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Tomato spotted wilt virus S-segment mRNAs have overlapping 3′-ends containing a predicted stem-loop structure and conserved sequence motif

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Abstract

The *Tomato spotted wilt virus* ambisense M- and S-RNA segments contain an A/U-rich intergenic region predicted to form a stable hairpin structure. The site of transcription termination of S-segment encoded N and NSs mRNAs synthesised in an in vitro transcription system was roughly mapped to the 3′-end of the intergenic hairpin, i.e. position 1568–1574 for N and position 1852–1839 for NSs, as determined by RT-PCR cloning and size estimation on Northern blots. This suggests that these viral transcripts contain a predicted stem-loop structure at their 3′-end. The potential involvement of the 3′-end structure in transcription termination is discussed.

Keywords: TSWV; Bunyaviridae; Transcription termination; Intergenic hairpin

1. Introduction

Bunyaviridae, like all segmented negative-strand RNA viruses, initiate transcription of their genome by a mechanism called cap snatching. In this process the viral polymerase cleaves a host mRNA at generally 10–20 nt from its 5′-capped end and subsequently uses the resulting capped leader to prime transcription of the viral genome (Dobie et al., 1997; Duijsings et al., 1999, 2001; Garcin et al., 1995). mRNAs of Bunyaviridae, a family of arthropod-borne negative and ambisense RNA viruses, are not polyadenylated and the processes involved in transcription termination of these viruses have remained largely unresolved.

Of the five genera comprising the Bunyaviridae, three are entirely negative sense coding (*Orthobunya-*, *Hanta-* and *Nairoviruses*). Transcription termination of these viruses occurs at a G/U-rich sequence (Patterson and Kolakofsky, 1984; Eshita et al., 1985; Hutchinson et al., 1996). The S-RNA segments of phleboviruses and the S- and M-RNAs of tospoviruses have an ambisense coding strategy (Fig. 1A) in which the intergenic region (IR) is expected to contain the transcription termination signals. The IRs of the phleboviruses *Sandfly fever sicilian* (SFSV), *Rift valley fever* (RVFV), and *Toscana* (TOSV) are G-rich and are not able to form stable secondary structures. Transcription termination of these viruses occurs near a CCGUCG sequence motif in the template, preceded by G- or C-tracts (Giorgi et al., 1991). The IRs of the *Punta toro* (PTV) and *Uukuniemi* (UUKV) phlebovirus S-RNA segments are predicted to form stable hairpin structures. Transcription termination of PTV mRNAs appears to occur near the top of a 100-bp-sized intergenic hairpin (Emery and Bishop, 1987), while termination of UUKV mRNAs is mapped to the 3′-end of the IR (Simons and Pettersson, 1991). In the latter case, transcription yields two mRNAs that possess a small 3′-stem-loop structure and are complementary at the entire 3′-untranslatable region (UTR) (Simons and Pettersson, 1991). Intriguingly, the hexanucleotide sequence (CCGUCG) that is conserved for TOSV, RVFV and SFSV is also found at the top of the intergenic hairpin of PTV, as

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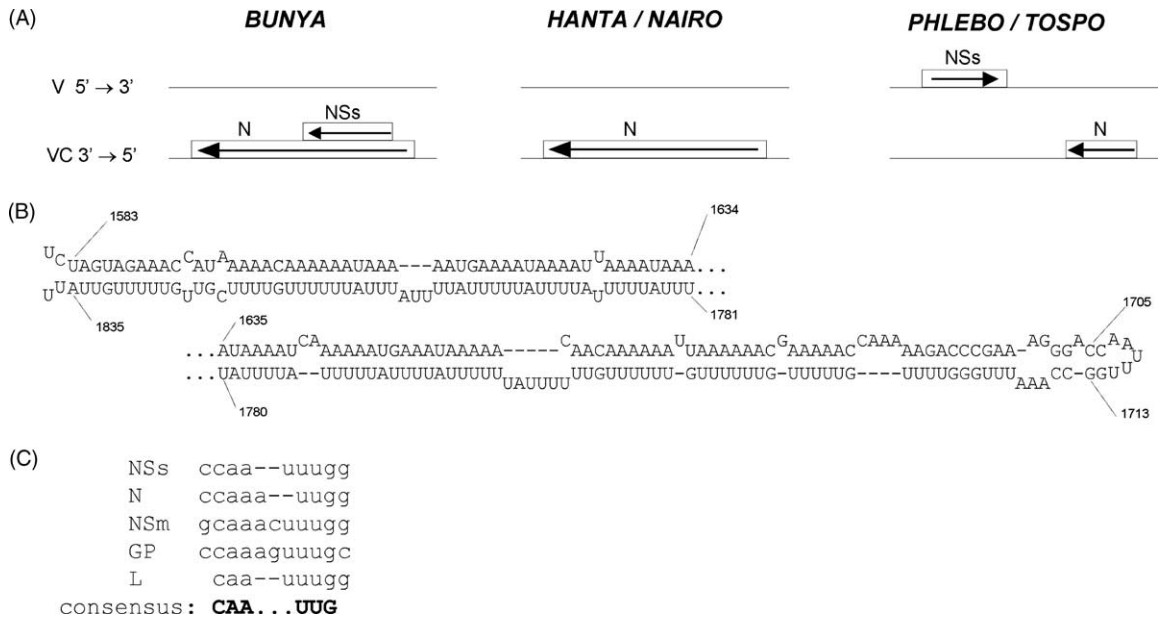

