and after being exposed to TSWV endonuclease cleavage during an in vitro transcription reaction. The site of endonuclease cleavage of the AMV3-N cap donor can be deduced by comparing the primer extension

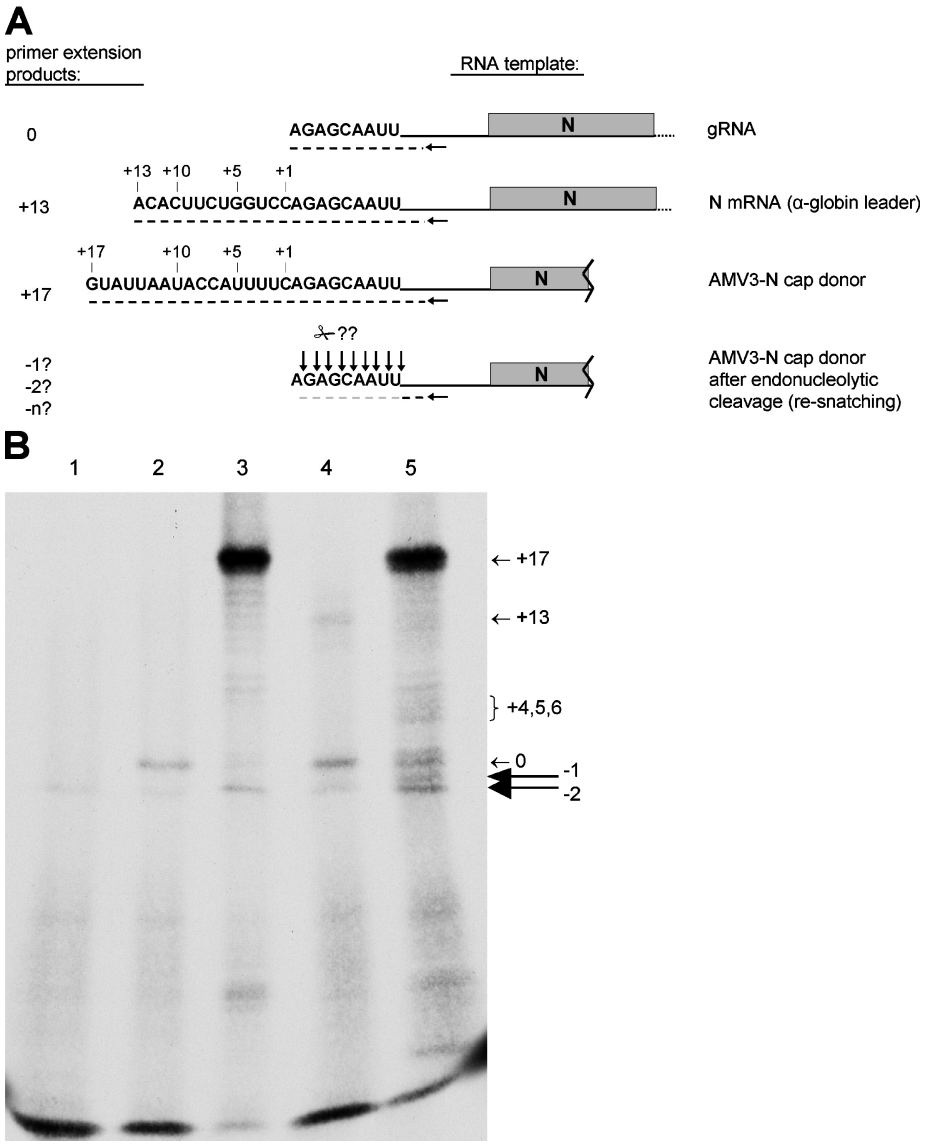

Fig. 2. Primer extension analyses on cap-donor RNA prior to or after endonuclease cleavage. (Panel A) Schematic representation of primer extension analysis. Primer ML01 (horizontal arrows) is aligned along RNA transcripts that are present in a TSWV transcription reaction. The expected sizes of the resulting primer extension products relative to extension on gRNA are indicated: 0 corresponds to run-off products on genomic RNA length; +13 and +17 correspond to run-off on N transcripts harboring leaders derived from globin mRNAs or AMV3-N, respectively; −1, −2, −*n* correspond to run-off products on cap donor AMV3-N after endonuclease cleavage. (Panel B) Primer extension analyses without template RNA (lane 1), on purified TSWV RNA (lane 2), on cap donor AMV3-N (lane 3), and on RNA products from in vitro transcription in the absence of added cap donor (lane 4) and in the presence of AMV3-N (lane 5). The sizes of the various primer extension products are indicated on the right and correspond to those as described in Panel A.

products to those obtained from TSWV genomic RNA, as depicted in Fig. 2A.

