Analysis of *Tomato spotted wilt virus* genome transcription

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It is the things we think we know that often present the greatest difficulties when we are actually challenged to explain them

Stephen Jay Gould

Aan mijn ouders

CONTENTS

CHAPTER 1

INTRODUCTION

Tomato spotted wilt virus

Tomato spotted wilt virus (TSWV, Fig. 1) is a plague with huge economic consequences. It is found on almost all continents and causes severe losses in several economically important food and ornamental crops such as lettuce, tomato, sweet pepper and chrysanthemum. TSWV has a very broad host range spanning both mono- and dicots and is transmitted by various species of thrips (*Thripidae*, main vector *Frankliniella occidentalis*). As no durable methods for combatting TSWV infection have yet been developed, TSWV remains an economic factor of importance.

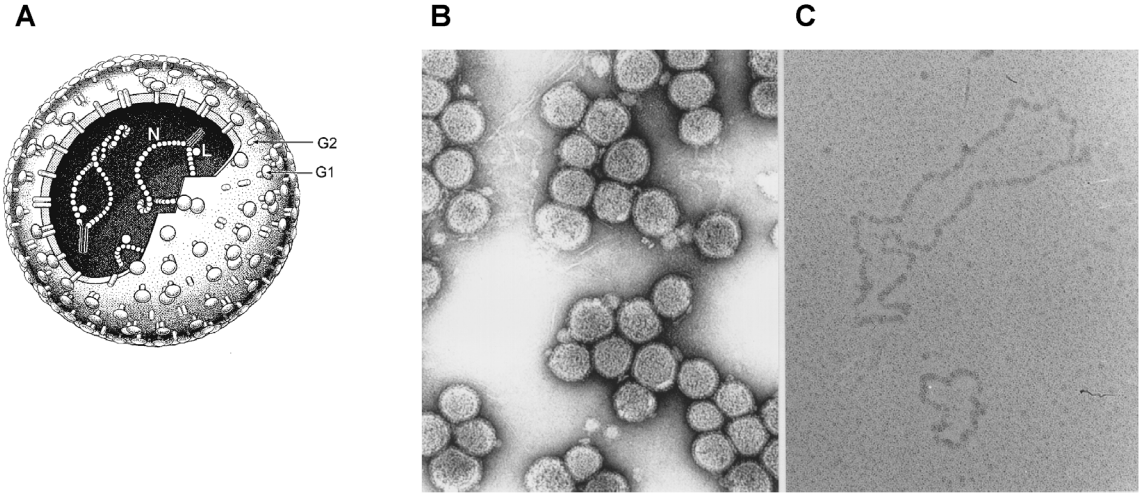

Figure 1: ***Tomato spotted wilt virus.***
Panel A, Schematic representation of a virus particle. The membrane envelope contains the viral glycoproteins (G1 and G2). Inside the envelope are the ribonucleoproteins (RNPs), consisting of viral RNA encapsidated by nucleoprotein (N) and polymerase (L). Panel B, Electron micrograph of purified virus particles. Panel C, Electron micrograph of purified RNPs.

Segmented negative strand RNA viruses

Within the large family of arthropod-borne *Bunyaviridae,* TSWV is the type species of the plant-infecting *Tospovirus* genus (Schmaljohn, 1996; Table 1). The other four genera in the family, *Orthobunyavirus*, *Hantavirus*, *Nairovirus* and *Phlebovirus,* comprise animal and human pathogens. Members of the *Bunyaviridae* are segmented negative strand RNA viruses, as are those belonging to the families *Orthomyxoviridae* (e.g. *Influenza A virus*) and *Arenaviridae* (e.g. *Lymphocytic choriomeningitis virus*), and the floating genera *Tenuivirus* (e.g. *Rice stripe virus*), *Ophiovirus* (e.g. *Citrus psorosis virus*) and *Deltavirus* (e.g. *Hepatitis delta virus*) (Table 1). These viruses replicate in the cytoplasm of infected cells, and their mRNAs are not polyadenylated, except for *Orthomyxoviridae* which replicate in the nucleus and have polyadenylated mRNAs.

Recently, a member of another floating genus of plant viruses (Varicosavirus), *Lettuce big-vein virus* (LBVV), was shown to have a segmented (bipartite) negative sense RNA genome (Sasaya *et al.*, 2001 and 2002). Intriguingly, this virus shares many characteristics of its replication and transcription strategies with the non-segmented Rhabdoviruses (Mononegavirales) (Sasaya *et al.*, 2004). LBVV virions are not enveloped, and its mRNAs are polyadenylated. This genus, therefore, would seem to represent the exception to the rule regarding segmented negative strand RNA viruses. The features of negative strand viruses discussed below appear not to apply to Varicosa viruses.

Table 1: Classification of negative strand RNA viruses.

The Segmented Negative Stranded ssRNA Viruses			
Family	*Genus*	*Type Species*	Host
Orthomyxoviridae			
	Influenzavirus A	*Influenza A virus*	Vertebrates
	Influenzavirus B	*Influenza B virus*	Vertebrates
	Influenzavirus C	*Influenza C virus*	Vertebrates
	Thogotovirus	*Thogoto virus*	Vertebrates
Bunyaviridae			
	Bunyavirus	*Bunyamwera virus*	Vertebrates
	Hantavirus	*Hantaan virus*	Vertebrates
	Nairovirus	*Dugbe virus virus*	Vertebrates
	Phlebovirus	*Rift Valey fever virus*	Vertebrates
	Tospovirus	*Tomato sp otted wilt virus*	Plants
Arenaviridae	*Arenavirus*	*Lymphocytic choriomeningitis virus*	Vertebrates
	Ophiovirus	*Citrus psorosis virus*	Plants
	Tenuivirus	*Rice stripe virus*	Plants
	Deltavirus	*Hepatitis delta virus*	Vertebrates

ICTV The International Committee on Taxonomy of Viruses

Segmented negative strand RNA viruses have a genome of two (*Arenaviridae*) to eight (*Orthomyxoviridae*) segments. These segments have inverted complementary termini, which can form a so-called panhandle structure thus circularising the linear RNA segments. The viral RNA is packed in ribonucleoprotein complexes (RNPs) which contain the viral polymerase and the RNA segment tightly encapsidated by nucleoprotein. The RNPs are enclosed by a lipid membrane envelope, which contains the

viral-encoded glycoproteins (as shown for TSWV in Fig. 1). One notable exception to this rule are the Tenuiviruses, which, intriguingly, lack an envelope but do contain an open reading frame (ORF) potentially coding for one or more glycoproteins (De Miranda *et al.,* 1996; Estabrook *et al.,* 1996; Takahashi *et al.,* 1993).

The term 'negative strand viruses' refers to the coding strategy of their genome segments. In positive strand viruses viral RNA is of coding or mRNA-sense polarity, which means that the infecting genomic RNA can readily be translated if provided with a 5' cap structure or internal ribosomal entry site (IRES). Negative strand RNA molecules must first be transcribed to yield the positive, coding strand. For the negative-strand RNA viruses transcription is thus the first step of the viral amplification cycle inside an infected cell, which explains the presence of viral polymerase inside virus particles (Van Poelwijk *et al.*, 1993).

Within the group of segmented negative sense RNA viruses a distinct sub-group is formed by the so-called ambisense viruses. These possess at least one genome segment that is of dual polarity, i.e. containing one reading frame in the viral-sense RNA and one in the viral-complementary RNA. *Arenaviridae*, *Tenuiviruses*, and the *Tospo*- and *Phlebovirus* genera of the *Bunyaviridae* are ambisense viruses.

Structural features of TSWV

The TSWV genome consists of the large (L), medium (M) and small (S) RNA segments (Fig. 2). The 5' and 3' terminal 8 nucleotides (nt) of each genomic RNA segment are complementary and conserved: 5'-AGAGCAAU and 3'-UCUCGUUA. The terminal complementarity for each individual segment is extended to ~ 65 nt, allowing formation of the characteristic panhandle structure (De Haan *et al.,* 1989, 1990; Kormelink *et al.,* 1992a; Fig. 1C).

The L RNA of 8997 nucleotides (nt) is entirely of negative polarity and codes for the viral RNA dependent RNA polymerase (RdRp) or L protein (De Haan *et al.,* 1991). This 331-kDa protein has been implied in several enzymatic activities such as transcriptase, replicase and endonuclease (Adkins *et al.,* 1995; Chapman *et al.,* 2003; Van Poelwijk*,* 1996). The 4821 nt M RNA segment is of ambisense polarity. The viral strand (vRNA) of the M RNA codes for the cell-to-cell movement protein NSm, and the viral complementary strand (vcRNA) codes for the glycoprotein precursor GP, which is post-translationally cleaved into the spike- or glycoproteins G1 and G2 (Kormelink *et al.,* 1992a). The ambisense S RNA segment of 2916 nt codes for a non-structural protein (NSs) in the viral strand, and for the nucleoprotein (N) in vc-sense (De Haan *et al.,* 1990). Recently, NSs has been shown to be involved in suppression of gene silencing (Bucher *et al.*, 2003; Takeda *et al.*, 2002), a postulated host defence mechanism against viruses.

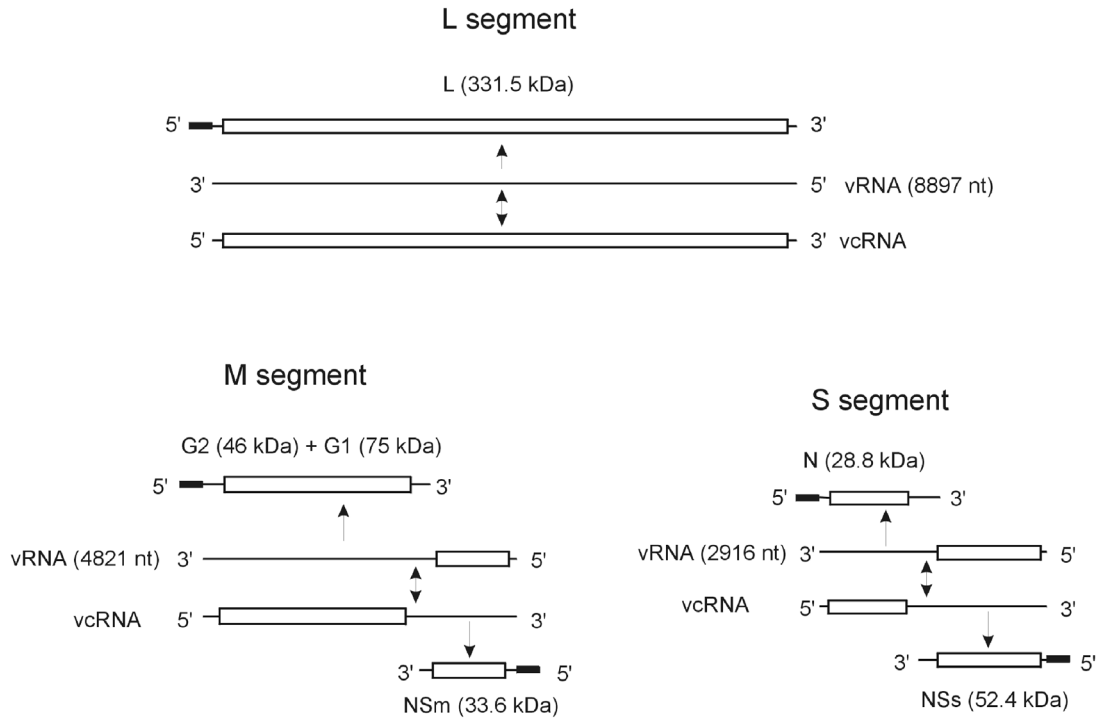

Figure 2: Genomic organisation and expression strategy of TSWV.
vRNA is viral sense RNA, vcRNA is viral complementary RNA. Open reading frames (ORFs) are indicated by white boxes, non-templated leader sequences by black bars.

The oppositely located ORFs on the ambisense S and M RNA segments are separated by intergenic regions (IRs) of several hundred nucleotides. Both IRs contain a long stretch of mainly A residues followed by a long stretch of mainly U residues, and are predicted to form large stable hairpin structures (~120 bp for the S RNA, Fig. 3; ~75 bp for the M RNA; De Haan *et al.*, 1990; Kormelink *et al.*, 1992a). In addition, a sequence (CCAAUUUGG for S and GCAAACUUUGG for M) that is conserved between different tospoviruses is located near the top of these intergenic hairpins (De Haan *et al.,* 1992; Kormelink *et al.,* 1992a; Maiss *et al.,* 1991).

Although not all steps of the TSWV infection cycle (Fig. 4; Adkins, 2000; Schmaljohn, 1996; and references therein) have been studied extensively, the cycle is thought to progress similarly to that of other negative strand RNA viruses. After introduction of TSWV into a plant cell by its biological vector (thrips), the viral membrane is removed and the nucleocapsids are released into the cytoplasm. The RNPs are the replicative and transcriptive units of TSWV, containing both the template and the enzyme for these processes. The RNPs inside virions contain mainly viral (v) sense RNA, although minor amounts of viral complementary (vc) sense RNA have also been detected in virus particles (Kormelink *et al.,* 1992b). Primary transcription by these

RNPs thus results primarily in viral mRNAs coding for the L protein, the glycoprotein precursor, and the N protein (Kormelink *et al.,* 1992b).

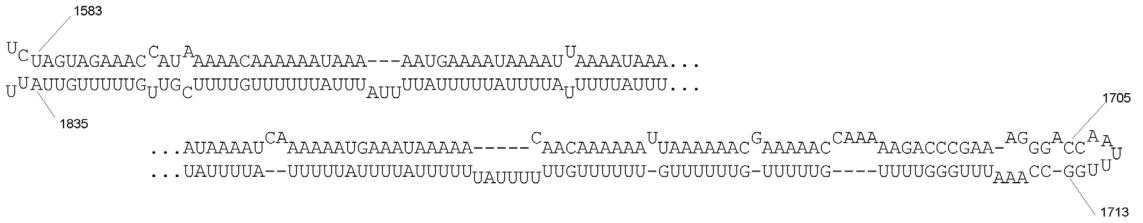

Figure 3: Predicted hairpin structure in the intergenic region (IR) of the TSWV S RNA segment.
The highly A/U-rich intergenic region (nt 1484-1986) is predicted to form a stable hairpin structure of nt 1583-1835. Nucleotides 1705-1713 form the conserved sequence motif.

TSWV infection cycle

After sufficient production of these proteins, replication of the viral genome is initiated, producing full length copies of the RNA genome segments. Similar to *Influenza A virus* (Portela & Digard, 2002), a pool of free soluble TSWV N protein may be required for anti-termination to allow replication of full-length genome copies, and may trigger the switch from transcription to replication. The vc-sense segments direct transcription of mRNAs for the NSs and NSm proteins, as a result of which the complete set of viral proteins are being synthesised. The role of the NSs protein has long been enigmatic, but recently NSs was shown to be a suppressor of gene silencing required to protect the virus against the plant's anti-viral response of post-transcriptional gene silencing (PTGS) (Bucher *et al.,* 2003; Takeda *et al.,* 2002). Other bunyaviral NSs proteins have also been reported to be involved in evading the host's anti-viral Interferon response, but in addition have been implied in translation (Di Bonito *et al.,* 1999; Simons *et al.,* 1992; Watkins & Jones, 1993) and, more recently, in down-regulating host mRNA translation (Bridgen *et al.,* 2001; Colón-Ramos *et al.,* 2003) and host cell RNA synthesis (Le May *et al.*, 2004).

The newly replicated genome segments are encapsidated by N protein and small amounts of L protein, forming new RNPs. These RNPs are transported to neighbouring cells by the aid of NSm, the viral movement protein which induces tubular structures at plasmodesmata to facilitate translocation of viral RNPs through the cell wall (Kormelink *et al.,* 1994; Storms *et al.*, 1995). In this way the viral infection spreads to neighbouring tissue, eventually leading to systemic infection.

The glycoprotein precursor is proteolytically cleaved to yield two mature proteins, G1 and G2, which are post-translationally glycosylated and targeted to the Golgi

apparatus (Kikkert *et al.*, 2001). Golgi membrane containing G1 and G2 is wrapped around newly formed RNPs to form new virus particles, which are enveloped by a double membrane and hence referred to as doubly enveloped virus particles (DEVs). Fusion of the outer membrane of these DEVs with the ER membrane finally leads to an accumulation of singly enveloped virus particles (SEVs) inside large vesicles (Kikkert *et al.,* 1999). These virions are ingested by feeding thrips, which become persistently infected as a result of virus replication inside the vector. Transmission of the virus through infection of new plants by feeding thrips will start a new infection cycle.

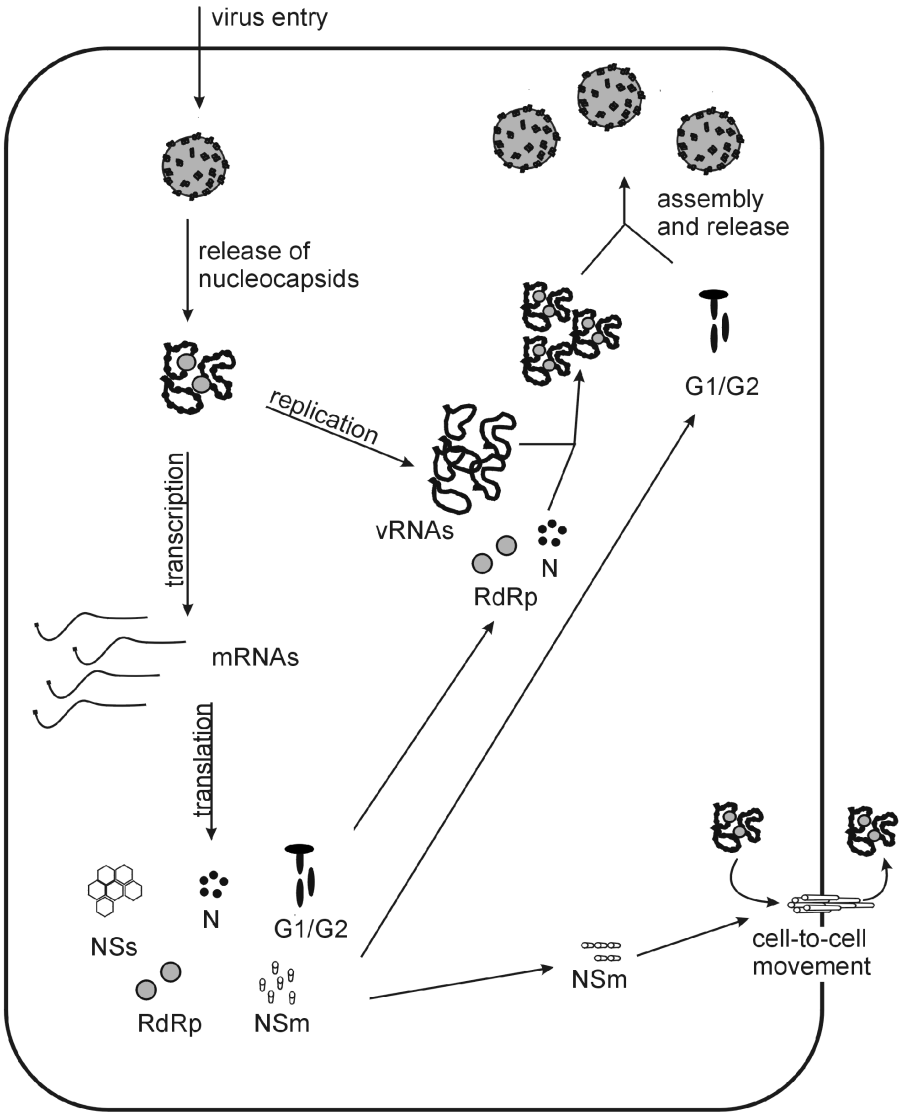

Figure 4: TSWV multiplication cycle inside a plant cell.

TSWV transcription

In order to be translated, viral mRNAs require either a 5' cap structure or an internal ribosomal entry site (IRES) (Gallie, 1998). To this end, TSWV has evolved a cap-stealing mechanism known as cap snatching, by which to initiate viral transcription. Cap snatching is common and exclusive to segmented negative strand RNA viruses (reviewed for ambisense viruses in Nguyen & Haenni, 2003). This mechanism was investigated for TSWV by extensive *in planta* studies (Duijsings *et al.,* 1999, 2001), resulting in the model for transcription initiation that is depicted schematically in Fig. 5. The capped 5'-end of a host mRNA with either an A or a G residue at 12-18 nt from the capped 5'-end is bound, presumably by the viral polymerase. This A or G is complementary to the ultimate U or penultimate C residue in the viral template, suggesting a requirement for a single basepairing interaction. The bound host mRNA is then endonucleolytically cleaved downstream of this A or G residue. The resulting capped leader, presumably positioned correctly by the basepairing interaction with the template, is then elongated to synthesise the viral mRNA. Not all details of this model have been confirmed for TSWV, but by analogy to *Influenza A virus* the cap-binding and endonuclease activity are thought to reside in the viral polymerase.