Fig. 1. Primary and secondary structure elements involved in TSWV S-RNA transcription. Panel A: genetic organization of the S-RNA segments of the five genera of the Bunyaviridae. V is viral sense and vc is viral complementary sense. Panel B: predicted hairpin structure within the intergenic region of the (viral sense) TSWV S-RNA. Panel C: conserved sequence motif following the stop-codon of the indicated TSWV genes, located near the top of the hairpin structure in the S and M-RNA segments (genes NSs, N, NSm and GP) and 103 bases beyond the L gene stop-codon.

well as in the template for transcription of the NSs mRNA of UUKV.

Tomato spotted wilt virus (TSWV) is the type species of the *Tospovirus* genus within the Bunyaviridae, and has two ambisense coding segments, the S- and M-RNA. The IRs of these segments are highly A/U-rich, and are each predicted to form a long stable hairpin structure as depicted for the S-RNA in Fig. 1B (de Haan et al., 1990; Kormelink et al., 1992). In addition, the IRs contain a conserved sequence motif (Fig. 1C) situated near the top of the hairpin. From the estimated sizes of the mRNAs, transcription is thought to terminate somewhere in the IR (de Haan et al., 1990; Kormelink et al., 1992), and it has been suggested that the hairpin structure or the conserved sequence motif may be involved in transcription termination.

Recently, an in vitro transcription assay has been established in which purified TSWV particles support transcription in the presence of rabbit reticulocyte lysate (Section 2). As cap donors, either globin mRNAs that were present in the lysate or exogenously added capped *Alfalfa mosaic virus* (AMV) RNAs were used, as demonstrated by RT-PCR amplification of de novo synthesised mRNAs. Furthermore, Northern blot analyses revealed that de novo synthesised mRNAs comigrated with viral mRNAs from total-RNA of infected plants, indicating that in vitro synthesised viral transcripts are indistinguishable from those synthesised in vivo.

To identify the transcription termination signals for TSWV S-RNA-derived mRNAs, the 3′-ends of in vitro synthesised NSs and N mRNAs were mapped. Cloning and sequence as well as Northern blot analyses of de novo synthesised TSWV transcripts revealed that transcription terminated near the 3′-end of the intergenic hairpin structure.

2. Materials and methods

2.1. RT-PCR cloning of in vitro synthesised TSWV mRNAs

TSWV in vitro transcription assays (in the presence of rabbit reticulocyte lysate) were performed as described previously (Section 2). After extraction and precipitation, mRNAs were amplified by RT-PCR as described previously (Duijsings et al., 1999) with the exception that this time expand long template PCR (Roche) was used. The primers that were used are as indicated in the text and are listed in Table 1. PCR products of expected size were gel-purified and cloned into pGEMTeasy for sequence analysis.

2.2. Northern blot hybridisation analysis

The plasmids for synthesis of the N-gene transcripts N-IR2 and N-IR7 were generated by RT-PCR cloning essentially as described in Section 2.1. The template for first strand synthesis was purified TSWV genomic RNA, the primer in the intergenic region was either IR2 or IR7 (Table 1), the forward primer consisted of the vc-sense genomic 5′-end sequence (underlined) preceded by the T7 promoter sequence (capitalised): 5′-cccgcggccgcggatccTAATACGACTCACTATAGagagcaatcgtg-3′. The PCR products were cloned into pUC19, from which (after linearisation) in vitro capped T7