Primer extension on purified TSWV RNA and on the capped AMV3-N transcript alone resulted in expected products that mapped to the beginning of the viral sequence, i.e., the 0 position (Fig. 2B, lane 2), and the very 5′-end of the AMV3 leader, i.e., the +17 position (Fig. 2B, lane 3), respectively. Primer extension on AMV3-N after exposure to TSWV endonuclease cleavage yielded several products (Fig. 2B, lane 5). Due to the large amount of AMV3-N cap donor present in the transcription reaction, the most predominant product was the result of primer extension on the uncleaved AMV3-N transcript (+17). In addition, products of −1 and −2 were observed, indicating cleavage had occurred 3′ of residues A18 and G19 of the AMV3-N transcript. The −1 product was absent from all other reactions, indicating clearly that re-snatching by cleavage of the AMV3-N donor at A18 had occurred. Although the −2 product was also present in extension reactions on the uncleaved AMV3-N transcript (Fig. 2B, lane 3), the −2 band seemed more intense in extension on the endonuclease-exposed AMV3-N (Fig. 2B, lane 5), suggesting that cleavage may also have occurred at G19.

No extension products shorter than the −2 band were observed. The primer extension product that terminated at position 0 was likely due to extension on gRNA present in

these reactions. In addition, products migrating at positions +1 and +4 to +6 were observed (Fig. 2B, lane 5). The product migrating at position +5 may reflect cleavage of the AMV3-N donor after residue A12, similar to previous observations (Van Knippenberg et al., 2002). The nature of the +1, +4, and +6 fragments remains unclear at present. As a control, primer extension was also performed on the products of an in vitro transcription reaction without exogenously added cap donor (Fig. 2B, lane 4). As expected, a band migrating at position +13 was observed, the result of primer extension on N mRNA transcripts harboring 13 nt leader sequences of globin mRNAs. The results, altogether, indicate that in the case of multiple basepairing cap donors cleavage seems to occur predominantly at position −1 and to a lesser extent at −2, but not further downstream.

Discussion

Cap snatching, the mechanism of stealing a capped leader sequence from a host mRNA to initiate viral transcription, was discovered over 25 years ago, first for FLUAV and then followed by other segmented (−)ssRNA viruses (Bouloy et al., 1978; Huiet et al., 1993; Plotch et al., 1979; Raju et al., 1990; Simons and Pettersson, 1991). Analysis of the requirements for cap donors has primarily been performed for FLUAV polymerase. Cleavage of a donor mRNA by FLUAV polymerase is thought to occur independently from basepairing interactions with the viral template, though such interactions are thought necessary for correct alignment of the cleaved leader on the template for priming of transcription (Hagen et al., 1995; Rao et al., 2003). Cleaved leaders have been observed to predominantly terminate in a 3′ CA dinucleotide (Beaton and Krug, 1981; Rao et al., 2003; Shaw and Lamb, 1984), and studies using recombinant FLUAV polymerase demonstrated that these leaders were far more efficiently elongated in vitro than other cleavage products (Rao et al., 2003). Moreover, a difference in endonuclease activity between purified viral cores and reconstituted recombinant polymerase complexes has been observed. Whereas earlier reports, using purified viral cores, had demonstrated the requirement for both 5′ and 3′ termini of the viral template to activate endonuclease activity, the presence of the 3′ terminus was not required for activation of endonuclease activity of recombinant polymerase cleavage of CA donors. This was suggested to reflect the existence of two different kinds of RNPs: those inside virus particles and those newly assembled inside infected cells.

Analyses of donor requirements for TSWV cap snatching have led to a model in which a cap donor requires a single basepairing interaction with the viral template, optimally at 16 nt from the 5′ capped end (Duijsings et al., 2001). Recently, indications for the possibility of extensive basepairing between cap donor and viral template were obtained from in vitro transcription experiments. Both α- and β-globin mRNAs, with the possibility for double basepairing to the viral RNA template, as well as synthetic capped transcripts resembling a viral mRNA, i.e., having a long stretch (14 nt) of complementarity to the template, were used as cap donors (Van Knippenberg et al., 2002). The results presented here show that, surprisingly, mRNAs with a long stretch of complementarity to the viral template are used in preference to those with shorter stretches of complementarity, suggesting that extensive basepairing interactions promote cap donor usage.