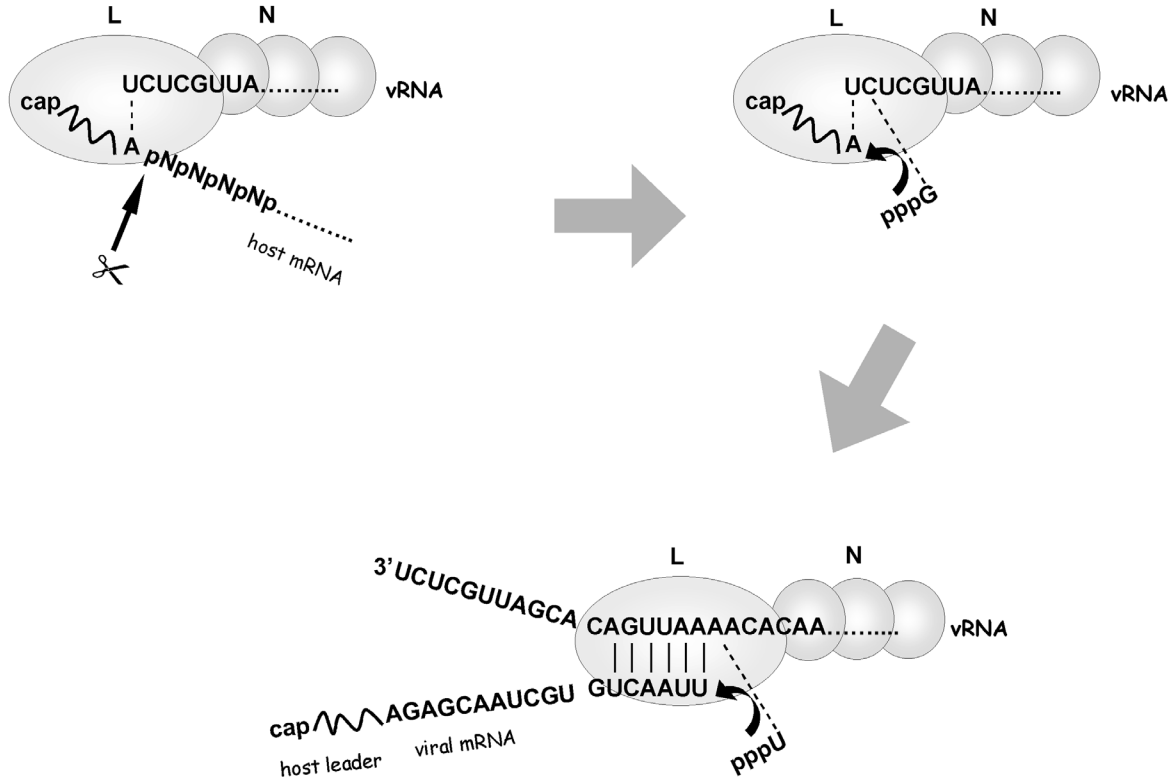

Figure 5: The model for cap snatching by TSWV as proposed by Duijsings *et al.*, 2001.

Unlike *Influenza A virus* mRNAs, bunyaviral mRNAs are synthesised in the cytoplasm and are generally not polyadenylated, indicating a distinct mechanism of transcription termination. For the entirely negative sense orthobunya-, nairo- and hantaviruses termination signals appear to be G- or C- rich stretches, with or without a consensus motif (Eshita *et al.,* 1985; Hutchinson *et al.,* 1996; Patterson & Kolakofsky, 1984). However, in most cases these signals have been studied only for the S segments of these viruses and therefore it has remained unknown whether they are conserved for all three segments. Only *Sin Nombre* hantavirus termination has been investigated for all three segments and, surprisingly, this virus would appear to use different strategies for each segment (Hutchinson *et al.,* 1996). Termination of the S-segment mRNA occurs just downstream of the sequence motif CCCACCC that is conserved between hanta- and orthobunyaviruses. This sequence motif could not be identified in the M and L genome segments. Mapping of the 3'-terminus of the L mRNA indicated it was not shorter than the genomic template RNA, suggesting this mRNA does not require a termination signal. The M-segment mRNA is polyadenylated at a polyadenylation-transcription termination signal (U_8) that is highly conserved among all characterised hantaviruses and closely resembles that of several other negative strand viruses (Hutchinson *et al.*, 1996). Whether this reflects a termination mechanism similar to that used by *Influenza A virus*, where stuttering of the viral polymerase complex at a U-tract (5 to 7 residues) results in synthesis of a poly(A) tail (Luo *et al.*, 1991; Zheng *et al.*, 1999), remains to be investigated.

Transcription termination of the ambisense S RNA segment of phleboviruses appears not be uniform. 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 IR of the *Punta toro* (PTV) phlebovirus S RNA segment is predicted to form a stable hairpin structure, and transcription termination appears to occur near the top of this 100-bp-sized hairpin (Emery & Bishop, 1987). *Uukuniemi virus* (UUKV) transcription terminates at the 3'-end of the IR, yielding two mRNAs that possess a small 3'-stem-loop structure and are complementary at the entire 3'-untranslatable region (UTR) (Simons & 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 well as in the template for transcription of the NSs mRNA of UUKV.

For TSWV, mRNA size estimation on Northern blots (~3.5 kb for GP, ~1.1 kb for NSm, ~1.7 kb for NSs and ~1.2 kb for N) suggests that termination occurs somewhere in the intergenic region (De Haan *et al.,* 1990; Kormelink *et al.,* 1992b). Therefore, the two

known features of the IR, the hairpin and the consensus sequence, have been suggested to be involved in transcription termination.

Methods to study transcription and replication of negative strand viruses

Molecular studies of transcription and replication of negative strand viruses have long been hampered by the fact that, unlike positive strand viruses, reverse genetics approaches (i.e. reconstructing viable, replicating genomes from full-length, cloned DNA copies) could not be directly applied due to the genomes being of negative polarity. Initiation of the genome amplification cycle requires (correctly assembled) RNPs, either reconstituted in cells (*in vivo*) by providing all the necessary components, or reconstituted *in vitro* using purified recombinant proteins or purified virus.

At the onset of the project described in this thesis, *in vivo* reconstitution of segmented negative strand viruses, allowing reverse genetics approaches, had recently been developed (reviewed in: Palese *et al.,* 1996; Pekosz *et al.,* 1999; Rose, 1996). These initial systems were based on infection of cultured cells with recombinant *Vaccinia virus* (vvT7) to obtain expression of bacteriophage T7 RNA polymerase. Upon co-transfection of these cells with plasmids containing T7 promoter-driven genes of the viral RdRp and N proteins, as well as a plasmid carrying a model RNA template (usually a reporter gene flanked by viral genomic terminal sequences), functional RNPs were reconstituted that could readily engage in transcription and/or replication. A disadvantage of these systems is that all cytoplasmic RNAs are capped by the *Vaccinia virus* capping enzyme. As a result, newly (T7) transcribed RNA is capped and can be directly translated, which hampers detection of genuine viral transcription. In addition, the cells can only be studied for a limited amount of time due to the cytopathogenic effect (CPE) of *Vaccinia virus* infection.

In a concurrent PhD project, a similar *in vivo* reconstitution system was developed to study TSWV replication and transcription (Duijsings, 2001). Active RNP complexes were successfully reconstituted in cultured murine cells, as measured by reporter gene (luciferase) activity. However, due to capping by *Vaccinia virus* capping enzyme, no distinction could be made between replicational and transcriptional activity. At the same time, for Uukuniemi phlebovirus (UUKV) the more elegant Pol I - Pol II system was developed which employs the mammalian promoters for RNA Polymerase I (Pol I) and RNA Polymerase II (Pol II), obviating the need for *Vaccinia virus* infection and thus avoiding *Vaccinia virus*-induced CPE (Flick *et al.,* 2001).

Alternatively, *in vitro* assays can be used to study the molecular details of virus multiplication. In these assays virus particles, purified from infected hosts, are stimulated to perform transcription and/or replication in a test tube by creating the appropriate reaction conditions. *In vitro* assays have provided much information about the

mechanisms of transcription and replication of segmented negative strand RNA viruses (Bellocq *et al.,* 1987; Bellocq & Kolakofsky, 1987; Garcin *et al.,* 1992; Nguyen *et al.,* 1997; Vialat *et al.,* 1992), in particular *Influenza A virus* (Nagata *et al.,* 1989; Peng *et al.,* 1996; Shapiro & Krug, 1988; Shi *et al.,* 1995; Skorko *et al.,* 1991; Toyoda *et al.,* 1994). An advantage of these assays is the high manipulability: many components of the reaction are under direct control, and can be added or omitted *ad libitum*.

Thesis outline

The research described in this thesis was aimed at further unravelling the mechanisms of TSWV transcription and replication. In particular, the project focussed on the process of transcription initiation, the potential translational dependence of transcription and the site and signal of transcription termination. Detailed investigation of these processes requires a manipulable system to allow the addition, deletion, or mutation of specific factors involved. A good starting point was provided by a previously developed *in vitro* assay based on gently lysed TSWV particles, which yielded measurable *in vitro* RdRp activity (Van Poelwijk, 1996). This assay was based on those previously established for other (positive strand) RNA plant viruses, and although RdRp activity could be demonstrated it remained unclear whether this was the result of replication or transcription.

As a first step, the available assay was further developed into a proper TSWV *in vitro* transcription assay (Chapter 2). The conditions required for transcription implied a translational dependence of transcription, as had been observed previously for several orthobunyaviruses. This potential translational dependence was further examined in Chapter 3. In order to identify the transcription termination signal(s), the site of termination of S-segment encoded mRNAs was investigated in Chapter 4, using the *in vitro* transcription assay. The requirements and restrictions of basepairing between cap donor and viral template to initiate transcription were further investigated in Chapter 5, as well as the exact endonuclease cleavage site in cap donors having a multiple base complementarity to the viral template. Finally, in Chapter 6 the experimental results obtained and their implications are discussed in relation to current knowledge of replication, transcription, and translation of other negative strand and ambisense RNA viruses. In particular, the model for the mechanism of cap snatching, as postulated by Duijsings *et al*. (2001), is fine-tuned in view of the findings reported in Chapter 5.

REFERENCES

Adkins, S., Quadt, R., Choi, T. J., Ahlquist, P. and German, T. (1995). An RNA-dependent RNA polymerase activity associated with virions of *Tomato spotted wilt virus*, a plant- and insect-infecting bunyavirus. *Virology* **207**, 308-311

Adkins, S. (2000). *Tomato spotted wilt virus*: Positive steps towards negative success. *Mol Pl Path* **1**, 151-157

Bellocq, C., Ramaswamy, R., Patterson, J. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: *In vitro* studies. *J Virol* **61**, 87-95

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: A possible mechanism. *J Virol* **61**, 3960-3967

Bridgen, A., Weber, F., Fazakaerly, J. K. and Elliott, R. M. (2001). *Bunyamwera bunyavirus* nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc Natl Acad Sci USA* **98**, 664-669

Bucher, E., Sijen, T., De Haan, P., Goldbach, R. and Prins, M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J Virol* **77**, 1329-1336

Chapman, E. J., Hilson, P. and German, T. L. (2003). Association of L protein and *in vitro Tomato spotted wilt virus* RNA-dependent RNA polymerase activity. *Intervirology* **46**, 177-181

Colón-Ramos, D. A., Irusta, P. M., Gan, E. C., Olson, M. R., Song, J. W., Morimoto, R. I., Elliott, R. M., Lombard, M., Hollingsworth, R., Hardwick, J. M., Smith, G. K. and Kornbluth, S. (2003). Inhibition of translation and induction of apoptosis by bunyaviral nonstructural proteins bearing sequence similarity to reaper. *Mol Biol Cell* **14**, 4162-4172

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1989). Molecular cloning and terminal sequence determination of the S and M RNAs of *Tomato spotted wilt virus*. *J Gen Virol* **70**, 3469-3473

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1990). The S RNA segment of *Tomato spotted wilt virus* has an ambisense character. *J Gen Virol* **71**, 1001-1007

De Haan, P., Kormelink, R., Resende, R. D., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). *Tomato spotted wilt virus* L RNA encodes a putative RNA polymerase. *J Gen Virol* **72**, 2207-2216

De Haan, P., De Avila, A. C., Kormelink, R., Westerbroek, A., Gielen, J. J. L., Peters, D. and Goldbach, R. (1992). The nucleotide sequence of the S RNA of *Impatiens necrotic spot virus*, a novel tospovirus. *FEBS Letters* **306**, 27-32

De Miranda, J. R., Munoz, M., Wu, R., Hull, R. and Espinoza, A. M. (1996). Sequence of *Rice hoja blanca tenuivirus* RNA-2. *Virus Genes* **12**, 231-237

Di Bonito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. and Giorgi, C. (1999). Immunological characterization of *Toscana virus* proteins. *Arch Virol* **144**, 1947-1960

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of *Nicotiana benthamiana*. *J Virol* **73**, 5172-5175

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 1-8

Duijsings, D. (2001). *Analysis of the transcription initiation mechanism of Tomato spotted wilt virus*. Thesis Wageningen University, The Netherlands.

Emery, V. C. and Bishop, D. H. (1987). Characterisation of *Punta toro* S mRNA species and identification of an inverted complementary sequence in the intergenic region of *Punta toro phlebovirus* ambisense S RNA that is involved in mRNA transcription termination. *Virology* **156**, 1-11

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D. H. (1985). Analyses of the mRNA transcription processes of *Snowshoe hare bunyavirus* S and M RNA species. *J Virol* **55**, 681-689

Estabrook, E. M., Suyenaga, K., Tsai, J. H. and Falk, B. W. (1996). *Maize stripe tenuivirus* RNA2 transcripts in plant and insect hosts and analysis of pvc2, a protein similar to the Phlebovirus virion membrane glycoproteins. *Virus Genes* **12**, 239-247

Flick, R. and Pettersson, R. F. (2001). Reverse genetics system for *Uukuniemi virus* (*Bunyaviridae*): RNA polymerase I-catalyzed expression of chimeric viral RNAs. *J Virol* **75**, 1643-1655

Gallie, D. R. (1998). A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**, 1-11

Garcin, D. and Kolakofsky, D. (1992). *Tacaribe arenavirus* RNA synthesis *in vitro* is primer dependent and suggests an unusual model for the initiation of genome replication. *J Virol* **66**, 1370-1376

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M. C., Takehara, K., Hilditch, C., Morikawa, S. and Bishop, D. H. (1991). Sequences and coding strategies of the S RNAs of *Toscana* and *Rift Valley fever* viruses compared to those of *Punta Toro, Sicilian Sandfly fever*, and *Uukuniemi* viruses. *Virology* **180**, 738-753

Hutchinson, K. L., Peters, C. J. and Nichol, S. T. (1996). *Sin Nombre virus* mRNA synthesis. *Virology* **224**, 139-149

Kikkert, M., van Lent, J., Storms, M., Bodegom, P., Kormelink, R. and Goldbach, R. (1999). *Tomato spotted wilt virus* particle morphogenesis in plant cells. *J Virol* **73**, 2288-2297

Kikkert, M., Verschoor, A., Kormelink, R., Rottier, P. and Goldbach, R. (2001). *Tomato spotted wilt virus* glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. *J Virol* **75**, 1004-1012

Kormelink, R., De Haan, P, Meurs, C., Peters, D., and Goldbach, R. (1992a). The nucleotide sequence of the M RNA segment of *Tomato spotted wilt virus*, a bunyavirus with two ambisense RNA segments. *J Gen Virol* **73**, 2795-2804

Kormelink, R., De Haan, P., Peters, D. and Goldbach, R. (1992b). Viral RNA synthesis in *Tomato spotted wilt virus*-infected *Nicotiana rustica* plants. *J Gen Virol* **73**, 687-693

Kormelink, R., Storms, M., van Lent, J., Peters, D. and Goldbach, R. (1994). Expression and subcellular location of the NSm protein of *Tomato spotted wilt virus* (TSWV), a putative viral movement protein. *Virology* **200**, 56-65

Le May, N., Dubaele, S., Proietti de Santis, L., Billecocq, A., Bouloy, M. and Egly, J.-M. (2004). TFIIH transcription factor, a target for the *Rift Valley hemorrhagic fever virus*. *Cell* **116**, 541-550

Luo, G., Luytjes, W., Enami, M. and Palese, P. (1991). The polyadenylation signal of *Influenza virus* RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. *J Virol* **65**, 2861-2867

Maiss, E., Ivanova, L., Breyel, E. and Adam, G. (1991). Cloning and sequencing of the S RNA from a Bulgarian isolate of *Tomato spotted wilt virus*. *J Gen Virol* **72**, 461-464

Nagata, K., Takeuchi, K. and Ishihama, A. (1989). *In vitro* synthesis of *Influenza* viral RNA: biochemical complementation assay of factors required for *Influenza virus* replication. *J Biochem* **106**, 205-208

Nguyen, M., Ramirez, B. C., Goldbach, R. and Haenni, A. L. (1997). Characterization of the *in vitro* activity of the RNA-dependent RNA Polymerase associated with the ribonucleoproteins of *Rice hoja blanca tenuivirus*. *J Virol* **71**, 2621-2627

Nguyen, M. and Haenni, A. L. (2003). Expression strategies of ambisense viruses. *Virus Res* **93**, 141-150

Palese, P., Zheng, H., Engelhardt, O. G., Pleschka, S. and García-Sastre, A. (1996). Negative-strand RNA viruses: Genetic engineering and applications. *Proc Natl Acad Sci USA* **93**, 11354-11358

Patterson, J. L. and Kolakofsky, D. (1984). Characterization of *La Crosse virus* small-genome transcripts. *J Virol* **49**, 680-685

Pekosz, A., He, B. and Lamb, R. A. (1999). Reverse genetics of negative-strand RNA viruses: Closing the cirlcle. *Proc Natl Acad Sci USA* **96**, 8804-8806

Peng, Q., Shi, J. M. G. and Summers, D. F. (1996). *Influenza A virus* RNA-dependent RNA polymerase cleaves *Influenza* mRNA *in vitro*. *Virus Res* **42**, 149-158

Portela, A. and Digard, P. (2002). The *Influenza virus* nucleoprotein: A multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* **83**, 723-734

Rose, J. K. (1996). Positive strands to the rescue again: A segmented negative-strand RNA virus derived from cloned cDNAs. *Proc Natl Acad Sci USA* **94**, 14998-15000

Sasaya, T., Ishikawa, K. and Koganezawa, H. (2001). Nucleotide sequence of the coat protein gene of *Lettuce big-vein virus*. *J Gen Virol* **82**, 1509-1515

Sasaya, T., Ishikawa, K. and Koganezawa, H. (2002). The nucleotide sequence of RNA1 of *Lettuce big-vein virus*, genus varicosavirus, reveals its relation to nonsegmented negative-strand RNA viruses. *Virology* **297**, 289-297

Sasaya, T., Kusaba, S., Ishikawa, K. and Koganezawa, H. (2004). Nucleotide sequence of RNA2 of *Lettuce big-vein virus* and evidence for a possible transcription termination/initiation strategy similar to that of rhabdoviruses. *J Gen Virol* **85**, 2709-2717

Shapiro, G. I. and Krug, R. M. (1988). *Influenza virus* RNA replication *in vitro*: Synthesis of viral template RNAs and virion RNAs in the absence of an added primer. *J Virol* **62**, 2285-2290

Shi, L., Summers, D. F., Peng, Q. and Galarza, J. M. (1995). *Influenza A virus* RNA polymerase subunit PB-2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. *Virology* **208**, 38-47

Simons, J. F. and Pettersson, R. F. (1991). Host-derived 5' ends and overlapping complementary 3' ends of the two messenger RNAs transcribed from the ambisense S segment of *Uukuniemi virus*. *J Virol* **65**, 4741-4748

Simons, J. F., Persson, R. and Pettersson, R. F. (1992). Association of the nonstructural protein NSs of Uukuniemi virus with the 40S ribosomal subunit. *J Virol* **66**, 4233-4241

Skorko, R., Summers, D. F. and Galarza, J. M. (1991). *Influenza A virus in vitro* transcription: roles of NS1 and NP proteins in regulating RNA synthesis. *Virology* **180**, 668-677

Storms, M. M. H., Kormelink, R., Peters, D., van Lent, J. W. M. and Goldbach, R. W. (1995). The nonstructural NSm protein of *Tomato spotted wilt virus* induces tubular structures in plant and insect cells. *Virology* **214**, 485-493

Takahashi, M., Toriyama, S., Hamamatsu, C. and Ishihama, A. (1993). Nucleotide sequence and possible ambisense coding strategy of *Rice stripe virus* RNA segment 2. *J Gen Virol* **74**, 769-773

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. and Okuno, T. (2002). Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. *FEBS Letters* **532**, 75-79

Toyoda, T., Kobayashi, M. and Ishihama, A. (1994). Replication *in vitro* of the *Influenza virus* genome: Selective dissociation of RNA replicase from virus-infected cell ribonucleoprotein complexes. *Arch Virol* **136**, 269-286

Van Poelwijk, F., Boye, K., Oosterling, R., Peters, D. and Goldbach, R. (1993). Detection of the L protein of *Tomato spotted wilt virus*. *Virology* **197**, 468-470

Van Poelwijk, F., Kolkman, J. and Goldbach, R. (1996). Sequence analysis of the 5' ends of *Tomato spotted wilt virus* N mRNAs. *Arch Virol* **141**, 177-184

Van Poelwijk, F. (1996). *On the expression strategy of the tospoviral genome*. Thesis Wageningen University, The Netherlands.