The observation that mRNAs, capable of basepairing with the viral template over a long stretch of nucleotide residues, were used as cap donors raised the question where the actual endonuclease cleavage would take place in these cases. Results from (preliminary) primer extension analyses indicated that cleavage of a multiple-basepairing donor (AMV3-N) seemed to occur after the first (A18) and maybe the second (G19) basepairing nucleotide. Cleavage after the third basepairing residue or even further downstream was not detected. Of the N and NSs transcripts with AMV3 leader sequences that have been cloned previously (Duijsings et al., 1999, 2001; Van Knippenberg et al., 2002), some lacked the first viral (A) residue or even the first two (AG) residues as a result of internal basepairing of the cap donor and priming of transcription at the second (C) or third (U) 3′ residue of the viral template. No transcripts were ever observed from which the first three (AGA) viral residues were absent. These data, altogether, suggest that TSWV endonuclease cleavage and/or elongation seem to be restricted to cap donor sequences basepairing to the first 3 viral template residues. This is similar to the observations made for FLUAV where cap donors with a tri-nucleotide (AGC) complementarity to the viral template are cleaved after the second complementary residue (Chung et al., 1994; Hagen et al., 1995; Rao et al., 2003). Moreover, priming of FLUAV transcription, i.e., elongation, by short (2–4 nt) synthetic oligonucleotides complementary to the template 3′-terminal bases was shown to be efficient with template-complementary di- and trinucleotides, but drastically reduced with a tetranucleotide (Honda et al., 1986). This suggests that, for elongation, the extent of complementarity between the viral template and the cleaved primer is restricted to 3 basepairs and that elongation can take place from position 2, 3, or 4 but not 5 on the viral template.

Since it is not yet possible to study the steps of TSWV endonuclease cleavage and elongation separately, it is not known whether cleavage of cap donor sequences occurs independently of basepairing to the viral template, as observed for FLUAV (Hagen et al., 1995). However, if basepairing would only be required for correct alignment of the cleaved primer to the viral template, it is enigmatic why donors with a longer stretch of complementarity would specifically be preferred. Thus, our results suggest that for TSWV, in contrast to FLUAV, basepairing interactions do play a role in selection of cap donor mRNAs during endonuclease

cleavage, and that, as for FLUAV, cleavage and subsequent elongation remain restricted to the first two complementary residues. This leads to a model of TSWV cap snatching in which multiple basepairing interactions enhance the selection of cellular/viral mRNAs as cap donor, as depicted in Fig. 3.

The initial finding that re-snatching of viral mRNAs occurs in vitro (Van Knippenberg et al., 2002) does not necessarily imply it also occurs in vivo. Although the presence of repeats of 5′ viral sequences in the leaders of some viral transcripts generated in vivo (Duijsings et al., 1999; Van Poelwijk et al., 1996) may point towards the occurrence of re-snatching, these repeats could equally well be the result of a prime-and-realign initiation mechanism (Estabrook et al., 1998; Garcin et al., 1995). Therefore, the finding that the viral polymerase has a clear preference for multiple basepairing cap donors reinforces the question whether re-snatching would be likely to occur in vivo. Re-snatching of viral mRNAs so far has only been reported for FLUAV, where viral transcripts were shown to be cleaved in vitro by the viral polymerase complex, albeit very inefficiently (Peng et al., 1996). Subsequent studies revealed that, in vitro, 'free' polymerase complexes, i.e., not bound to the viral template, could protect these transcripts from the endonuclease activity by binding to them (Shih and Krug, 1996). This may require only a limited pool of free polymerase since protection would only be required from the moment of mRNA synthesis to the moment of transport out of the nucleus. Such unbound polymerase complexes have indeed been detected in the nuclei of FLUAV-infected cells (Detjen et al., 1987; Shih and Krug, 1996).