Vialat, P. and Bouloy, M. (1992). *Germiston virus* transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66**, 685-693

Zheng, H. Y., Lee, H. A., Palese, P. and García-Sastre, A. (1999). *Influenza A virus* RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. *J Virol* **73**, 5240-5243

CHAPTER 2

Purified *Tomato spotted wilt virus* particles support both genome replication and transcription *in vitro*

SUMMARY

Purified *Tomato spotted wilt virus* particles were shown to support either genome replication or transcription *in vitro*, depending on the conditions chosen. Transcriptional activity was observed only upon addition of rabbit reticulocyte lysate, indicating a dependence on translation. Under these conditions RNA molecules of subgenomic length were synthesised that hybridised to strand specific probes for the N and NSs genes. Cloning of these transcripts demonstrated the presence of non-viral leader sequences at their 5'-ends, confirming the occurrence of genuine viral transcription initiation known as cap snatching. Sequence analyses revealed that both α- and β-globin mRNAs, present in the reticulocyte lysate, as well as added *Alfalfa mosaic virus* (AMV) RNAs were utilised as cap donors. Moreover, an artificially produced N mRNA containing an AMV-derived leader was shown to be used as cap donor, indicating that re-snatching of viral mRNAs takes place *in vitro*.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the plant-infecting genus *Tospovirus* within the arthropod borne *Bunyaviridae* (Van Regenmortel *et al.*, 2000). Like all members of this family, TSWV has enveloped spherical particles, the membrane containing two types of viral glycoproteins, denoted G1 and G2. The core of the virion contains ribonucleoproteins (RNPs), that consist of linear, single-stranded RNA segments tightly encapsidated by the nucleoprotein (N) and a few copies of the viral RNA-dependent RNA polymerase (RdRp; also denoted L protein). The TSWV genome is tripartite and consists of ambisense S and M RNA segments, and a negative sense L RNA. The S RNA (2.9 kb) codes for the nucleoprotein (N) in viral complementary (vc) sense and a non-structural protein (NSs), of as yet unknown function, 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; Kormelink *et al*., 1994). The L RNA (8.9 kb) codes for the viral RdRp in vc-sense (De Haan *et al*., 1991). Due to base pairing of the conserved and complementary 5' and 3' termini the genomic RNA segments form panhandle structures and as a result, the RNPs appear as pseudo-circular structures in electron micrographs (De Haan *et al*., 1989).

All TSWV genes are expressed by the synthesis of mRNAs that can be discriminated from the (anti) genomic RNA strands by the presence of non-viral leader sequences (Kormelink *et al.*, 1992b and 1992c). These leader sequences are the result of cap snatching, a mechanism used by all segmented negative strand RNA viruses to initiate transcription of their genome and first described for *Influenza virus* (Plotch *et al.*, 1981). During this process the viral RdRp, encompassing an endonuclease activity, cleaves a host mRNA at a position 10-20 nucleotides from the capped 5'-end, and uses the resulting leader sequence to prime transcription of its genome (Kormelink *et al.,* 1992c; Van Poelwijk *et al*., 1996). More recently, it has been demonstrated that *Alfalfa mosaic virus* (AMV) RNAs can be utilised by TSWV as cap donors during a mixed infection of *Nicotiana benthamiana* (Duijsings *et al.*, 1999). Furthermore, it was shown that suitable cap donors require a single base complementarity to the ultimate or penultimate residue of the TSWV template (Duijsings *et al.*, 2001).

Most details concerning replication and transcription of the ambisense TSWV genome have remained unknown. This is partly due to the fact that, compared to most negative strand RNA viruses, e.g. *Influenza virus* (Beaton and Krug, 1986; Shapiro *et al*., 1988), *La Crosse virus* (Bellocq *et al.*, 1987b) and *Germiston virus* (Vialat *et al*., 1992), purified TSWV particles show only limited *in vitro* RdRp activity (Adkins *et al*., 1995; Van Poelwijk, 1996). These initial *in vitro* studies using purified TSWV, moreover, have

not elucidated whether genome transcription or replication, or both, took place. Besides a majority of smaller products, only a single distinct RNA species of approximately 3 kb was observed (Adkins *et al*., 1995) but this could equally well represent full-length S RNA (2.9 kb) which would be indicative of replication, or the subgenomic mRNA for the glycoproteins (approximately 3.5 kb; Kormelink *et al*., 1992b), indicative of transcription.

In this chapter the *in vitro* RdRp activity of purified TSWV particles has been further investigated. It is shown that, depending on buffer conditions and the presence or absence of rabbit reticulocyte lysate (RRL), TSWV particles support either genome replication or genome transcription. Evidence for genuine viral transcription is provided by the synthesis of RNA molecules containing 5' non-viral leader sequences. These non-viral leader sequences originated not only from endogenous RNA present in RRL, but also from exogenously added cap donor RNAs. In addition, the RdRp activity of purified cytoplasmic RNPs has been investigated, since these form the minimal viral transcription and replication units. Surprisingly, the cytoplasmic RNPs demonstrated a slightly different RNA synthetic activity compared to virions.

RESULTS

RNA synthesis in the absence of reticulocyte lysate

For a first product analysis of *in vitro* synthesised RNA, purified TSWV was incubated in the presence of NTPs (including radiolabelled CTP) under conditions as reported by Adkins *et al.* (1995). The RNA produced was used as a probe to screen a Northern blot containing all TSWV genome segments (Fig. 1, lane 1) and the results showed that RNA molecules specific for all three segments were synthesised (Fig. 1, lane 2).

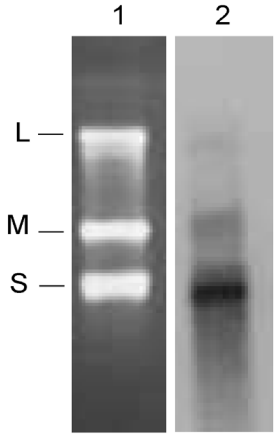

Figure 1: Northern blot analysis using *in vitro* synthesised RNA as a probe.
Lane 1, Ethidiumbromide stained RNA profile of purified TSWV RNA resolved on a 1.5% agarose gel; lane 2, Northern blot hybridisation of *in vitro* synthesised radiolabelled products to TSWV RNA. The positions of the L, M, and S RNA segments are indicated.

In order to identify the nature of the RNA molecules synthesised, *in vitro* reaction products from several independent experiments were resolved by RNA gel electrophoresis. The results from these analyses consistently revealed a major distinct product of approximately 3 kb (Fig. 2A, lane 2), as reported earlier by Adkins *et al.* (1995), which was absent from the control reaction (Fig. 2A, lane 1). The 3 kb product hybridised with a strand specific probe for the vc-sense S RNA (Fig. 2B, lane 2), and comigrated with the viral S RNA isolated from TSWV-infected *Nicotiana rustica* (Fig. 2B, lane 4) or purified virions (Fig. 2B, lane 1). This indicated that the 3 kb RNA band represented full length S RNA, apparently synthesised as a result of replication. Occasionally, an additional distinct RNA product of 5 kb was observed (Fig. 2A, lane 3), matching in size with the viral genomic M RNA segment from purified RNPs. No subgenomic length RNA products with a size that could correspond to TSWV mRNAs (N mRNA, 1.2 kb; NSs mRNA, 1.7 kb) were observed (Fig. 2, compare lane 2 of panel A with lane 4 of panel B).

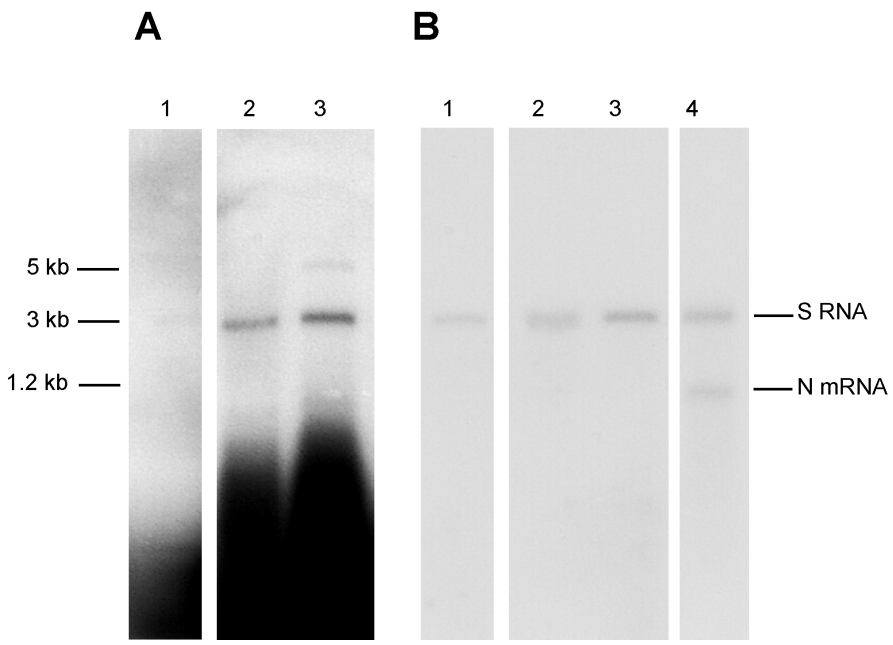

Figure 2: *In vitro* RNA synthesis directed by TSWV virions in the absence of reticulocyte lysate.
TSWV RNA synthesised *de novo* in the presence of ^{32}P-CTP was resolved on a 1.5% agarose gel and subsequently blotted to Hybond-N. Panel A, Northern blot of radiolabelled RNA synthesised *in vitro* by TSWV. Lane 1, heat inactivated virus; lane 2, RNA synthesised *in vitro* in the absence of reticulocyte lysate; lane 3, RNA synthesised *in vitro* in the absence of reticulocyte lysate and in the presence of exogenous AMV RNA4. Panel B, Northern blot hybridisation of panel A with a strand specific probe detecting vc-sense S RNA and N mRNA. Lanes 1-3 as in panel A; lane 4, total RNA of TSWV-infected *Nicotiana rustica*. Hybridisation was performed only after full decay of the radioactive signal from panel A.

Since TSWV transcription is known to be initiated by cap snatching (Kormelink *et al*., 1992c; Van Poelwijk *et al*., 1996), the absence of a suitable cap donor could have been a limiting factor for the occurrence of transcription. To test this hypothesis, exogenous AMV which has previously been shown to be a suitable cap donor for TSWV transcription initiation *in vivo* (Duijsings *et al*., 1999), was added to the *in vitro* reaction. Although a 5 kb band may seem more prevalent in the profile of RNA products (Fig. 2A, lane 3), in general, during repeated analyses the profile did not differ from that of reactions that were performed in the absence of a cap donor (Fig. 2A, lane 2). Moreover, no potential N or NSs transcripts could be observed after Northern blot hybridisation (Fig. 2B, lane 3), indicating that the additional presence of a cap donor was not sufficient to stimulate transcription.

Altogether, the results indicated that in the absence of reticulocyte lysate purified virions are only capable of genome replication, as demonstrated by the production of genome length S and M RNA and (visual) absence of subgenomic length mRNAs.

RNA synthesis in the presence of reticulocyte lysate

For *Germiston virus* and *La Crosse virus*, both representing animal-infecting members of the *Bunyaviridae*, addition of reticulocyte lysate (RRL) has been reported to lead to a significant stimulation of transcription *in vitro*, as demonstrated by the synthesis of subgenomic RNA species (Bellocq *et al.*, 1987b; Vialat *et al.*, 1992). To test whether the presence of RRL would also stimulate TSWV transcription *in vitro*, RdRp assays were performed containing RRL as described in Materials and Methods.

In the presence of RRL, the total rate of RNA synthesis was increased considerably (Fig. 3A, compare lanes 2 and 3). While hardly any or no genome length RNA molecules were visible, high amounts of distinct smaller products were observed (Fig. 3A, lane 3*), potentially representing the N and NSs mRNAs of approximately 1.2 kb and 1.7 kb, respectively (Kormelink *et al*., 1992b). After decay of the radioactive signal, Northern blot hybridisation showed that the major 1.2 kb band reacted with a strand specific probe for the N gene (Fig. 3B, lane 3), and comigrated with the N mRNA isolated from TSWV-infected *N. rustica* (Fig. 3B, lane 5) indicating that it likely represented the N mRNA. The minor 1.7 kb band did not show hybridisation with a strand specific probe for the NSs gene, although it did comigrate with the NSs mRNA isolated from TSWV-infected *N. rustica* (Fig. 3C, lane 5). The absence of a direct hybridisation signal was most likely due to the low amount of *de novo* synthesised product, as compared to the intensity of the band of the putative N mRNA. Addition of exogenous AMV RNA4 did not alter the profile of RNA molecules synthesised in the presence of RRL (Fig. 3A, lane 4 and 4*).

These results demonstrated that addition of RRL highly stimulated RNA synthesis and appeared to induce transcription, as demonstrated by the production of subgenomic viral RNA molecules.

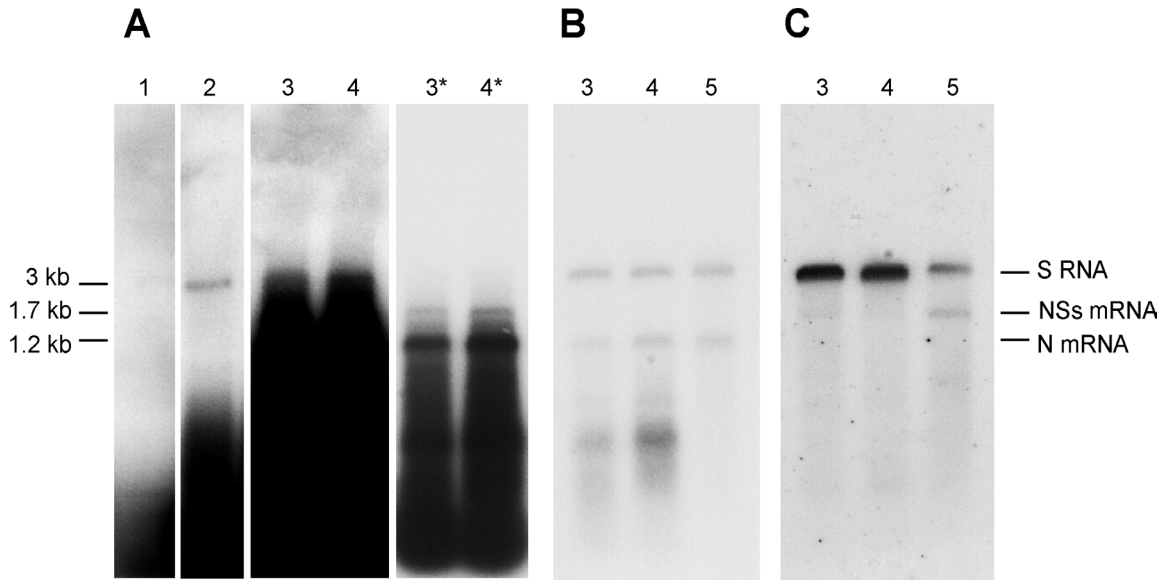

Figure 3: Effect of RRL on *in vitro* RNA synthesis directed by TSWV virions.
TSWV RNA synthesised *de novo* in the presence of ^{32}P-CTP was resolved on a 1.5% agarose gel and subsequently blotted to Hybond-N. Panel A, Northern blot of radiolabelled RNA synthesised *in vitro* by TSWV. Lane 1, heat-inactivated virus; lane 2, RNA synthesised *in vitro* in the absence of RRL; lane 3, RNA synthesised *in vitro* in the presence of RRL; lane 4, RNA synthesised *in vitro* in the presence of both RRL and AMV RNA4. Lanes marked with *: shorter exposure. Panels B and C; Hybridisation signal of the lanes in panel A with strand specific probes detecting vc-sense S RNA and N mRNA (B) and v-sense S RNA and NSs mRNA (C). Lanes 3-4 as in panel A; Lane 5, total RNA from TSWV-infected *Nicotiana rustica*.

In the presence of RRL, in vitro *RNA synthesis coincides with cap snatching*

To verify whether RNA synthesis in the presence of RRL was indeed genuine viral transcription, the RNA products were analysed for the presence of non-viral sequences at their 5' termini, which would be indicative of cap snatching. This process requires the presence of capped leader donors, which could be present in the RRL as fragmented (micrococcal endonuclease-cleaved) globin mRNAs.

Previously, an RT-PCR protocol was developed to selectively amplify viral mRNAs with a non-viral leader sequence, and discriminate these from genomic viral RNAs (Duijsings *et al.*, 1999). Using this approach, purified RNA products from the *in vitro* transcription reaction were reverse transcribed using a specific internal primer for the TSWV N, NSm, and GP genes respectively, followed by PCR with a gene-specific primer and a primer matching the 5' first 11 nt of the α- or β-globin mRNA (see

Materials and Methods). By this procedure, products of expected sizes were amplified (Fig. 4, lanes 1-3) corresponding to potential TSWV mRNAs. This was further supported by the absence of PCR products from control reactions using heat-inactivated virus (Fig. 4, lanes 4-6). Cloning and sequencing of the PCR products obtained from the N gene transcripts showed that they indeed were preceded by the first 13-14 nucleotides of the α- and β-globin mRNAs (Table 1), indicating that transcription initiation by cap snatching took place *in vitro*.

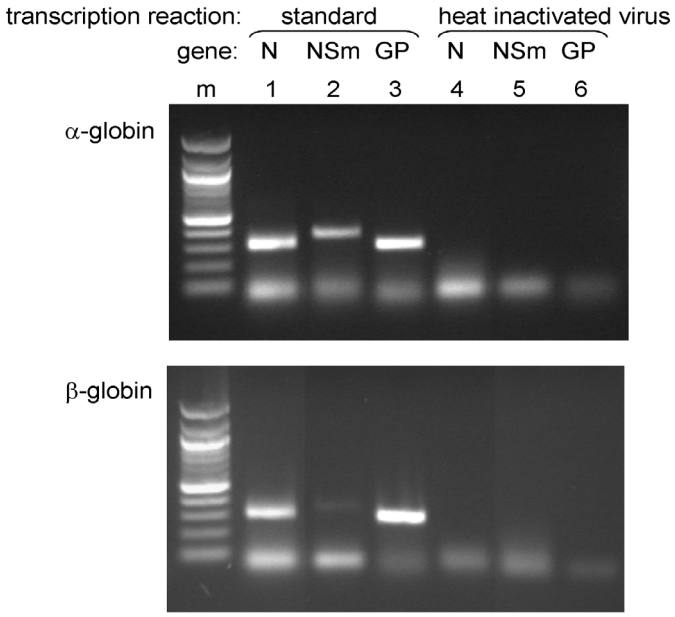

Figure 4: RT-PCR analysis of *in vitro* synthesised TSWV RNA.
RNA synthesised *in vitro* in the presence of RRL (using active virus in lanes 1-3 and heat inactivated virus in lanes 4-6) was reverse transcribed using internal primers for the TSWV genes N, NSm, or GP, followed by PCR using internal primers for the same genes in combination with a primer specific for the 5' 11 nt of either the α-globin (upper panel) or β-globin (lower panel) mRNA. The virus used and the TSWV genes are indicated above the lanes. Lane m: 100 bp size marker.

Previous studies already had demonstrated a requirement for a single base complementarity between the cap donor RNA and the TSWV template (Duijsings *et al*., 2001). A preference was observed for an A residue in the cap donor between positions 12 and 18 from the capped 5'-end, with position 16 being optimal. This A residue can basepair with the ultimate U residue or, though less efficiently, with the antepenultimate U residue in the TSWV template. Moreover, a G residue instead of an A is also accepted, resulting in potential internal basepairing with the penultimate C residue of the viral template.

Interestingly, the α-globin mRNA contains a dinucleotide AG at positions 14 and 15, offering the possibility of double basepairing (Fig. 5). This basepairing is observed both to the ultimate UC dinucleotide and internally to the second UC dinucleotide of the template (Table 1 and Fig. 5). Since the α-globin mRNA was used by TSWV as cap donor, complementarity is apparently not restricted to single basepairing. However, it remains to be determined whether cleavage of this donor took place after the A or after the G residue.

More intriguing is the situation for the β-globin mRNA, which also offers the possibility of double basepairing, though in this case with a dinucleotide GA at positions 13 and 14 (Fig. 5). With this cap donor both single basepairing, of A14 with the ultimate U residue of the TSWV template, and double basepairing, of G13A14 with the penultimate and antepenultimate CU dinucleotide, was observed (Table 1 and Fig. 5). The presence of the G residue preceding A14 may be the reason that A14 is preferred over A16, the position that was previously found to be optimal (Duijsings *et al*., 2001).

Table 1: 5'-terminal sequences of viral N gene transcripts synthesised *in vitro*.

Cap donor	5'-terminal RNA sequence obtained	Number of clones
α-globin mRNA	5' *ACACUUCUGGUCCA* GAGCAA...	10/11
	5' *ACACUUCUGGUCCA* GCAA...	1/11
β-globin mRNA	5' *ACACUUGCUUUUGA* GAGCAA...	5/9
	5' *ACACUUGCUUUUGA* GCAA...	4/9
AMV RNA4	5' *GUUUUUAUUUUUAA* GAGCAA...	2/9
	5' *GUUUUUAUUUUUAA* GCAA...	5/9
	5' *GUUUUUAUUUUUA*GAGAGCAA...	2/9
AMV RNA3	5' *GUAUUAAUACCAUUUUCA* GAGCAA...	5/5
Synthetic AMV RNA3 (wild type)	5' *GUAUUAAUACCAUUUUCA* GAGCAA...	10/10
Synthetic AMV RNA3 (mutant C17G)	5' *GUAUUAAUACCAUUUUGA* GAGCAA...	6/7
	5' *GUAUUAAUACCAUUUUGA* GCAA...	1/7

Sequence results showing *in vitro* synthesised TSWV N mRNA 5' leader sequences derived from exogenous (AMV4, AMV3) or endogenous (α-globin, β-globin) mRNAs. Non-TSWV leader sequences are shown in italics. The donor residue that is assumed to basepair with the viral template is represented in bold. Sequences underlined represent extra inserted residues.

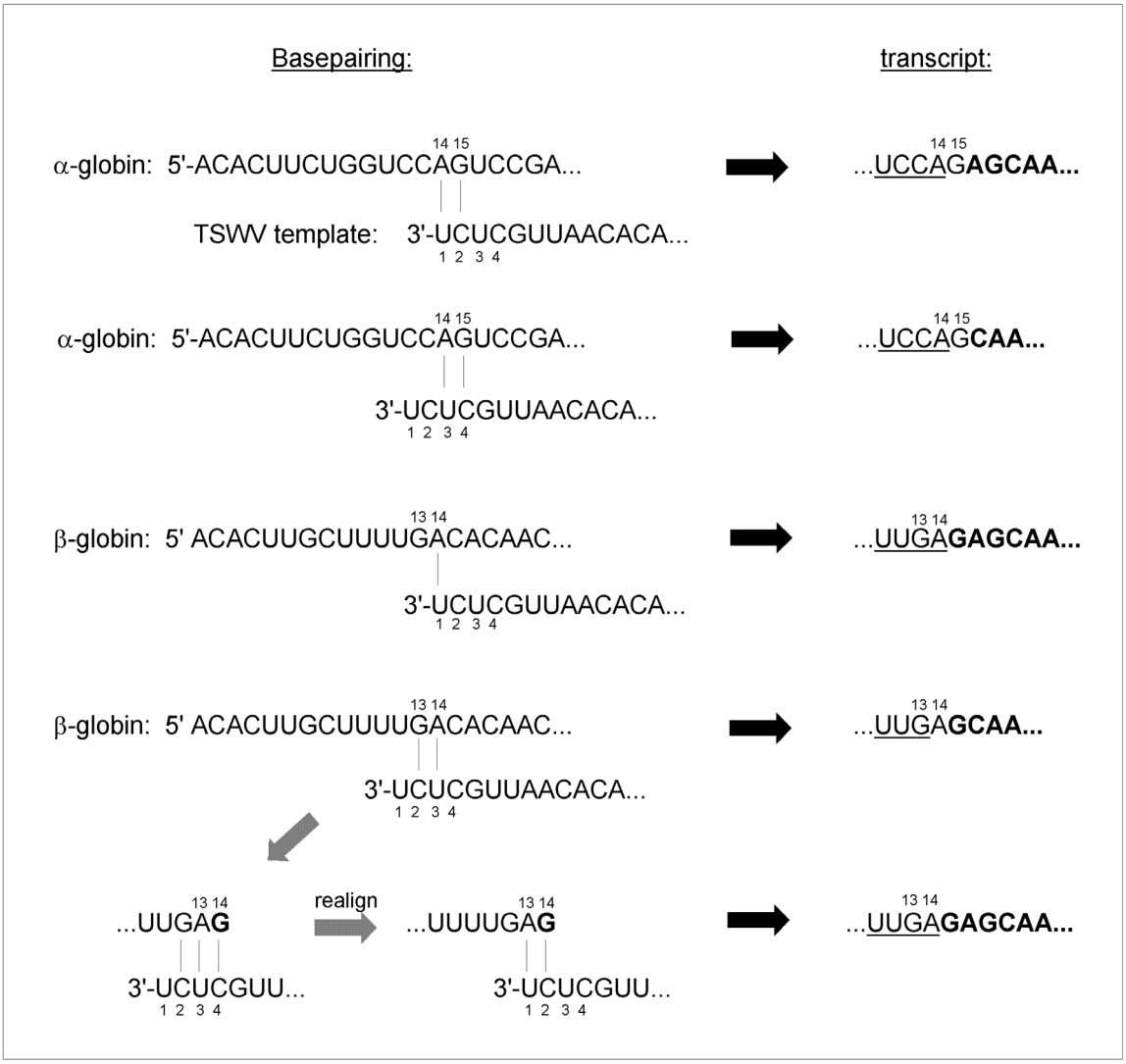

Figure 5: Possible basepairing interactions between globin mRNA cap donors and the TSWV template and the transcript sequences obtained.
Leader sequence derived from the cap donor is underlined, the nucleotides in bold represent viral sequence. Striped arrows indicate a possible prime-and-realign mechanism.

Exogenously added capped RNAs are accepted as cap donors

To investigate whether the process of cap snatching *in vitro* faithfully resembles that *in vivo*, AMV RNA was tested as exogenous cap donor *in vitro*. RNA products synthesised *in vitro* in the presence of an added mixture of AMV RNA3 and 4 (kindly provided by Prof. J. Bol) were reverse transcribed with a primer for the TSWV N gene, followed by PCR using primers matching the 5' first 11 nt of AMV RNA3 and 4, respectively, in combination with a nested internal primer for the N gene. Products of expected size were cloned and sequenced, and indeed were shown to consist of the AMV RNA3 and RNA4 5' terminal sequences preceding the N gene transcript (Table 1). The cleavage sites as

observed from several independent experiments (Table 1) reflected those found for *in vivo* cap snatching of AMV RNA3 and 4 (Duijsings *et al*., 1999).

Since exogenous AMV RNA was shown to be accepted as cap donor for *in vitro* transcription initiation, the absence of transcription in RdRp assays without RRL was verified. To this end, the RT-PCR reactions to detect AMV4-primed N mRNA were performed on RNA synthesised *in vitro* in the presence of exogenous AMV RNA4 but in the absence of RRL. No PCR products were obtained (data not shown), confirming that viral transcription *in vitro* required the presence of RRL.

The potential of the *in vitro* transcription assay as a tool to study the process of cap snatching was further investigated. To this end, capped T7 transcripts of wild type AMV RNA3 or mutant RNA3-C17G were provided as cap donor in a TSWV *in vitro* transcription reaction. RT-PCR and cloning analysis demonstrated that both transcripts were indeed used as cap donor (Table 1). Wild-type AMV3 transcript, like purified AMV RNA3, consistently showed basepairing to the ultimate template residue (Table 1). However, the C17G mutant also showed internal double basepairing analogous to the situation described for the β-globin cap donor (see previous paragraph). These results indicate that the *in vitro* transcription assay could be used as a reliable system to further study the process of cap snatching by testing mutable cap donors.

Re-snatching of TSWV mRNA

The possibility that the viral transcription complex was capable of re-using (*de novo* synthesised) viral mRNAs as cap donors was examined. To this end, capped T7 transcripts consisting of the AMV3 leader sequence fused to the N-gene transcript sequence were synthesised, and tested as cap donor. It was anticipated that if viral mRNAs were re-used as cap donors *in vitro*, the AMV3 leader sequence would be cleaved from the AMV3-N gene transcript and used to prime transcription of, for example, the NSs gene.

RT-PCR analyses of RNA extracted from *in vitro* reactions in which capped AMV3-N gene transcripts were added indeed revealed the synthesis of NSs transcripts preceded by the AMV3 leader sequence, while control reactions using heat-inactivated virus yielded no RT-PCR products (data not shown). Re-snatching of viral mRNAs was confirmed by subsequent cloning and sequence analysis (Table 2). Two of the retrieved sequences confirmed that basepairing took place as expected at A18 of the donor, followed by cleavage and elongation of the mRNA. However, one of the sequences resulted from basepairing at A12, and incorporation of an extra G residue.

Table 2: 5'-terminal sequences of viral NSs gene transcripts synthesised *in vitro* in the presence of a capped AMV3 - N gene transcript as potential cap donor.

Cap donor	5'-terminal NSs mRNA sequence obtained	Number of clones
AMV3-N transcript	5′ *GUAUUAAUACCAUUUUC***A** GAGCAA...	2/3
	5′ *GUAUUAAUACC* G GAGAGCAA...	1/3

Sequence results showing *in vitro* synthesised TSWV NSs mRNA 5' leader sequences derived from a capped T7 transcript of AMV3-N mRNAs. AMV3 leader sequences are shown in italics. The donor residue that is assumed to basepair with the viral template is represented in bold. The underlined residue is extra inserted sequence.

In vitro *RdRp activity of purified cytoplasmic RNPs*

To investigate the RdRp activity of purified cytoplasmic RNPs, *in vitro* replication and transcription assays were performed comparing the activity of cytoplasmic RNPs and virions. In the absence of RRL, purified virus particles synthesised the 3kb product representing S RNA (Fig. 6A, lane 2). In contrast, RNPs demonstrated a much higher rate of RNA synthesis and consistently were observed to generate both a 3 kb and a 5 kb RNA product (Fig. 6A, lane 3), and occasionally even a 9 kb product (Fig. 6B, lane 2). The 3 kb product comigrated with the S RNA as determined by Northern hybridisation (results not shown). Since this RNA product profile is similar to the profile of purified TSWV RNA (Fig. 1, lane 1), the three discrete products most likely represented replication products of the three genomic RNA segments.

In the presence of RRL, RNPs synthesised hardly any RNA (Fig. 6B, lane 3), in contrast to purified virions. When these RNA products were further analysed by RT-PCR for the presence of globin leader sequences, the reaction with virions, as expected, resulted in a very clear PCR product indicating cap snatching of globin mRNAs (Fig. 6C, lane 2), whereas only a faint band was observed for the reaction products of RNPs (Fig. 6C, lane 4). These data suggested that cytoplasmic RNPs may possess some transcriptional activity, but only at a very low level compared to virions.

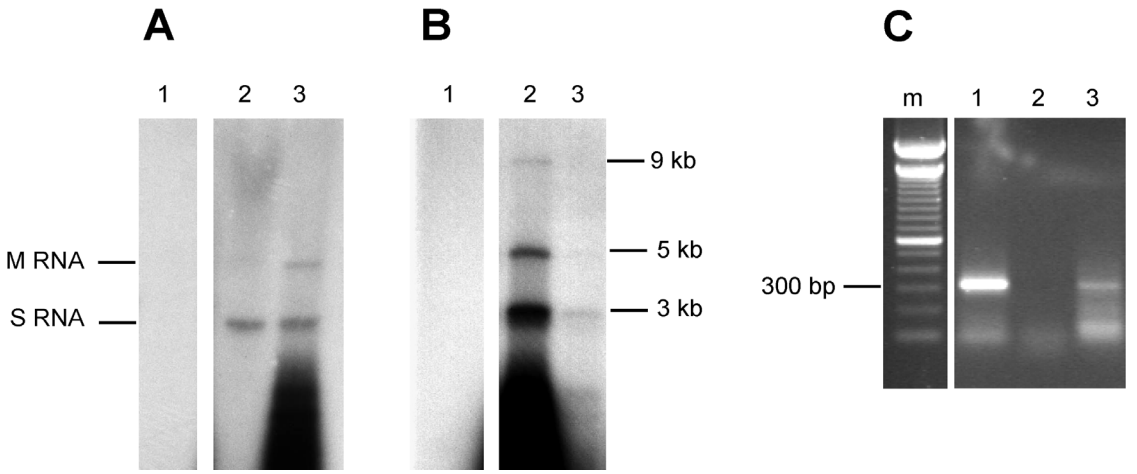

Figure 6: *In vitro* RNA synthesis directed by TSWV nucleocapsids in the absence or presence of RRL.

TSWV RNA synthesised *de novo* in the presence of ^{32}P-CTP was resolved on a 1.5% agarose gel and subsequently blotted to Hybond-N. Panel A, Comparison of transcriptional activity between TSWV virions and cytoplasmic nucleocapsids in the absence of RRL. Lane 1, heat inactivated virus; lane 2, RNA synthesised *in vitro* by purified virions; lane 3, RNA synthesised *in vitro* by purified cytoplasmic nucleocapsids. Panel B, comparison of transcriptional and replicational activity of purified cytoplasmic nucleocapsids. Lane 1, heat inactivated nucleocapsids; lane 2, RNA synthesised *in vitro* in the absence of RRL; lane 3, RNA synthesised *in vitro* in the presence of RRL. Panel C, RT-PCR analysis of *in vitro* synthesised TSWV RNA. RNA synthesised *in vitro* in the presence of RRL was reverse transcribed using internal primers for the TSWV N gene, followed by PCR using an internal primer for the N gene in combination with a primer specific for the 5' 11 nt of the α-globin mRNA. Lane 1, active virus; lane 2, heat inactivated virus; lane3, nucleocapsids. Lane m: 100 bp size marker.

DISCUSSION

The results presented in this chapter demonstrated that purified TSWV particles are capable of supporting transcription and replication, as shown by the *de novo* synthesis of genomic and subgenomic length RNA molecules and the occurrence of cap snatching. Transcription could only be observed in the presence of RRL. Our findings indicate that reaction conditions initially used by Adkins *et al.* (1995) and Van Poelwijk (1996) to study TSWV RdRp activity *in vitro* support only replication. Under these conditions, only genomic length RNA molecules were synthesised, whereas subgenomic length RNA molecules remained (visually) absent from autoradiographic analyses of radiolabelled RNA molecules. Additional Northern blot hybridisation and RT-PCR cloning analyses confirmed these observations.

Not only purified virus particles but also purified cytoplasmic RNPs were demonstrated to possess *in vitro* RNA synthetic activity, albeit somewhat different from virions. Whereas virions show limited replicational activity but high transcriptional

activity, the situation for cytoplasmic RNPs is reversed, i.e. they are hardly transcriptionally active but highly active in replication. It would therefore seem that cytoplasmic RNPs and RNPs inside virus particles exist in a different mode of RNA synthetic activity. This may be understandable in view of the viral life cycle. When virions enter a cell, their first process to support is transcription in order to provide all necessary viral proteins for reproduction, while RNPs found in the cytoplasm may in majority be active in copying the viral genome for viral reproduction. This difference in mode of RdRp activity may be the result of some associated factor.

The switch between transcription and replication may be constituted by the nucleoprotein, similar to *Influenza A virus*, for example, for which *in vitro* synthesis of full-length vRNA was found to depend on a pool of free soluble nucleoprotein (NP) (Shapiro & Krug, 1988). The low replicational activity of purified TSWV virions observed here may thus be explained by the absence of a pool of free soluble N protein. The purified cytoplasmic RNPs, however, are highly active in replication, while the purification method would not seem to allow co-purification of free soluble N protein. Studies with *Influenza virus* NP purified from virions demonstrated that, when incubated at 4ºC in the absence of (viral) RNA, this protein forms multimers which are indistinguishable from viral RNPs in electron micrographs (Ruigrok & Baudin, 1995). For TSWV, RNP-like aggregates of N protein have been observed when the protein was expressed in the baculovirus system (Kormelink, unpublished results), and it cannot be excluded that these structures are also formed during a natural infection cycle. If this would be the case, such RNP-like N multimers could be co-purified with viral RNPs and may account for the high rate of replication observed with purified cytoplasmic RNPs.

In vitro transcription in the presence of RRL has also been reported for *Germiston virus* and *La Crosse virus* (Vialat *et al.*, 1992; Bellocq & Kolakofsky, 1987; Bellocq *et al.*, 1987), animal-infecting members of the *Bunyaviridae*. For these viruses, the translational dependence of transcription was further investigated with the use of translation inhibitors, and these analyses showed that transcription did not require viral protein synthesis. Instead, the stimulating effect was explained by stabilisation of the nascent transcript by scanning ribosomes, inhibiting basepairing interactions of the nascent strand, presumably with its template, and thereby preventing premature termination of transcription that normally took place in the absence of RRL. This was further confirmed by assays in the absence of RRL, but with GTP replaced by ITP (inosine triphosphate), which weakens RNA-RNA interactions. This abolished premature termination at the typical termination sites that were commonly observed in the absence of RRL (Bellocq & Kolakofsky, 1987). In the studies reported here for TSWV, the translational dependence of transcription was not further investigated, but a similar explanation for such closely related viruses is likely. In contrast, *in vitro* transcription of

the tenuivirus *Rice hoja blanca* (RHBV), also an ambisense RNA plant virus, was found to be independent of translation (Nguyen *et al*., 1997). Apparently, processes like transcription and replication seem more conserved within families than between plant-infecting ambisense RNA viruses.

The occurrence of transcription in the presence of RRL was not only supported by Northern blot analyses, but also by cloning of molecules harbouring non-TSWV leader sequences from products of an *in vitro* transcription reaction. Non-viral leader sequences present at the 5'-end of TSWV RNA molecules are the result of cap snatching, a process by which an endonuclease activity, encompassed by the viral polymerase, cleaves off capped leader sequences of host mRNAs to prime transcription on the viral genome. Recent *in vivo* studies have shown that AMV RNAs can be utilised as cap donor (Duijsings *et al*., 1999), and that the 5'-leader sequence of the AMV RNA requires the presence of a residue at approximately 16 nt from the 5'-end that allows basepairing to the ultimate or penultimate residue of the TSWV RNA template (Duijsings *et al.*, 2001). Hence, the presence of AMV leader sequences at the 5'-end of N and NSs RNA molecules cloned from *in vitro* transcription assays to which AMV RNA was supplied as cap donor, indicates that these molecules result from genuine viral RdRp-governed *de novo* transcription. Sequence data, moreover, showed that the endonuclease cleavage of AMV RNA3 and 4 leaders *in vitro* (Table 1) yields similar cleavage profiles as obtained from *in vivo* studies (Duijsings *et al.*, 1999 and 2001).

The appearance of similar RNA profiles in the absence or presence of additional AMV RNA as cap donor was not surprising, as the RRL, although micrococcal nuclease treated, likely still contained sufficient amounts of (fragmented) endogenous cap donors. Previously, 5' RACE cloning of *Germiston virus* mRNAs synthesised *in vitro* in the presence of untreated RRL, revealed 5' leader sequences derived from α- and β-globin mRNAs (Vialat *et al*., 1992). When primers for these leader sequences were used in RT-PCR analyses of TSWV mRNAs synthesised *in vitro*, cap snatching of both globin mRNAs was indeed demonstrated.

The results obtained with α- and β-globin mRNAs and with a mutant AMV3 (C17G) RNA as cap donor, indicate that the presence of two basepairing residues does not exclude these RNAs from being used as cap donor. However, the effect of two basepairing residues in a leader on (the efficiency of) its use as cap donor and, more importantly, on the site of endonuclease cleavage still remains to be investigated. In the case of single basepairing, cleavage is expected to occur after the basepairing A residue (Duijsings *et al*., 2001). In the case of double basepairing, two possibilities exist: cleavage after the first or after the second basepairing residue. Although it may be tempting to assume that cleavage will occur after the most downstream positioned (second) basepairing residue, this scenario does not seem likely for the observed re-

snatching of viral mRNAs, which can have up to 100% complementarity. The observation of re-snatching *in vitro* favours the hypothesis that cleavage takes place after one of the first basepairing residues. This would be analogous to and in agreement with observations for *Influenza A virus*, where elongation of the transcription-initiation complex has been demonstrated to occur from the second, third and fourth viral residue, although with decreasing efficiencies, but not from the fifth viral residue (Honda *et al.*, 1986). It will therefore be important to determine the actual cleavage site of multiple-basepairing cap donors, in order to elucidate further the mechanism of cap snatching.