Extensive re-snatching in vivo would result in a very low level of net synthesis of viral mRNAs, so it is likely that TSWV also protects its mRNAs from being re-snatched. However, since TSWV replicates in the cytoplasm, a role for the viral polymerase in mRNA protection seems unlikely as it would amount to synthesis of an extra copy of polymerase for every single mRNA synthesized. Two features are present in TSWV mRNAs that allow the virus to discriminate them from cellular mRNAs, i.e., the conserved 8 viral residues immediately downstream of the (cellular) capped-RNA leader sequence, and a 3′-end hairpin structure (Van Knippenberg et al., 2005). As demonstrated here, the mere presence of the 3′-end hairpin in viral mRNAs does not confer such protection by itself in vitro. The hairpin structure may be involved in ensuring efficient translation by circularization of the mRNA, possibly through specific binding of a viral

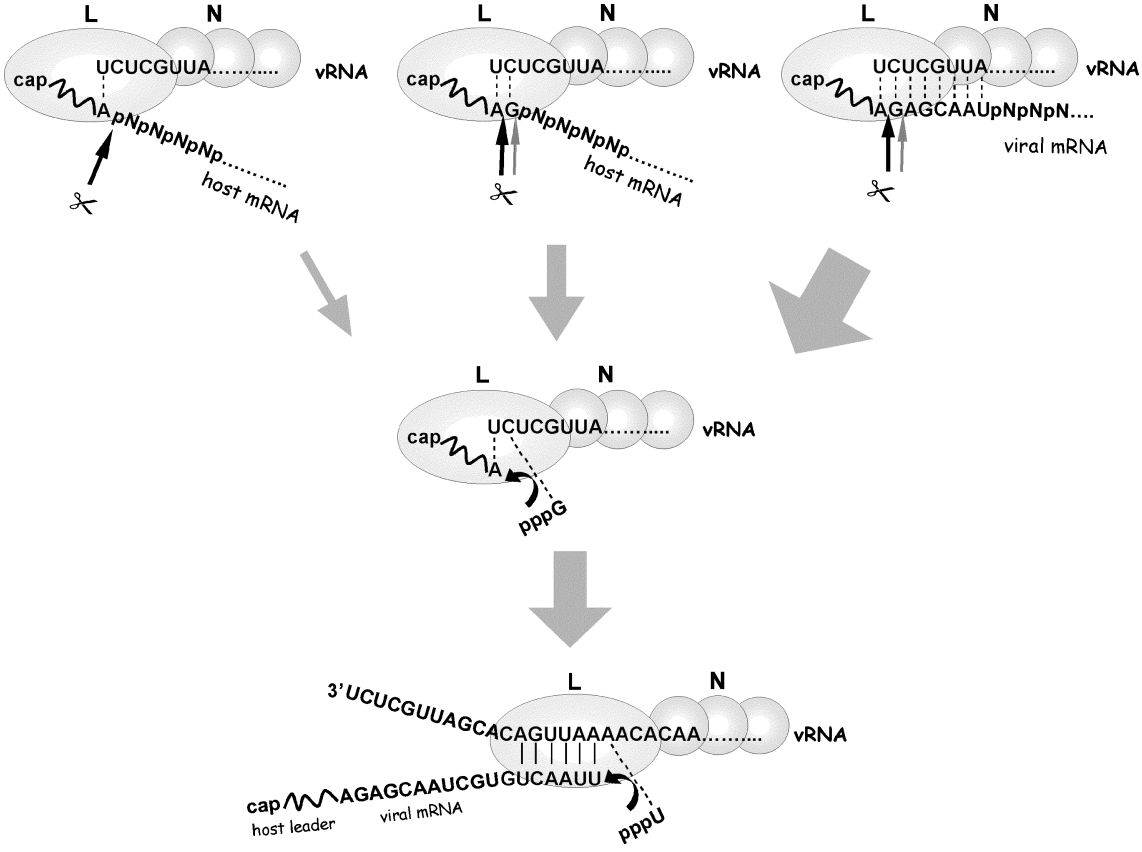

Fig. 3. Adjusted model for TSWV cap snatching in which multiple basepairing interactions enhance the selection of cellular/viral mRNAs as cap donor. When TSWV in vitro is offered the possibility to choose between several cap donor molecules, i.e., a cellular mRNA with one or two residues or a viral mRNA with a long stretch of residues basepairing to the viral template, the virus preferentially selects cap donors with longer stretches of base complementarity. The size of the down-pointing arrows reflects the preference of TSWV for certain cap donors. After alignment of the cap donor to the viral template, endonuclease cleavage is taking place 3′ of the first or, to a lesser extent, second basepairing residue followed by elongation.

protein, as previously reported for other viruses (Fabian and White, 2004; Gallie, 1998; Gallie and Kobayashi, 1994; Leonard et al., 2004; Matsuda and Dreher, 2004; Meulewaeter et al., 2004; Neeleman et al., 2001). Hence, it is postulated that the presence of a viral protein (e.g., NSs) in the complex of translation initiation factors bound to the 5′ and 3′ termini of the mRNA may provide the distinction between viral and host mRNAs, and thereby prevent re-snatching in vivo. Whether TSWV mRNAs are indeed protected by a viral translation-enhancer protein remains to be elucidated.