Interestingly, basepairing of the β-globin mRNA to the TSWV template was found to occur exclusively on A14 (with or without concurrent basepairing of G13), whereas A16, which was previously found to be the optimal basepairing nucleotide, was not used (Table 1). It is tempting to conclude that the presence of G13 influences the site of basepairing, leading to internal double basepairing of G13A14. However, half of the clones obtained seemed to indicate single basepairing of A14 to viral residue U1 (Table 1 and Fig. 5). A possible alternative explanation for this observation is that the presence of the G13 residue does indeed influence the site of basepairing, resulting in exclusively internal (double) basepairing which, in combination with a "prime-and-realign" mechanism, would result in transcripts indiscernible from mRNAs resulting from single basepairing of A14-U1 (Fig. 5). Such a prime-and-realign mechanism has previously been described for *Hantaan virus* transcription initiation (Garcin *et al.*, 1995).

It is unlikely that re-snatching of viral mRNAs would also occur *in vivo*. Since there is no sequence restriction to protect cap snatching from TSWV mRNAs, the virus may have some protection mechanism to avoid cleavage of functional viral mRNAs. This may require the presence of an additional viral protein not present in the virion, and for this function NSs could be a potential candidate.

MATERIALS & METHODS

Virus purification
TSWV isolate BR-01 was purified from systemically infected leaves of *Nicotiana rustica* as described by Kikkert *et al.* (1997), with the modification that the resuspension buffer consisted of 0.01 M phosphate, pH 7.0 and 0.01 M Na_2SO_3.

Purification of cytoplasmic RNPs
Nucleocapsids of TSWV isolate BR-01 were purified from systemically infected leaves of *Nicotiana rustica* essentially as described by De Avila *et al.* (1990), with the modification that the extraction buffer contained of 0.1 M Tris-HCl instead of 0.01 M. After centrifugation on the sucrose cushion the nucleocapsids were resuspended in sterile H_2O, aliquoted and stored at -80ºC.

***In vitro* TSWV RdRp activity assay**
In vitro RdRp assays were performed using 10-15 µg of purified TSWV or nucleocapsids in a final volume of 25 µl. Assays without RRL were done as described by Adkins *et al*. (1995) with slight modifications, and contained 20 mM HEPES pH7.4, 0.5 mM MgAc, 5 mM $MnCl_2$, 2.5 mM DTT, 1 mM of each NTP, 0.1% NP-40 and 0.8 U/µl RNasin. Assays in the presence of RRL were performed according to Nguyen *et al*. (1997). These assays contained 4 mM MgAc, 1 mM of each NTP, 0.1% NP-40, 0.8 U/µl RNasin, 60 ng/µl tRNA, and were supplemented by the AP-Biotech rabbit reticulocyte lysate system according to the manufacturer's procedures. *In vitro* RdRp assays were incubated at 30^{o}C for 1.5 h. For visualisation of the RNA products, 2 µl of [α-^{32}P] CTP (800 Ci/mmol) was added instead of CTP. When indicated, AMV RNA (75% RNA4, 25% RNA3; J. F. Bol, personal communication) was added to a final concentration of 0.1 µg/µl. RNA products were phenol-chloroform extracted, ethanol precipitated and resuspended in sterile ddH_2O. Radiolabelled RNA products were resolved by electrophoresis on a 1.5% agarose gel, followed by downward Northern blotting in 10x SSC for 1.5 h. The RNA was cross-linked to the filter by exposure to UV-light for 10 min prior to autoradiography.

Riboprobe synthesis and hybridisation
Synthesis of strand-specific riboprobes and hybridisation analyses were performed as described by Kormelink *et al*. (1992b). For detection of v-sense (sub)genomic S RNA molecules (NSs mRNA and S RNA) vc-sense probe S1 was used, and for vc-sense (sub)genomic S RNA molecules (N mRNA and S RNA) v-sense probe S2 was used. Hybridisation of Northern blots was performed only after full decay of the radioactive signal from the *in vitro* synthesised ^{32}P-labelled RNA present on these blots.

RT-PCR and cloning analyses
To amplify *in vitro* synthesised N and NSs gene transcripts containing 5' terminal sequences from AMV RNAs 3 or 4, RNA extracted from RdRp assays was reverse transcribed and PCR amplified as described by Duijsings et al. (1999). For RT and PCR amplification of the NSm gene transcript, primer Z23 (5'-CCCTTCTGACTCTGTGATC-3', complementary to nt 322-339 of the TSWV M RNA) was used, and for the GP transcript, primer J16 (5'-GTTGAATCGATGCAG-3', identical to nt 4548-4562 of the TSWV M RNA) was used. To demonstrate the presence of globin leader sequences, primers p141 (5'-CCCGGATCCACACUUCUGGU-3': the 5' 11 nt of the α-globin mRNA preceded by a *Bam*HI site) and p142 (5'-CCCGGATCCACACUUGCUUU-3': the 5' 11 nt of the β-globin mRNA preceded by a *Bam*HI site) were used. PCR products obtained were subsequently cloned and sequenced.

Construction and T7-directed *in vitro* transcription of (mutant) AMV RNA3 constructs
AMV RNA3 transcripts were obtained by run-off transcription with T7 RNA polymerase of pXO32NcoP3, containing a full-length cDNA clone of AMV RNA3 (Neeleman *et al*., 1993). Transcription was performed in the presence of cap-analogue m7G(5')ppp(5')G according to the manufacturer's procedures (Ambion

mMESSAGE mMACHINE). Prior to transcription, the DNA template was linearised with *Ava*II, finally resulting in run-off transcripts of 472 nt. Construction of AMV RNA 3 mutant C17G was done as described in detail for other RNA3 mutants by Duijsings *et al.* (2001). AMV3-N gene constructs were obtained as described in the previous section. To allow *in vitro* synthesis of capped AMV RNA 3 mutant C17G and AMV3-N transcripts, T7 promoter sequences were introduced upstream the AMV RNA leader sequence by PCR amplification.

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REFERENCES

Adkins, S., Quadt, R., Choi, T. J., Ahlquist, P. and German, T. (1995). An RNA-dependent RNA polymerase activity associated with virions of *Tomato spotted wilt virus*, a plant- and insect-infecting bunyavirus. *Virology* **207,** 308-311

Beaton, A. R. and Krug, R. M. (1986). Transcription antitermination during *Influenza* viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proc Natl Acad Sci USA* **83,** 6282-6286

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: a possible mechanism. *J Virol* **61,** 3960-3967

Bellocq, C., Ramaswamy, R., Patterson, J. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: *In vitro* studies. *J Virol* **61,** 87-95

Chung, T. D., Cianci, C., Hagen, M., Terry, B., Matthews, J. T., Krystal, M. and Colonno, R. J. (1994). Biochemical studies on capped RNA primers identify a class of oligonucleotide inhibitors of the *Influenza virus* RNA polymerase. *Proc Natl Acad Sci USA* **91,** 2372-2376

De Avila, A. C., Huguenot, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W. and Peters, D. (1990). Serological differentiation of 20 isolates of *Tomato spotted wilt virus*. *J Gen Vir* **71**, 2801-2807

De Haan, P., Kormelink, R., de Oliveira Resende, R., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). *Tomato spotted wilt virus* L RNA encodes a putative RNA polymerase. *J Gen Virol* **72,** 2207-2216

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1989). Molecular cloning and terminal sequence determination of the S and M RNAs of *Tomato spotted wilt virus*. *J Gen Virol* **70,** 3469-3473

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1990). The S RNA segment of *Tomato spotted wilt virus* has an ambisense character. *J Gen Virol* **71,** 1001-1007

Di Bonito, P., L. Nicoletti, S. Mochi, L. Accardi, A. Marchi and C. Giorgi. (1999). Immunological characterization of *Toscana virus* proteins. *Arch Virol* **144,** 1947-1960

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of Nicotiana benthamiana. *J Virol* **73,** 5172-5175

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20,** 1-8

Galarza, J. M., Peng, Q. G., Shi, L. C. and Summers, D. F. (1996). *Influenza A* virus RNA-dependent RNA polymerase: Analysis of RNA synthesis *in vitro*. *J Virol* **70,** 2360-2368

Garcin, D., Lezzi, M., Dobbs, M., Elliot, R. M., Schmaljohn, C., Kang, C. Y. and Kolakofsky, D. (1995). The 5' ends of *Hantaan virus* (*Bunyaviridae*) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. *J Virol* **69**, 5754-5762

Honda, A., Mizumoto, K. and Ishihama, A. (1986). RNA polymerase of *Influenza virus*: dinucleotide-primed initiation of transcription at specific positions on viral RNA. *J Biol Chem* **261**, 5987-5991

Kikkert, M., Van Poelwijk, F., Storms, M., Bloksma, H., Karsies, W., Kormelink, R. and Goldbach, R. (1997). A protoplast system for studying *Tomato spotted wilt virus* infection. *J Gen Virol* **78**, 1755-1763

Kormelink, R., De Haan, P., Meurs, C., Peters, D. and Goldbach, R. (1992a). The nucleotide sequence of the M RNA segment of *Tomato spotted wilt virus*, a bunyavirus with two ambisense RNA segments. *J Gen Virol* **73,** 2795-2804

Kormelink, R., De Haan, P., Peters, D. and Goldbach, R. (1992b). Viral RNA synthesis in *Tomato spotted wilt virus*-infected *Nicotiana rustica* plants. *J Gen Virol* **73,** 687-693

Kormelink, R., Kitajima, E. W., De Haan, P., Zuidema, D., Peters, D. and Goldbach, R. (1991). The nonstructural protein (NSs) encoded by the ambisense S RNA segment of *Tomato spotted wilt virus* is associated with fibrous structures in infected plant cells. *Virology* **181,** 459-468

Kormelink, R., Van Poelwijk, F., Peters, D. and Goldbach, R. (1992c). Non-viral heterogeneous sequences at the 5' ends of *Tomato spotted wilt virus* mRNAs. *J Gen Virol* **73,** 2125-2128

Kormelink, R., Storms, M., van Lent, J., Peters, D. and Goldbach, R. (1994). Expression and subcellular location of the NSm protein of *Tomato spotted wilt virus* (TSWV), a putative viral movement protein. *Virology* **200,** 56-65

Neeleman, L., van der Vossen, E.A.G. and Bol, J.F. (1993). Infection of tobacco with *Alfalfa mosaic virus* cDNAs sheds light on the early function of the coat protein. *Virology* **196**, 883-887

Nguyen, M., Ramirez, B. C., Goldbach, R. and Haenni, A. L. (1997). Characterization of the *in vitro* activity of the RNA-dependent RNA polymerase associated with the ribonucleoproteins of *Rice Hoja Blanca tenuivirus*. *J Virol* **71,** 2621-2627

Plotch, S. J., Bouloy, M., Ulmanen, I. and Krug, R. M. (1981). A unique cap (m^7GpppX_m) – dependent *Influenza* virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23,** 847-858

Ruigrok, R. W. H. and Baudin, F. (1995). Structure of *Influenza virus* ribonucleoprotein particles: II. Purified RNA-free *Influenza virus* ribonucleoprotein forms structures that are indistinguishable from the intact *Influenza virus* ribonucleoprotein particles. *J Gen Virol* **76** (4) 1009-1014

Shapiro, G. I. and Krug, R. M. (1988). *Influenza virus* RNA replication *in vitro*: Synthesis of viral template RNAs and virion RNAs in the absence of an added primer. *J Virol* **62** (7) 2285-2290

Van Poelwijk, F. (1996). *On the expression strategy of the tospoviral genome*. Thesis Wageningen University, The Netherlands

Van Poelwijk, F., Kolkman, J. and Goldbach, R. (1996). Sequence analysis of the 5' ends of *Tomato spotted wilt virus* N mRNAs. *Arch Virol* **141,** 177-184

Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. and Wickner, R. B. (2000). Virus Taxonomy, Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA. pp 1-1162

Vialat, P. and Bouloy, M. (1992). *Germiston virus* transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66,** 685-693

CHAPTER 3

Tomato spotted wilt virus transcription *in vitro* is independent of translation

SUMMARY

Ongoing TSWV transcription *in vitro* was previously demonstrated to require the presence of reticulocyte lysate. This dependence was further investigated by testing the occurrence of transcription in the presence of two translation inhibitors: edeine, which still allows scanning of nascent mRNAs by the 40S ribosomal subunit, and cycloheximide, which completely blocks translation including ribosome scanning. Neither of these inhibitors blocked TSWV transcription initiation or elongation *in vitro*, as demonstrated by *de novo* synthesised viral mRNAs with globin mRNA-derived leader sequences, suggesting that TSWV transcription *in vitro* requires the presence of (a component within) reticulocyte lysate rather than a viral protein resulting from translation.

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INTRODUCTION

Transcription of segmented negative strand RNA viruses is initiated by a mechanism known as cap snatching, in which the viral polymerase cleaves a host mRNA generally at 10-20 nt from its 5' capped end and uses the resulting leader to prime viral transcription. This also applies to *Tomato spotted wilt virus* (TSWV), the type species of the Tospovirus genus within the *Bunyaviridae*. Previously, *Alfalfa mosaic virus* (AMV) RNAs have been shown to be utilised by TSWV as cap donors during a mixed infection of *Nicotiana benthamiana* (Duijsings *et al.*, 1999). Furthermore, it was demonstrated that suitable cap donors require a single base complementarity to the ultimate or penultimate residue of the TSWV template (Duijsings *et al.*, 2001).

More recently, an *in vitro* assay has been developed in which purified TSWV particles were shown to support either viral transcription or replication, depending on the presence or absence of rabbit reticulocyte lysate (RRL), respectively (Chapter 2; see also Fig. 1A). In the presence of RRL, viral transcription was observed, as demonstrated by *de novo* synthesis of subgenomic RNA molecules (Fig. 1A, lane 3) that hybridised to strand-specific probes for the N and NSs genes and comigrated with these mRNAs as present in total RNA from infected plants (Chapter 2). Moreover, evidence for genuine viral transcription initiation *in vitro* was obtained by RT-PCR cloning of these mRNAs, revealing the presence of non-templated leader sequences at the 5'-ends. These RNA leader sequences were derived either from globin mRNAs present in RRL or from exogenously added AMV RNA.

The requirement for RRL for ongoing viral transcription *in vitro* has previously been reported for *La Crosse* (Bellocq & Kolakofsky, 1987; Bellocq *et al.*, 1987) and *Germiston* (Vialat & Bouloy, 1992) orthobunyaviruses. Although this observation suggested a translational dependence, no actual viral protein products were required. Instead, analyses using translation inhibitors as well as *in vitro* assays using a nucleotide analogue led to the hypothesis that RRL was required for stabilisation of the nascent transcript by scanning ribosomes. The observation that TSWV *in vitro* transcription also requires the presence of RRL (Chapter 2) prompted the question whether the underlying mechanism for this dependence is similar to what has been suggested for *La Crosse* and *Germiston* viruses. To investigate this requirement for RRL, the effect of translation inhibitors on *in vitro* TSWV transcription and transcription initiation, i.e. cap snatching, was analysed.

RESULTS

TSWV transcription* in vitro *is not affected by translation inhibitors

TSWV transcription *in vitro* (i.e. in the presence of RRL) was performed as described previously (Chapter 2) in the absence or presence of edeine and cycloheximide, respectively. The translation inhibitor cycloheximide stalls the ribosomes on the transcript by blocking elongation (peptidyl transferase) (Godchaux *et al*., 1967; Jaye *et al*., 1982). Edeine prevents association of the 60S ribosomal subunit to the 40S subunit but, unlike cycloheximide, still allows scanning of the nascent transcript by the 40S subunit (Kozak & Shatkin, 1978).

When either of these inhibitors was added to the TSWV *in vitro* transcription reaction, no change in the RNA product profile was observed (Fig. 1B, lanes 3 and 6) compared to a profile of the standard reaction (Fig. 1B, lane 2). Increasing concentrations of the inhibitors, even levels far exceeding those reportedly necessary for complete inhibition of protein synthesis (Bellocq *et al*., 1987; Vialat & Bouloy, 1992), did not affect TSWV transcription (Fig. 1B, lanes 4-5 and 7-8). These findings indicated that viral transcription did not depend on RRL for translation of a viral protein, nor for ribosome scanning of the nascent transcript.

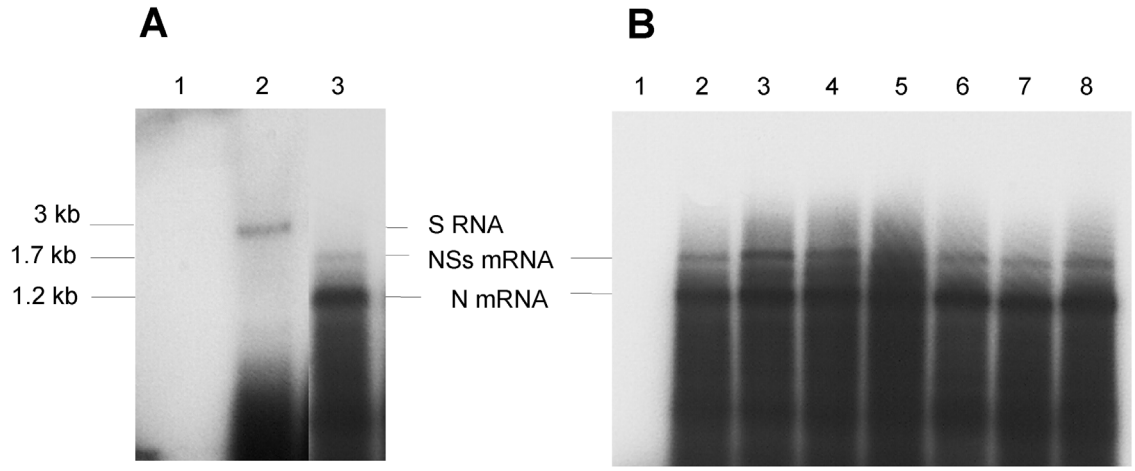

Figure 1: Northern blot of radiolabelled TSWV *in vitro* transcription products.
Panel A, RdRp activity of TSWV in the presence or absence of RRL. Lane 1: negative control reaction with heat inactivated virus; lane 2: RdRp activity in the absence of RRL; lane 3: RdRp activity in the presence of RRL. Panel B, TSWV transcriptional activity in the presence of translation inhibitors. Lane 1, control reaction with heat inactivated virus; lane 2, standard transcription reaction in absence of inhibitors; lanes 3-5, transcription in the presence of cycloheximide at concentrations of 50, 100 and 250 µg/ml, respectively; lanes 6-8, transcription in the presence of edeine at concentrations of 10, 50 and 80 µM, respectively.

No detectable protein synthesis occurs during transcription in the presence of RRL

In order to verify that cycloheximide and edeine were indeed functional in blocking *in vitro* translation, protein synthesis from control RNA templates was analysed by 12% SDS-PAGE. In the presence of translation inhibitors, translation of control RNA was effectively blocked already at the lowest concentration used (Fig. 2B), indicating that cycloheximide and edeine indeed were fully effective in blocking translation.

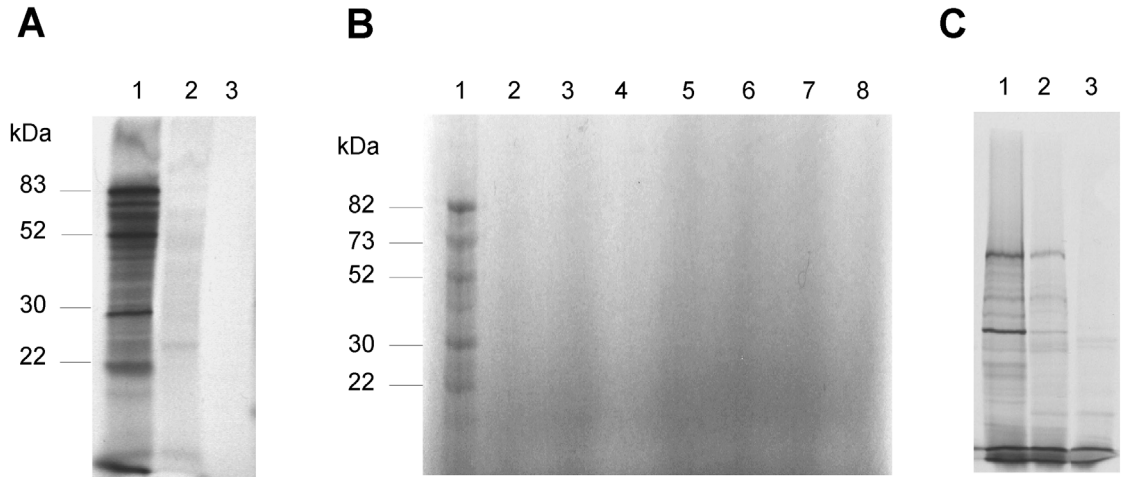

Figure 2: SDS-PAGE of *in vitro* synthesised ^{35}S-labelled products.
Panel A, Lane 1, standard translation of control RNA; lane 2, standard translation of control RNA in the presence of purified TSWV; lane 3, TSWV *in vitro* transcription reaction. Panel B, Standard translations of control RNA in the absence (lane 1) or presence (lanes 2-8) of translation inhibitors. Lanes 2-4, translation in the presence of cycloheximide at concentrations of 50, 100 and 250 µg/ml, respectively; lanes 5-8, translation in the presence of edeine at concentrations of 5, 10, 50 and 80 µM, respectively. Panel C, Translation of control RNA. Lane 1, in the presence of 0.5 mM MgAc and 170m M KAc; lane 2, in the presence of 2.5 mM MgAc and 170 mM KAc; lane 3, in the presence of 0.5 mM MgAc and 70 mM KAc.