Materials and methods

Plasmid construction and synthesis of cap donors

Cap donor RNAs were synthesized in vitro using the Ambion T7 mMessage mMachine kit according to the manufacturer's instructions. Wt AMV RNA3 transcripts were generated from plasmid pXO32*Nco*P3, and AMV3-N transcripts were synthesized as described previously (Van Knippenberg et al., 2002). Mutant AMV RNA3 (mut-AMV3) was constructed by PCR amplification of (part of) the AMV RNA3 sequence on pXO32*Nco*P3 using primer p85 (5′-CCCGAATTCGAAGAGTACGAATTACGCG, complementary to nt 339–313 of the AMV RNA3 sequence), in combination with primer mut-AMV3 (5′- cccggatcc**TAATACGACTCACTATAG**UAUUAAUACCCUUUUCAGCATATTCC, mutated residues are underlined, bold residues are T7 promoter sequence) corresponding to the AMV RNA3 5′ sequence. After cloning of the PCR product into pUC19 and linearization, run-off capped T7 transcripts could be made.

The construct for full-length AMV3-N mRNA without 3′-end hairpin (plasmid pUC/IR2, to yield transcript m-IR2) was generated by RT-PCR amplification of N mRNAs synthesized in an in vitro TSWV transcription assay in the presence of capped AMV RNA3 (Van Knippenberg et al., 2002) using primer IR2 (5′-CCCGGATCCGCACAACACACAGAAAGC, underlined sequence identical to S RNA nt 1852–1869, preceded by *Bam*HI site) in combination with primer pT7-AMV3 (5′-cccggatcc**TAATACGACTCACTATAG-**UAUUAAUACCAUUUUC, AMV RNA3 nt 1–17, preceded by the T7 promoter sequence in bold and a 5′ *Bam*HI linker sequence). The PCR product was cloned into pGEMTeasy and subcloned into pUC19 using the *Sph*I and *Bam*HI restriction sites, yielding plasmid pUC/IR2.

For transcription of the full-length AMV3-N mRNA with 3′-end hairpin (m-IR7), TSWV genomic RNA was RT-PCR amplified using primer IR7 (5′-CCCGGATCCCTCTGTTTGTCATCTCTTTC, underlined sequence identical to S RNA nt 1548–1568, preceded by *BamH*I site) in combination with primer p009 (5′-cccggatcc-**TAATACGACTCACTATAG**AGAGCAATCGTG, identical to S RNA vc-sense nt 1–12, preceded by the T7 promoter sequence in bold and a 5′ *BamH*I linker sequence). The PCR product was digested with *Bam*HI and cloned into the *Bam*HI-site of pUC19. To generate pUC/IR7, containing the sequence of the full N mRNA with 5′ AMV3 leader and 3′ hairpin, the 3′-hairpin sequence was transferred from this plasmid to pUC/IR2 by cloning the *Pac*I–*Kpn*I restriction fragment of this plasmid into *Pac*I–*Kpn*I-digested pUC/IR2. The *Pac*I site is located in the TSWV S RNA sequence between the N stop codon and the beginning of the hairpin sequence, the *Kpn*I site in the MCS of the pUC vector.

In vitro cap donor-competition assays

TSWV in vitro transcription assays (in the presence of rabbit reticulocyte lysate) were performed as described previously (Van Knippenberg et al., 2002). Cap donors were added to the reactions as specified in the text. After extraction and precipitation, mRNAs were amplified by RT-PCR as described previously (Duijsings et al., 1999) using nested primers specific for the N or NSs gene (as indicated) in combination with a primer for the 5′ 11 nt of AMV RNA3 (Van Knippenberg et al., 2002). PCR products of expected size were gel-purified and cloned into pGEM-Teasy for sequence analysis.

Primer extension analyses

Primer extension analyses were performed as described by Kormelink et al. (1992b). Primer ML01 (5′ gtaagactgagtgttaagg; identical to S RNA sequence 2869–2887) was end-labeled with ^{32}P using polynucleotide kinase. The primer was extended on the indicated RNA samples by reverse transcription using AMV RT. Samples were analyzed by electrophoresis on a 6% sequencing gel.

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