In addition, protein synthesis in *in vitro* TSWV transcription reactions was analysed by supplying ^{35}S-labelled methionine to the assay instead of ^{32}P-labelled CTP. Strikingly, although the control RNA was properly translated, no detectable protein synthesis was observed in the ^{35}S-labelled TSWV transcription reaction (Fig. 2A, lanes 1 and 3). The absence of a clear visual protein product from the *in vitro* transcription assay was in part due to the salt conditions applied in this assay, since closer examination showed that these deviated from the conditions recommended for optimal translation (AP-Biotech, manufacturer's recommendations). Whereas TSWV transcription was found to be optimal at 5.2 mM Mg^{2+} and 30 mM K^{+}, translation is regarded optimal at 1.7 mM Mg^{2+} and 130 mM K^{+} (all final concentrations). Higher Mg^{2+} (2.5 mM) or lower K^{+} (70 mM) concentrations severely reduced translation efficiency of the control RNA (Fig. 2C),

indicating that the circumstances used for viral transcription indeed hampered translation. Moreover, it was observed that translation of a control RNA in the presence of purified TSWV was severely reduced (Fig. 2A, lane 2), although not completely abolished. This decrease in translation activity was not due to the addition of Nonidet P-40, a detergent that was added to disrupt the viral membrane, since addition of this component alone had no effect on translation of the control RNA (not shown).

Transcription initiation by cap snatching is not affected by translation inhibitors

To further substantiate the evidence that translation inhibitors did not affect TSWV transcription *in vitro*, viral RNA synthesised *in vitro* in the presence of cycloheximide or edeine was analysed by RT-PCR using a viral N gene-specific primer in combination with a primer corresponding to the 5' leader sequence of α-globin mRNA. Using this approach, viral mRNAs can be specifically amplified and discriminated from (anti)genomic RNA molecules, as previously shown by Duijsings *et al*. (1999, 2001) and in Chapter 2. PCR fragments of expected sizes were obtained (Fig. 3A, lanes 2 and 3), and subsequent cloning and sequence analyses (Fig. 3B) confirmed their identity as N gene transcripts with the α-globin mRNA leader sequence, indicating that TSWV transcription initiation was not affected by inhibition of translation.

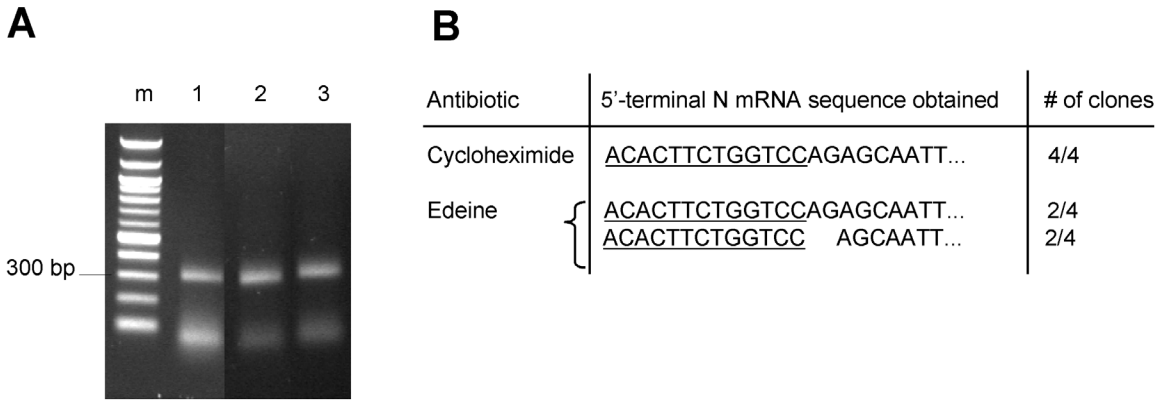

Figure 3: The occurrence of cap snatching in the presence of translation inhibitors.
Panel A, RT-PCR amplification of N-gene transcripts with α-globin leader sequences synthesised in the TSWV *in vitro* transcription assay in the absence of inhibitors (lane 1), in the presence of 100 µg/ml cycloheximide (lane 2), and in the presence of 10 µM edeine (lane 3). Lane m: 100-bp molecular weight marker. Panel B, Sequence data. The PCR products of lanes 2 and 3 of panel A were cloned and sequenced, revealing the presence of α-globin leader sequences (underlined) at the 5'-end of N mRNA transcripts.

DISCUSSION

Stimulation of *in vitro* transcription by addition of RRL has previously been reported for *La Crosse* (Bellocq *et al*., 1987; Bellocq & Kolakofsky, 1987) and *Germiston* (Vialat & Bouloy, 1992) orthobunyaviruses, for which *in vitro* transcription in the absence of RRL always resulted in prematurely terminated transcripts. It was demonstrated that RRL most likely was required for ribosome scanning of nascent transcripts, preventing premature termination as a result of secondary structures, and not for viral protein synthesis. This hypothesis was supported by the observation for *La Crosse virus* that replacing GTP with ITP, a GTP-analogue that is unable to basepair with C-residues, was sufficient to compensate for the absence of RRL. For *Germiston virus* the scanning ribosomes-hypothesis was supported by the observation that ongoing transcription was still detected in the presence of the translation inhibitor edeine, when scanning ribosomal subunits occupy the RNA transcript and so prevent structural folding.

Recently, TSWV *in vitro* transcription was also demonstrated to require the addition of RRL (Chapter 2). The experiments described here demonstrated that addition of translation inhibitors had no effect on either elongation or initiation of transcription. Moreover, whereas the *in vitro* transcription reactions of *La Crosse* and *Germiston* virus are really coupled transcription-translation reactions, no visible protein synthesis could be detected for TSWV *in vitro* transcription. These results seem to indicate that the RRL-dependence of TSWV *in vitro* transcription is similar to that of *Germiston* and *La Crosse* viruses in that no actual viral protein synthesis is required, yet differs from it with respect to exactly what factor of the lysate confers stimulation of transcription.

The only other segmented ambisense RNA plant virus for which an *in vitro* transcription assay was established is the tenuivirus *Rice hoja blanca virus* (Nguyen *et al*., 1997). This virus, although a member of a genus sharing many characteristics with the *Bunyaviridae*, does not share the requirement for RRL for *in vitro* transcription. In the past, cellular factors (e.g. tubulin) have been demonstrated to be required for transcription of negative strand RNA viruses such as *Sendai virus, Vesicular stomatitis virus, Human parainfluenza virus type 3* and *Measles virus* (De *et al.*, 1990; Hill *et al*., 1986; Moyer *et al.*, 1986; Ray & Fujinami, 1987). The identity of the factor required for TSWV transcription remains to be determined.

MATERIALS & METHODS

TSWV *in vitro* transcription assays and RT-PCR and cloning analyses of RNA products were performed as described in Chapter 2. *In vitro* translations were performed using Promega rabbit reticulocyte lysate (RRL) according to the manufacturer's recommendations. The control RNA used was supplied with the RRL kit and is post-translationally autocatalytically cleaved into products of 82, 73, 52, 30 and 22 kDa.

REFERENCES

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: A possible mechanism. *J Virol* **61**, 3960-3967

Bellocq, C., Ramaswamy, R., Patterson, J. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: *In vitro* studies. *J Virol* **61**, 87-95

De, B., Galinski, M. S. and Banerjee, A. K. (1990). Characterization of an *in vitro* system for the synthesis of mRNA from *human parainfluenza virus type 3*. *J Virol* **64**, 1135-1142

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of Nicotiana benthamiana. *J Virol* **73**, 5172-5175

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 1-8

Godchaux, W., Adamson, S.O. and Herbert, E. (1967)**.** Effects of cycloheximide on polyribosome function in reticulocytes. *J Mol Biol* **27,** 57-72

Hill, V. M., Harmon, S. A. and Summers, D. F. (1986). Stimulation of *Vesicular stomatitis virus in vitro* RNA synthesis by microtubule-associated proteins. *Proc Natl Acad Sci USA* **83**, 5410-5413

Jaye, M.C., Godchaux, W. and Lucas-Lenard, J. (1982). Further studies on the inhibition of cellular protein synthesis by *Vesicular stomatitis virus*. *Virology* **116,** 148-162

Kozak, M. and Shatkin, A. J. (1978). Migration of 40S ribosomal subunits on messenger RNA in the presence of edeine. *J Biol Chem* **253,** 6568-6577

Moyer, S. A., Baker, S. C., and Lessard, J.L. (1986). Tubulin: a factor necessary for the synthesis of both *Sendai* and *Vesicular stomatitis virus* RNAs. *Proc Natl Acad Sci USA* **83**, 5405-5409

Nguyen, M., Ramirez, B. C., Goldbach, R. and Haenni, A. L. (1997). Characterization of the *in vitro* activity of the RNA-dependent RNA Polymerase associated with the ribonucleoproteins of *Rice Hoja Blanca tenuivirus*. *J Virol* **71**, 2621-2627

Ray, J. and Fujinami, R. S. (1987). Characterization of *in vitro* transcriptional products of *Measles virus*. *J Virol* **61**, 3381-3387

Vialat, P. and Bouloy, M. (1992). *Germiston virus* transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66**, 685-693

CHAPTER 4

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

SUMMARY

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.

A slightly modified version of this chapter has been submitted as: van Knippenberg, I., Goldbach, R. and Kormelink, R. *Tomato spotted wilt virus* S-segment mRNAs have overlapping 3'-ends containing a predicted stem-loop structure and conserved sequence motif.

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 & 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 & Bishop, 1987), while termination of UUKV mRNAs is mapped to the 3'-end of the IR (Simons & 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 & 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 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.

Figure 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, 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 (S RNA nt 1705-1713, M RNA nt 1151-1161) and 103 bases beyond the L gene stop codon (nt 8765-8772).

Recently, an *in vitro* transcription assay has been established in which purified TSWV particles support transcription in the presence of rabbit reticulocyte lysate (Chapter 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.

RESULTS

RT-PCR amplification of N and NSs mRNAs

To roughly map the 3'-ends of the TSWV N and NSs mRNAs, transcripts synthesised in the TSWV *in vitro* transcription assay (Chapter 2) were RT-PCR amplified using a primer for the α-globin-derived 5'-leader sequence (p141) in combination with a primer located in the IR. Two complementary sets of primers (IR1-IR8 and IR9-IR16; Table 1) were designed for this purpose, spanning the intergenic region (IR) beyond the N and NSs ORFs, respectively (Figs. 2A and 3A). RT-PCR reactions were expected to yield a series of products of increasing size up to the last primer to specifically anneal to the 3'-end of the mRNA.

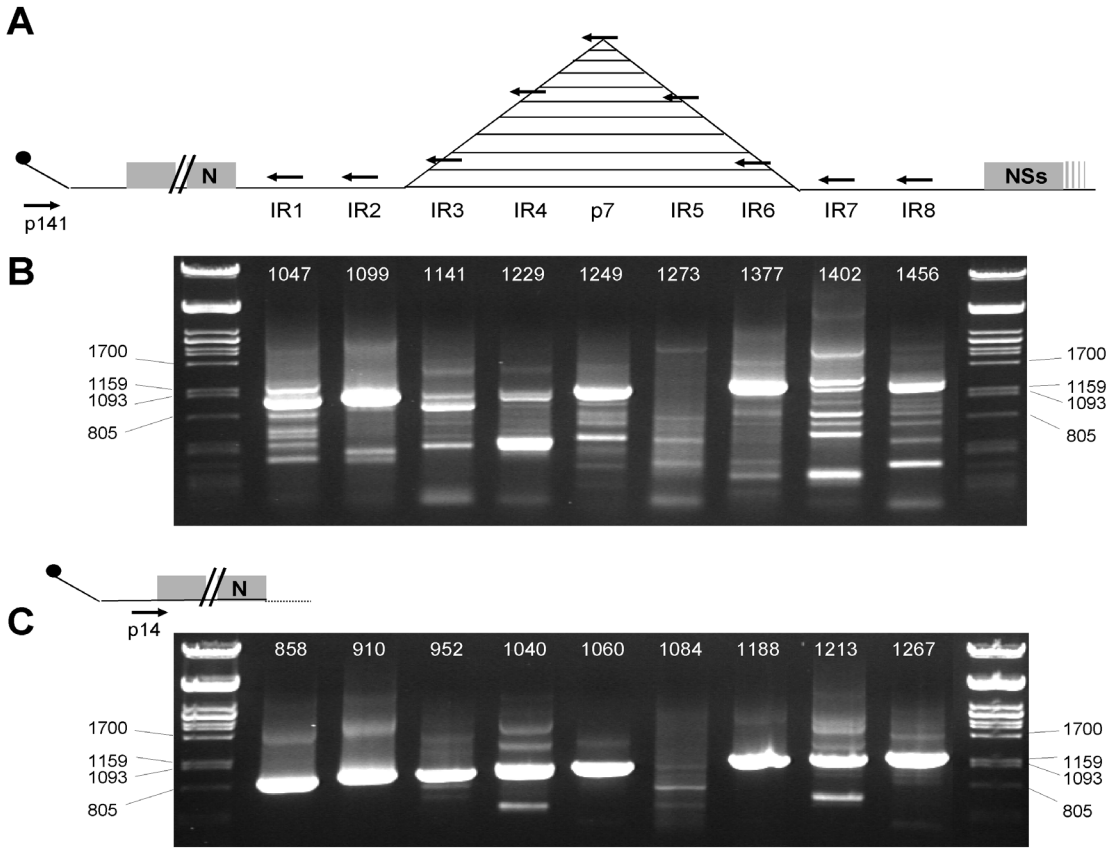

Figure 2: 3'-end mapping of *in vitro* synthesised TSWV N mRNA.
Panel A, Diagram of the intergenic region of the S vcRNA, with arrows indicating the location of 3'-end primers used to amplify N mRNAs. The pyramid-like feature represents the intergenic hairpin structure. Panel B, Agarose gel electrophoresis of RT-PCR products; the fragments in each lane are amplified by the primer indicated above the lane in panel A in combination with a primer for the cap snatch-derived α-globin leader. The expected sizes of the products are indicated at the top of the gel. Panel C, Positive control RT-PCR products obtained on genomic RNA with the same 3'-end primers as in panel B, but now in combination with an upstream (internal) N gene primer (p14, Table 1).

Control RT-PCR analyses for the N-gene sequence using a primer at the N start codon (p14, Table 1) in combination with the IR-primers, to amplify the genomic RNA sequence, showed products of increasing size (Fig. 2C), indicating the IR-primers were functional with the exception of IR5. Likewise, control reactions for the NSs-gene sequence (Fig. 3C; primer p18, Table 1) demonstrated the IR-primers for NSs were functional.

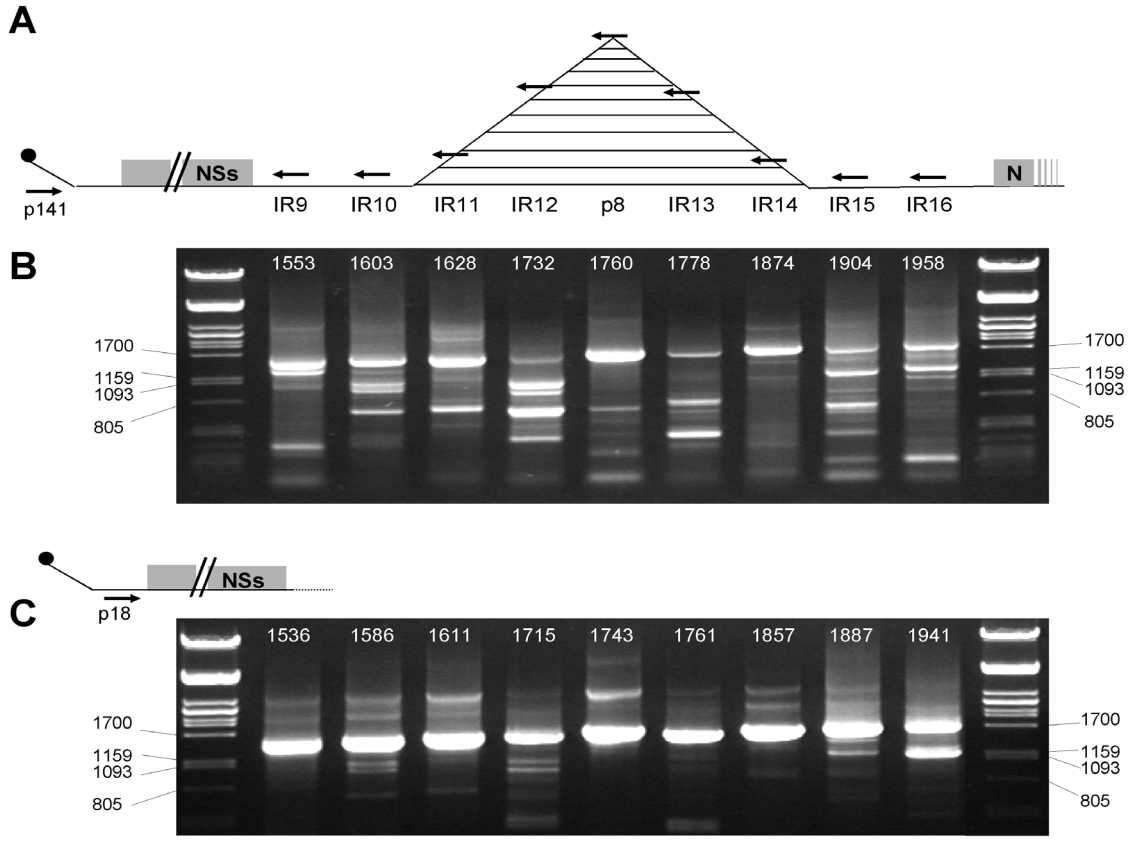

Figure 3: 3'-end mapping of *in vitro* synthesised TSWV NSs mRNA.
Panel A, Diagram of the intergenic region of the S vRNA, with arrows indicating the location of 3'-end primers used to amplify NSs mRNAs. The pyramid-like feature represents the intergenic hairpin structure. Panel B, Agarose gel electrophoresis of RT-PCR products; the fragments in each lane are amplified by the primer indicated above the lane in panel A in combination with a primer for the cap snatch-derived α-globin leader. The expected sizes of the products are indicated at the top of the gel. Panel C, Positive control RT-PCR products obtained on genomic RNA with the same 3'-end primers as in panel B, but now in combination with an upstream primer corresponding to the viral 5'-end sequence (p18, Table 1).

When N-gene transcripts were amplified using the α-globin primer (p141) in combination with primers IR1-8 (Fig. 2B), a great diversity of products was observed for most of the reactions, making the results more ambiguous. However, PCR fragments of the expected sizes were obtained and those of the reactions with primers IR1, IR2, p7,

IR6, and IR7 were cloned for sequence analysis. Cloning and sequencing of the band of the reaction with primer IR8 failed at several attempts. The products of primers IR1, IR2, p7 and IR6 were confirmed to be N-gene mRNAs with the α-globin-derived leader. The IR7 clone (the upper band of the doublet of PCR products), however, did not contain the N-gene sequence but appeared to result from non-specific amplification of glycoprotein sequence. These results, although not allowing precise mapping of transcription termination, indicate that N mRNAs at their 3'-end contain the intergenic sequence predicted to form a stable hairpin structure.

When primers IR9-16 were used in combination with a primer for the α-globin leader sequence (p141; Fig. 3B) to amplify NSs mRNAs, products of expected increasing size were observed in addition to non-specific products. The bands of expected size of the reactions with primers p8 and IR14 were cloned and sequenced, revealing the expected α-globin-primed NSs mRNA sequences. Cloning of the bands of IR15 and IR16 failed at several attempts. Although these results, like those for the N-gene transcript, do not allow precise mapping of the transcription termination site, they indicate that transcription termination of the NSs mRNA occurs at least beyond the position of primer IR14. Thus, these results point to a similar termination strategy for both v and vc-sense transcripts of the S RNA, yielding mRNAs that have a predicted hairpin structure at their 3'-end.

Size estimation by Northern blot hybridisation analysis

To obtain further evidence that termination of the N mRNA occurs at the 3'-end of the intergenic hairpin, a size comparison was performed by Northern analysis. To this end, synthetic capped T7 transcripts N-IR2 an N-IR7 were synthesised, corresponding to the TSWV N-gene transcript up to primer IR2 and IR7, thus representing the N mRNA without and with 3'-end hairpin, respectively. These transcripts, along with total RNA isolated from TSWV-infected *Nicotiana rustica*, were analysed by Northern blot hybridisation using an N-gene specific probe (Fig. 4).

The results showed that the N mRNA from infected plants (Fig. 4, lane 2) migrated slower than the N-IR2 transcript (Fig. 4, lane 3) and slightly faster than the N-IR7 transcript (Fig. 4, lane 1). When either N-IR2 or N-IR7 was mixed with the total RNA (results not shown) the size differences persisted, indicating that the apparent migration differences were not an artefact resulting from varying salt concentrations in the samples. These results are in agreement with transcription termination near the 3'-end of the hairpin.

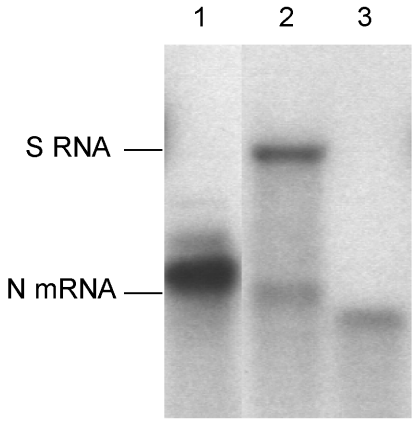

Figure 4: Northern blot size comparison of TSWV N mRNAs.
Lane 1, synthetic T7 transcript representing an N mRNA terminating at the position of primer IR7 (N-IR7). Lane 2, total RNA of TSWV-infected *N. rustica*. Lane 3, synthetic transcript representing an N mRNA terminating at the position of primer IR2 (N-IR2). (The location of primers IR2 and IR7 within the intergenic region of the S RNA is shown in figure 2A).

DISCUSSION

The RT-PCR cloning and sequence analysis data presented here indicate that transcription of TSWV S-segment derived mRNAs terminates near the 3'-end of the intergenic hairpin. Termination of the N mRNA appears to occur between the positions of primers IR6 and IR7, i.e. between S-RNA nt 1568 and 1574, which is supported by size estimation of cloned transcripts in Northern blot hybridisation studies that showed that the N mRNA is slightly smaller than the 1.38 kb-sized IR7 synthetic RNA. For NSs, termination occurs beyond primer IR14, i.e. beyond nt 1839. By analogy to the N mRNA, termination for NSs is likely to occur before the position of primer IR15. The NSs mRNA would thus be estimated to be slightly smaller than 1.9 kb which is in agreement with earlier estimations from Northern blot analyses of TSWV-infected leaf material (De Haan *et al.,* 1990; Kormelink *et al.,* 1992), and would correspond with termination occurring near the 3'-end of the hairpin. Mapping of the 3' ends by RT-PCR amplification has been hampered by the high A/U-content of the intergenic region which complicated design of proper primers. In addition, the primer for the snatched leader sequence (p141) can by default only be very short (13 nt), necessitating a low annealing temperature for the first few cycles of PCR which leads to increased amounts of non-specific amplification products. Attempts at more accurate determination of the 3' ends, e.g. by transcript circularisation, immunocapture RT-PCR, or 3'-RACE have not been successful, presumably due to the small amount of viral transcripts synthesised in the *in vitro* reaction as compared to the amount of input gRNA.

The results described here point to a transcription termination strategy for TSWV S RNA similar to that of TOS and UUK phleboviruses, also yielding mRNAs with overlapping 3'-ends. The TSWV template sequence, however, does not contain the conserved motif (CCGUCG) allegedly involved in TOSV termination. Although the TSWV IR does contain a conserved sequence motif that has been suggested to play a role

in transcription termination, termination appears to occur at a considerable distance (~130 nt) from this sequence. Both UUKV S-segment mRNAs have a small stem-loop structure at their 3'-end, which may constitute the termination signal (Simons & Pettersson, 1991). This may indicate a termination mechanism based on the formation of a hairpin structure in the nascent transcript, as also suggested for *Tacaribe virus* (Iapalucci *et al.,* 1991) and *Lymphocytic choriomeningitis virus* (LCV) (Meyer & Southern, 1993), members of the *Arenaviridae*, another virus family of ambisense coding ssRNA viruses. In view of the site of termination, i.e. at the end of the hairpin sequence and quite distant from the conserved sequence motif, termination by hairpin folding in the nascent transcript seems a plausible hypothesis for TSWV transcription termination.

Both the hairpin structure and the conserved sequence motif, as present in the ambisense TSWV S RNA, are also found in the IR of the M RNA segment, suggesting that transcription termination for this segment is regulated similarly to that of the S segment. Although the L RNA segment is entirely negative sense coding and thus lacks an intergenic region, both intergenic features can also be found in this genome segment. The conserved sequence motif is located at position 8765 (103 nt beyond the L ORF stop codon; Fig. 1C). In addition, folding predictions indicated formation of a (bulged) hairpin structure consisting of at least residues 8807-8829 (146-168 nt from the stop codon; Fig. 5). It is thus indeed possible that termination of all viral mRNAs is regulated in a similar fashion, although this remains to be elucidated in particular for the L mRNA.

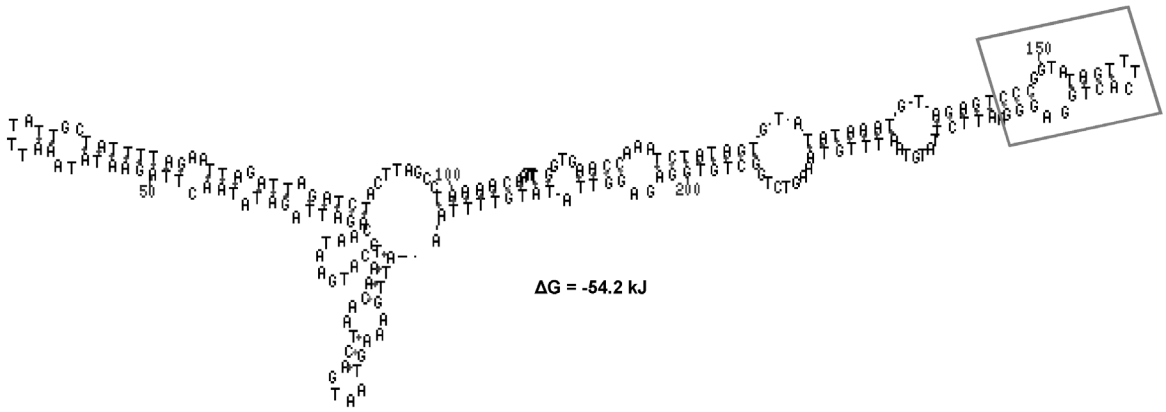

Figure 5: Folding prediction for the 3' UTR of the TSWV L RNA.
The sequence submitted for folding is residues 8662-8879, corresponding to the sequence between the L ORF stop-codon and the genomic panhandle. Shown is the most energetically stable structure. The boxed stem-loop structure is present in all folding predictions of this sequence.

Like many plant-viral mRNAs, those of TSWV are not polyadenylated, but instead appear to contain a specific 3'-end structure, i.e. the intergenic hairpin sequence. This 3'-end hairpin may be involved in stimulation of viral mRNA translation through 5'-3' terminal interaction, as has been observed for the 3'-end structural feature of several plant-infecting RNA viruses (Fabian & White, 2004; Gallie, 1998; Gallie & Kobayashi, 1994; Leonard *et al*., 2004; Matsuda & Dreher, 2004; Meulewaeter *et al*., 2004; Neeleman *et al*., 2001). This circularisation would likely be mediated by a viral protein, for which NSs may be a suitable candidate as the NSs proteins of several bunyaviruses have been implicated in translation (Di Bonito *et al.,* 1999; Simons *et al.,* 1992; Watkins & Jones, 1993).

MATERIALS & METHODS

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 (Chapter 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.

Table 1: Primers used to amplify *in vitro* synthesised TSWV transcripts.

Primer name	5' →3' corresponds to	Sequence
p141	α-globin mRNA 5' 13 nt	acacttctggtcc
IR1	S RNA nt 1904-1923	cacactaagcaagcacaagc
IR2	S RNA nt 1852-1869	gcacaacacacagaaagc
IR3	S RNA nt 1810-1832	attttttgttttcgttgtttttg
IR4	S RNA nt 1721-1742	gggttttgtttttgttttttg
p7	S RNA nt 1702-1724	ggaccaatttggccaaatttggg
IR5	S RNA nt 1678-1697	cgaaaaaccaaaaagacccg
IR6	S RNA nt 1574-1593	cctcctgtctagtagaaacc
IR7	S RNA nt 1548-1568	ctctgtttgtcatctctttc
IR8	S RNA nt 1498-1518	gctttttctaattatgttatg
IR9	S RNA nt 1518-1498	cataacataattagaaaaagc
IR10	S RNA nt 1568-1548	gaaagagatgacaaacagag
IR11	S RNA nt 1593-1574	ggtttctactagacaggagg
IR12	S RNA nt 1697-1678	cgggtctttttggtttttcg
p8	S RNA nt 1724-1702	cccaaatttggccaaattggtcc
IR13	S RNA nt 1742-1721	caaaaaacaaaaacaaaaccc
IR14	S RNA nt 1839-1807	aaaataacaaaaacaacgaaaac
IR15	S RNA nt 1869-1852	gctttctgtgtgttgtgc
IR16	S RNA nt 1923-1904	gcttgtgcttgcttagtgtg
p14	S RNA nt 2769-2739	tctaaggttaagctcactaagg
p18	S RNA nt 1-50	agagcaattgtgtcagaattttgttcataatcaaacctcacttagaaaat

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 the previous paragraph. 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 transcription yielded capped RNA transcripts resembling the N-gene mRNA with (N-IR7) or without (N-IR2) intergenic hairpin, but without cap snatch-derived leader. These transcripts, along with a sample of total RNA from TSWV-infected *N. rustica* plants, were analysed by Northern blot hybridisation using a probe to detect the N-gene sequence as described previously (Chapter 2).

Folding predictions for the L RNA 3' terminus.

Folding predictions were performed at 25ºC, using MFold (D. Stewart and M. Zuker, © 2003 Washington University; http://mfold.burnet.edu.au). The sequence submitted for folding is residues 8662-8879, corresponding to the sequence between the L ORF stop-codon and the genomic panhandle.

REFERENCES

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. and Girard, M. (1990). Characterization of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with *Germiston virus* (Bunyavirus). *Virology* **175**, 50-58

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1990). The S RNA segment of *Tomato spotted wilt virus* has an ambisense character. *J Gen Virol* **71**, 1001-1007

Di Bonito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. and Giorgi, C. (1999). Immunological characterization of Toscana virus proteins. *Arch Virol* **144**, 1947-1960

Dobie, D. K., Blair, C. D., Chandler, L. J., Rayms-Keller, A., McGaw, M. M., Wasieloski, L. P. and Beaty, B. J. (1997). Analysis of *La Crosse virus* S mRNA 5' termini in infected mosquito cells and *Aedes triseriatus* mosquitoes. *J Virol* **71**, 4395-4399

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of Nicotiana benthamiana. *J Virol* **73**, 5172-5175

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap-snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 2545-2552

Emery, V. C. and Bishop, D. H. (1987). Characterisation of *Punta toro* S mRNA species and identification of an inverted complementary sequence in the intergenic region of *Punta toro phlebovirus* ambisense S RNA that is involved in mRNA transcription termination. *Virology* **156**, 1-11

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D. H. (1985). Analyses of the mRNA transcription processes of *Snowshoe hare bunyavirus* S and M RNA species. *J Virol* **55**, 681-689

Fabian, m. R. and White, K. A. (2004). 5'-3' RNA-RNA interaction facilitates cap- and poly(A) tail-independent translation of *Tomato bushy stunt virus* mRNA: a potential common mechanism for *Tombusviridae*. *J Biol Chem* **279**, 28862-28872

Gallie, D. R. (1998). A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**, 1-11

Gallie, D. R. and Kobayashi, M. (1994). The role of the 3' untranslated region of non-polyadenylated plant viral mRNAs in regulating translational efficiency. *Gene* **142**, 159-165

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M. C., Takehara, K., Hilditch, C., Morikawa, S. and Bishop, D. H. (1991). Sequences and coding strategies of the S RNAs of *Toscana* and *Rift Valley fever* viruses compared to those of *Punta Toro, Sicilian Sandfly fever,* and *Uukuniemi* viruses. *Virology* **180**, 738-753

Hutchinson, K. L., Peters, C. J. and Nichol, S. T. (1996). *Sin Nombre virus* mRNA synthesis. *Virology* **224**, 139-149

Iapalucci, S., López, N. and Franze-Fernández, M. T. (1991). The 3' end termini of the *Tacaribe arenavirus* subgenomic RNAs. *Virology* **182**, 269-278

Kormelink, R., De Haan, P., Peters, D. and Goldbach, R. (1992). Viral RNA synthesis in *Tomato spotted wilt virus*-infected *Nicotiana rustica* plants. *J Gen Virol* **73**, 687-693

Leonard, S., Viel, C., Beauchemin, C., Daigneault, N., Fortin, M. G. and Laliberte, J. F. (2004). Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein in planta. *J Gen Virol* **85**, 1055-1063

Matsuda, D. and Dreher, T. W. (2004). The tRNA-like structure of *Turnip yellow mosaic virus* RNA is a 3'-translational enhancer. *Virology* **321**, 36-46

Meulewaeter, F., van Lipzig, R., Gultayev, A. P., Pleij, C. W., van Damme, D., Cornelissen, M. and van Eldik, G. (2004). Conservation of RNA structures enables TNV and BYDV 5' and 3' elements to cooperate synergistically in cap-independent translation. *Nucleic Acids Res* **32**, 1721-1730

Meyer, B. J. and Southern, P. J. (1993). Concurrent sequence analysis of 5' and 3' RNA termini by intramolecular circularization reveals 5' nontemplated bases and 3' terminal heterogeneity for *Lymphocytic choriomeningitis virus* mRNAs. *J Virol* **67**, 2621-2627

Neeleman, L., Olsthoorn, R. C. L., Linthorst, H. J. M. and Bol, J. F. (2001). Translation of a nonpolyadenylated viral RNA is enhanced by binding of viral coat protein or polyadenylation of the RNA. *Proc Natl Acad Sci USA* **98**, 14286-14291

Patterson, J. L. and Kolakofsky, D. (1984). Characterization of *La Crosse virus* small-genome transcripts. *J Virol* **49**, 680-685

Simons, J. F., Persson, R. and Pettersson, R. F. (1992). Association of the nonstructural protein NSs of *Uukuniemi virus* with the 40S ribosomal subunit. *J Virol* **66**, 4233-4241

Simons, J. F. and Pettersson, R. F. (1991). Host-derived 5' ends and overlapping complementary 3' ends of the two messenger RNAs transcribed from the ambisense S segment of *Uukuniemi virus*. *J Virol* **65**, 4741-4748

Watkins, C. A. and Jones, I. M. (1993). Association of the 40S ribosomal subunit with the NSs nonstructural protein of *Punta Toro virus*, p 136. *International Congress of Virology, Glasgow 1993*

CHAPTER 5

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

SUMMARY

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 analysed in pair-wise competition in TSWV *in vitro* transcription assays. A strong preference was observed for multiple-basepairing donors.

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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* (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.,* 1992; Raju *et al.*, 1990; Simons & 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 & Elliott 1993a,b). However, the host mRNAs from which the capped leader sequences had been snatched, and therefore the actual cleavage site, remained unknown. For *Influenza A virus*, 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, *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 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 & Elliott, 1993a; Vialat & Bouloy, 1992).

Tomato spotted wilt virus (TSWV) is the type species of the plant-infecting *Tospovirus* genus within the *Bunyaviridae*. After the initial finding of 5' non-templated sequences on TSWV mRNAs (Kormelink *et al.*, 1992, 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 and 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; see also Chapter 1). 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; Chapter 2). 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 end that may be involved in basepairing to 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*. 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 (Chapter 2), 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), harbouring 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 analysed

for *de novo* synthesised 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 (Chapter 2), titration experiments demonstrated that in fact 8 ng/µl was already sufficient (results not shown).

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.

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* synthesised N gene transcripts revealed that 19 out of the 23 clones analysed contained the marker nucleotide at position 12 (Table 2), indicative for 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). In the case of the 5:1 ratio, RT-PCR cloning of *de novo* synthesised N gene transcripts showed that 5 out of 6 clones represented mRNAs with a leader derived from mut-AMV3, this was 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.

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 (Chapter 2) that viral mRNAs are re-snatched *in vitro*. To answer this question, a competition experiment was performed analogous to the 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 to 31 are complementary to template 3' residues 1-14).

Table 2: N and NSs mRNA 5'-end sequences resulting from cap snatching of the indicated cap donors.

cap donor	retrieved N mRNA 5' sequence	N°. 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.

When equimolar amounts (80 ng/µl each) of both cap donors were added, RT-PCR cloning of *de novo* synthesised NSs gene transcripts revealed that 13 out of 14 clones contained an AMV3 leader lacking the marker nucleotide as present in mut-AMV3, thus represented NSs 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 (Chapter 2) 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 (Chapter 4). To analyse 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. 1A) were tested as cap donors *in vitro*. For both m-IR2 and m-IR7 donors, RT-PCR cloning (Fig. 1B) and sequence analyses of *de novo* synthesised NSs 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.

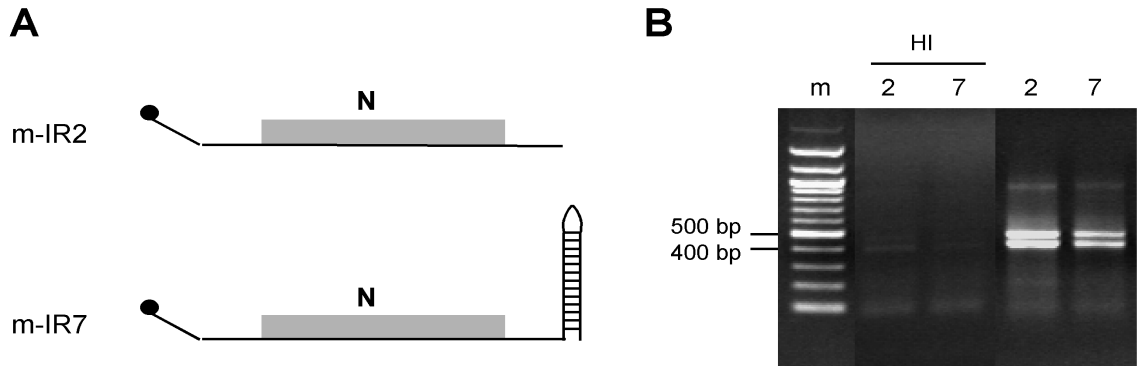

Figure 1: Re-snatching of viral transcripts containing or lacking a 3'-terminal hairpin.
Panel A, Schematic representation of full length N mRNA transcripts m-IR2 and m-IR7 containing a capped AMV RNA3-derived leader sequence and with (m-IR7) or without (m-IR2) the 3'-end hairpin structure. Panel B, 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 and nested PCR products, respectively, and are attributed to an excess of RT primer in the reaction.

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 analysed 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 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. 2, lane 2), and the very 5'-end of the AMV3 leader, i.e. the +17 position (Fig. 2, lane 3), respectively. Primer extension on AMV3-N after exposure to TSWV endonuclease cleavage yielded several products (Fig 3, 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 2, lane 3), the -2 band seemed more intense in extension on the endonuclease-exposed AMV3-N (Fig 2, 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 (Chapter 2). 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. 2, lane 4). As expected, a band migrating at position +13 was observed, the result of primer extension on N mRNA transcripts harbouring 13 nt leader sequences of globin mRNAs (Chapter 2). 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.

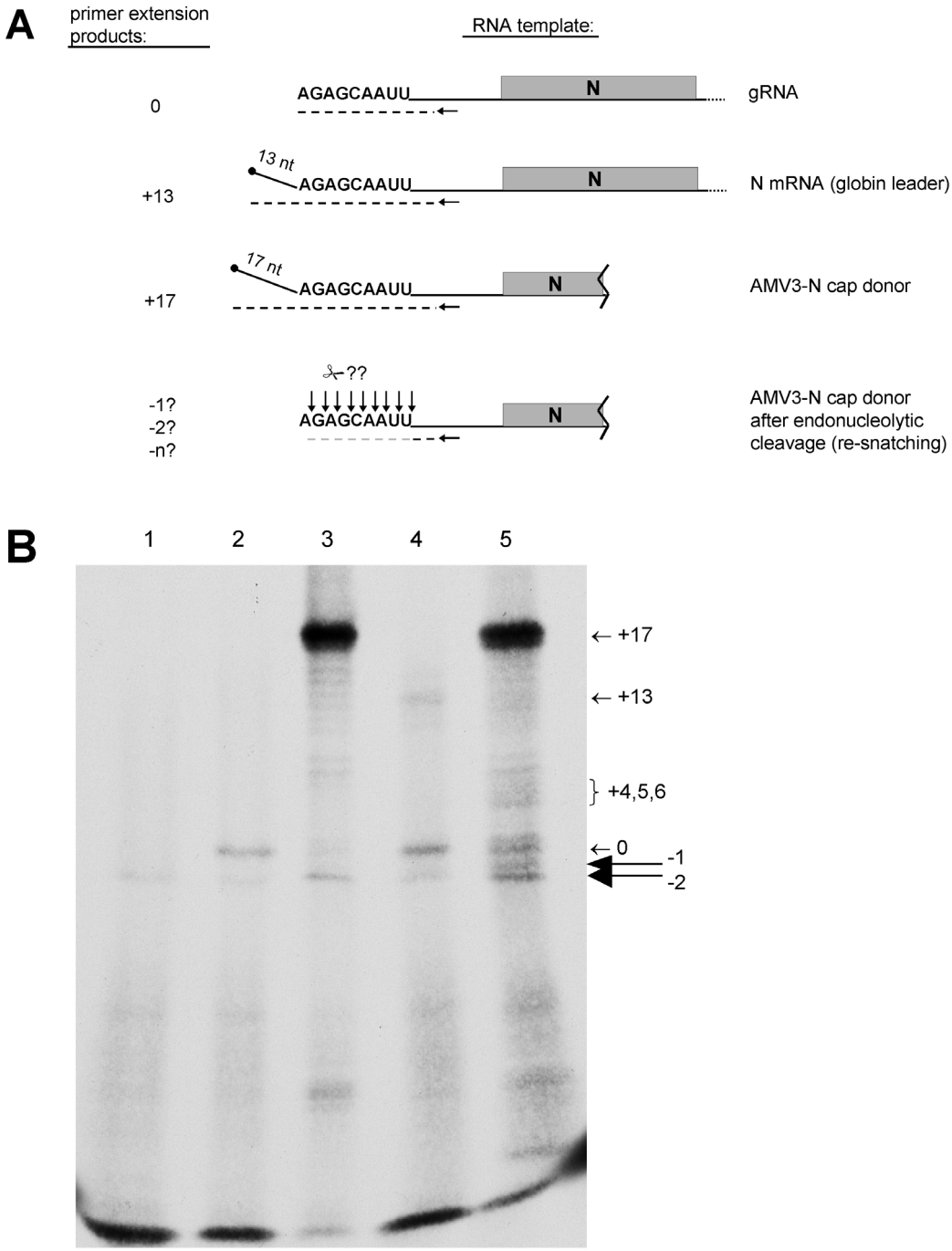

Figure 2: Primer extension analyses on cap-donor RNA prior to, or after endonuclease cleavage.
Panel A, Schematic representation of primer extension analysis. Primer ML01 is aligned along RNA transcripts as present in a TSWV transcription reaction and the expected sizes of the resulting primer extension products are indicated.
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 at the right: +1 corresponds to run-off products on genomic RNA length; +13/+14 and +17 corresponds to run-off on N transcripts harbouring leaders derived from globin mRNAs, or AMV3-N respectively; -1 and -2 correspond to run-off products on cap donor AMV3-N after endonuclease cleavage.

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 *Influenza A virus* 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 & Pettersson 1991). Analysis of the requirements for cap donors has primarily been performed for *Influenza A virus*. Cleavage of a donor mRNA by *Influenza* polymerase is thought to occur independent 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 & Krug, 1981; Rao *et al.*, 2003; Shaw & Lamb, 1984), and studies using recombinant *Influenza* 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 (Chapter 2). 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; Chapter 2), 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 *Influenza A virus* 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 *Influenza A virus* 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 independent of basepairing to the viral template, as observed for *Influenza A virus* (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 *Influenza virus*, basepairing interactions do play a role in selection of cap donor mRNAs during endonuclease cleavage, and that, as for *Influenza virus*, cleavage and subsequent elongation remain restricted to the first two complementary residues.

The initial finding that re-snatching of viral mRNAs occurs *in vitro* (Chapter 2) 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 *Influenza A virus*, 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 & 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 *Influenza A virus*-infected cells (Detjen *et al.*, 1987; Shih & 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 synthesised. 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 (Chapter 4). 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 circularisation of the mRNA, possibly through specific binding of a viral protein, as previously reported for other viruses (Fabian & White, 2004; Gallie, 1998; Gallie & Kobayashi, 1994; Leonard *et al*., 2004; Matsuda & 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 & METHODS

Plasmid construction and synthesis of cap donors

Cap donor RNAs were synthesised *in vitro* using the Ambion T7 mMessage mMachine kit according to the manufacturer's instructions. Wt AMV RNA3 transcripts and AMV3-N transcripts were synthesised as described previously (Chapter 2). were generated from plasmid pXO32NcoP3, as described previously (Chapter 2). Mutant AMV RNA3 (mut-AMV3) was constructed by PCR amplification of (part of) the AMV RNA3 sequence on pXO32NcoP3 using primer p85 (5'-CCCGAATTCGAAGAGTACGAATTACGCG, complementary to nts 339-313 of the AMV RNA3 sequence), in combination with primer mut-AMV3 (5'- cccggatcc**TAATACGACTCA-CTATAG**GUAUUAAUACCCUUUUCAGCATATTCC, 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 linearisation, 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 synthesised in an *in vitro* TSWV transcription assay in the presence of capped AMV RNA3 (Chapter 2), using primer IR2 (5'-CCCGGATCCGCACAACACACAGAAAGC, underlined sequence identical to S RNA nts 1852-1869, preceded by BamHI site), in combination with primer pT7-AMV3 (5'-cccggatcc**TAATACGACTCACTATAG-**GUAUUAAUACCAUUUUC, AMV RNA3 nts 1-17, preceded by the T7 promoter sequence in bold and a 5' BamHI linker sequence). The PCR product was cloned into pGEMTeasy and subcloned into pUC19 using the SphI and BamHI 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 nts 1548-1568, preceded by BamHI site) in combination with primer p009 (5'-cccggatcc**TAATACGACTCACTATAG**AGAGCAATCGTG, identical to S RNA vc-sense nts 1-12, preceded by the T7 promoter sequence in bold and a 5' BamHI linker sequence). The PCR product was digested with BamHI and cloned into the BamHI-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 PacI-KpnI restriction fragment of this plasmid into PacI-KpnI-digested pUC/IR2. The PacI site is located in the TSWV S RNA sequence between the N stop codon and the beginning of the hairpin sequence, the KpnI 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 (Chapter 2). 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 (Chapter 2). PCR products of expected size were gel-purified and cloned into pGEMTeasy for sequence analysis.

Primer extension analyses

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

REFERENCES

Aragón, T., De La Luna, S., Novoa, I., Carrasco, L., Ortín, J. and Nieto, A. (2000). Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of *Influenza virus*. *Mol Cell Biol* **20**, 6259-6268

Beaton, A. R. and Krug, R. M. (1981). Selected host cell capped RNA fragments prime *Influenza* viral RNA transcription *in vivo*. *Nucleic Acids Res* **9**, 4423-4436

Bellocq, C., Ramaswamy, R., Patterson, J. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: *In vitro* studies. *J Virol* **61**, 87-95

Bergmann, M., García-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P. and Muster, T. (2000). *Influenza virus* NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* **74**, 6203-6206

Bishop, D. H., Gay, M. E. and Matsuoko, Y. (1983). Nonviral heterogeneous sequences are present at the 5' ends of one species of *Snowshoe hare bunyavirus* S complementary RNA. *Nucleic Acids Res* **11**, 6409-6418

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. and Haller, O. (2001). Genetic evidence for an interferon-antagonistic function of *Rift Valley fever virus* nonstructural protein NSs. *J Virol* **75**, 1371-1377

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. and Girard, M. (1990). Characterization of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with *Germiston virus* (Bunyavirus). *Virology* **175**, 50-58

Bouloy, M., Plotch, S. J. and Krug, R. M. (1978). Globin messenger RNA are primers for the transcription of *Influenza viral* RNA in-vitro. *Proc Natl Acad Sci USA* **75**, 4886-4890

Bucher, E., Hemmes, H., De Haan, P., Goldbach, R. and Prins, M. (2004). The *Influenza A virus* NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* **85**, 983-991

Bucher, E., Sijen, T., De Haan, P., Goldbach, R. and Prins, M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J Virol* **77**, 1329-1336

Burgui, I., Aragón, T., Ortín, J. and Nieto, A. (2003). PABP1 and eIF4G associate with *Influenza virus* NS1 protein in viral mRNA translation intiation complexes. *J Gen Virol* **84,** 3263-3274

Chen, Z. Y., Li, Y. Z. and Krug, R. M. (1999). *Influenza A virus* NS1 protein targets poly(A)-binding protein II of the cellular 3 '-end processing machinery. *Embo J* **18**, 2273-2283

Chung, T. D., Cianci, C., Hagen, M., Terry, B., Matthews, J. T., Krystal, M. and Colonno, R. J. (1994). Biochemical studies on capped RNA primers identify a class of oligonucleotide inhibitors of the *Influenza virus* RNA polymerase. *Proc Natl Acad Sci USA* **91**, 2372-2376

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1990). The S RNA segment of *Tomato spotted wilt virus* has an ambisense character. *J Gen Virol* **71**, 1001-1007

De La Luna, S., Fortes, P., Beloso, A. and Ortín, J. (1995). *Influenza virus* NS1 protein enhances the rate of translation initiation of viral mRNAs. *J Virol* **69**, 2427-2433

Detjen, B. M., Angelo, C. S. T., Katze, M. G. and Krug, R. M. (1987). The three *Influenza virus* polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J Virol* **61**, 16-22

Di Bonito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. and Giorgi, C. (1999). Immunological characterization of *Toscana virus* proteins. *Arch Virol* **144**, 1947-1960

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of *Nicotiana benthamiana*. *J Virol* **73**, 5172-5175

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap-snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 1-8

Estabrook, E. M., Tsai, J. and Falk, B. W. (1998). *In vivo* transfer of *Barley stripe mosaic hordeivirus* ribonucleotides to the 5' terminus of *Maize stripe tenuivirus* RNAs. *Proc Natl Acad Sci USA* **95**, 8304-8309

Fabian, m. R. and White, K. A. (2004). 5'-3' RNA-RNA interaction facilitates cap- and poly(A) tail-independent translation of *Tomato bushy stunt virus* mRNA: a potential common mechanism for *Tombusviridae*. *J Biol Chem* **279**, 28862-28872

Gallie, D. R. (1998). A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**, 1-11

Garcin, D., Lezzi, M., Dobbs, M., Elliott, R. M., Schmaljohn, C., Kang, C. Y. and Kolakofsky, D. (1995). The 5' ends of *Hantaan virus* (*Bunyaviridae*) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. *J Virol* **69**, 5754-5762

Hagen, M., Tiley, L., Chung, T. D. and Krystal, M. (1995). The role of template-primer interactions in cleavage and initiation by the *Influenza virus* polymerase. *J Gen Virol* **76**, 603-611

Honda, A., Mizumoto, K. and Ishihama, A. (1986). RNA polymerase of *Influenza virus* dinucleotide-primed initiation of transcription at specific positions on viral RNA. *J Biol Chem* **261**, 5987-5991

Huiet, L., Feldstein, P. A., Tsai, J. H. and Falk, B. W. (1993). The *Maize stripe virus* major noncapsid protein messenger RNA transcripts contain heterogeneous leader sequences at their 5' termini. *Virology* **197**, 808-812

Jin, H. and Elliot, R. M. (1993). Non-viral sequences at the 5' ends of *Dugbe nairovirus* S mRNAs. *J Gen Virol* **74**, 2293-2297

Jin, H. and Elliott, R. M. (1993). Characterization of *Bunyamwera virus* S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. *J Virol* **67**, 1396-1404

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. and Elliott, R. M. (2003). *Bunyamwera virus* nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death. *J Virol* **77**, 7999-8008

Kormelink, R., Van Poelwijk, F., Peters, D. and Goldbach, R. (1992). Non-viral heterogeneous sequences at the 5' ends of *Tomato spotted wilt virus* mRNAs. *J Gen Virol* **73**, 2125-2128

Krug, R. M., Yuan, W., Noah, D. L. and Latham, A. G. (2003). Intracellular warfare between human *Influenza* viruses and human cells: the roles of the NS1 protein. *Virology* **309**, 181-189

Leonard, S., Viel, C., Beauchemin, C., Daigneault, N., Fortin, M. G. and Laliberte, J. F. (2004). Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein in planta. *J Gen Virol* **85**, 1055-1063

Li, W.-X., Li, H., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E. W. A., Johnson, K. L., García-Sastre, A., Ball, L. A., Palese, P. and Ding, S.-W. (2004). Interferon antagonist proteins of *Influenza* and *vaccinia* viruses are suppressors of RNA silencing. *Proc Natl Acad Sci USA* **101**, 1350-1355

Marión, R. M., Aragón, T., Beloso, A., Nieto, A. and Ortín, J. (1997). The N-terminal half of the *Influenza virus* NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. *Nucleic Acids Res* **25**, 4271-4277

Matsuda, D. and Dreher, T. W. (2004). The tRNA-like structure of *Turnip yellow mosaic virus* RNA is a 3'-translational enhancer. *Virology* **321**, 36-46

Meulewaeter, F., van Lipzig, R., Gultayev, A. P., Pleij, C. W., van Damme, D., Cornelissen, M. and van Eldik, G. (2004). Conservation of RNA structures enables TNV and BYDV 5' and 3' elements to cooperate synergistically in cap-independent translation. *Nucleic Acids Res* **32**, 1721-1730

Neeleman, L., Olsthoorn, R. C. L., Linthorst, H. J. M. and Bol, J. F. (2001). Translation of a nonpolyadenylated viral RNA is enhanced by binding of viral coat protein or polyadenylation of the RNA. *Proc Natl Acad Sci USA* **98**, 14286-14291

Peng, Q., Shi Jose, M. G. and Summers Donald, F. (1996). *Influenza A virus* RNA-dependent RNA polymerase cleaves *Influenza* mRNA *in vitro*. *Virus Res* **42**, 149-158

Plotch, S., Bouloy, M. and Krug, R. M. (1979). Transfer of 5-prime terminal cap of globin messenger RNA to *Influenza* viral complementary RNA during transcription *in-vitro*. *Proc Natl Acad Sci USA* **76**, 1618-1622

Qiu, Y. and Krug Robert, M. (1994). The *Influenza virus* NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol* **68**, 2425-2432

Raju, R., Raju, L., Hacker, D., Garcin, D., Compans, R. and Kolakofsky, D. (1990). Nontemplated bases at the 5' ends of *Tacaribe virus* messenger RNA. *Virology* **174**, 53-59

Rao, P., Yuan, W. M. and Krug, R. M. (2003). Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis. *EMBO J* **22**, 1188-1198

Salvatore, M., Basler, C. F., Parisien, J. P., Horvath, C. M., Bourmakina, S., Zheng, H. Y., Muster, T., Palese, P. and García-Sastre, A. (2002). Effects of *Influenza A virus* NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. *J Virol* **76**, 1206-1212

Shaw, M. W. and Lamb, R. A. (1984). A specific subset of host-cell messenger RNA prime *Influenza virus* messenger RNA synthesis. *Virus Res* **1**, 455-468

Shih, S.-R. and Krug, R. M. (1996). Surprising function of the three *Influenza* viral polymerase proteins: Selective protection of viral mRNAs against the cap-snatching reaction catalyzed by the same polymerase proteins. *Virology* **226**, 430-435

Simons, J. F., Persson, R. and Pettersson, R. F. (1992). Association of the nonstructural protein NSs of *Uukuniemi virus* with the 40S ribosomal subunit. *J Virol* **66**, 4233-4241

Simons, J. F. and Pettersson, R. F. (1991). Host-derived 5' ends and overlapping complementary 3' ends of the two messenger RNAs transcribed from the ambisense S segment of *Uukuniemi virus*. *J Virol* **65**, 4741-4748

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. and Okuno, T. (2002). Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. *FEBS letters* **532**, 75-79

Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P. and García-Sastre, A. (2000). Activation of interferon regulatory factor 3 is inhibited by the *Influenza A virus* NS1 protein. *J Virol* **74**, 7989-7996

Van Poelwijk, F., Kolkman, J. and Goldbach, R. (1996). Sequence analysis of the 5' ends of *Tomato spotted wilt virus* N mRNAs. *Arch Virol* **141**, 177-184

Vialat, P. and Bouloy, M. (1992). *Germiston virus* transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66**, 685-693

Wang, X. Y., Li, M., Zheng, H. Y., Muster, T., Palese, P., Beg, A. A. and García-Sastre, A. (2000). *Influenza A virus* NS1 protein prevents activation of NF-kappa B and induction of alpha/beta interferon. *J Virol* **74**, 11566-11573

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. and Elliott, R. M. (2002). *Bunyamwera bunyavirus* nonstructural protein NSs counteracts the induction of alpha/beta interferon. *J Virol* **76**, 7949-7955

CHAPTER 6

GENERAL DISCUSSION

***TSWV transcription* in vitro**

Although much progress has been made in the past decade to characterise TSWV genome transcription and replication (Duijsings, 2001; Kormelink, 1994; Van Poelwijk, 1996), many details of these processes have thus far remained elusive. To gain more insight into these processes, the research described in this thesis was firstly directed toward developing a functional *in vitro* transcription assay. As described in Chapter 2, purified virions as well as purified cytoplasmic RNPs of TSWV are observed to perform both genome replication and transcription in *vitro* (Chapter 2). Which of these activities occurs appears to depend on the assay conditions, i.e. on the absence or presence of rabbit reticulocyte lysate (RRL).

Although TSWV transcription is initiated by cap snatching, the lack of transcription in the absence of RRL, apparently, was not due to a lack of capped RNA molecules since addition of capped RNA molecules alone was unable to induce transcription (Chapter 2). It follows, therefore, that stimulation of transcription by the addition of RRL is not due solely to the presence of (fragmented) mRNAs in this lysate. The use as cap donors of globin mRNAs present in the lysate as well as exogenously added capped RNAs indicated that the observed transcription was indeed genuine cap snatch-initiated viral transcription. Moreover, this demonstrated that the *in vitro* transcription assay could be used to investigate the mechanism of transcription initiation using mutable synthetic capped RNA transcripts.

Dependence of transcription *in vitro* on the presence of RRL has previously been observed for *La Crosse* and *Germiston* orthobunyaviruses, and was suggested to be based on a requirement for stabilisation of the nascent transcript by scanning ribosomes (Bellocq & Kolakofsky, 1987; Vialat & Bouloy, 1992). For TSWV, however, no reduction of transcription was observed when transcript scanning by ribosomes was inhibited the addition of translation inhibitors (Chapter 3). Moreover, no protein synthesis could be observed in the *in vitro* transcription reaction, since the reaction conditions did not allow for translation to take place. These findings indicated that, in contrast to *La Crosse* and *Germiston* viruses, TSWV transcription *in vitro* is not coupled to translation. In this respect TSWV may more closely resemble *Snowshoe hare* orthobunyavirus for which transcription *in vivo* was found to be unaffected by the addition of translation inhibitors (Eshita *et al*., 1985). The factor in the RRL that stimulates TSWV transcription has not been identified. However, one of the host factors found associated to *Vesicular stomatitis virus* (VSV) transcriptional RNPs (see below) was translation elongation factor-1alpha, suggesting it may be this or a similar factor in the RRL that is responsible for stimulating TSWV transcription *in vitro*.

Replicational activity of TSWV virion-derived and cytoplasmic RNPs: the switch between transcription and replication

In the absence of RLL, TSWV virions only show low levels of replicational activity, whereas cytoplasmic RNPs appear to have increased replicational activity, as observed by the synthesis of not only genomic S RNA but also M and L RNA molecules (Chapter 2). In the presence of RRL the situation is reversed, i.e. high transcriptional activity is observed with purified virions whereas hardly any RNA synthesis can be observed for cytoplasmic RNPs. It would seem therefore, that RNPs may exist in two different conformations: transcriptional RNPs as present inside virions and replicational RNPs in the cytoplasm. The existence of two functionally different RNP complexes has been suggested previously for *Influenza virus*, based on similar observations of different levels of RNA synthesis between virion-derived RNPs and cellular RNPs (Skorko *et al*., 1991).

After cell entry, the RNPs must first engage in transcription to synthesise viral proteins, but later switch to replication of the viral genome. For negative sense RNA viruses, in general, this switch has been suggested to be constituted by the viral nucleocapsid protein which is required to act as antiterminator during replication. Recently, however, studies on *Lymphocytic Choriomeningitis arenavirus* (LCMV) have demonstrated that although NP indeed fulfils a crucial stimulatory role in both processes, elevated concentrations of NP did not result in increased levels of replication compared to transcription (Pinschewer *et al*., 2003). Thus, for LCMV the switch between these processes is not constituted by cellular concentrations of NP. Interestingly, two different kinds of RNPs have recently been isolated from cells infected with VSV, a non-segmented negative strand RNA virus (Qanungo *et al.*, 2004). One was a transcriptase complex consisting of viral RNA, polymerase and nucleocapsid protein, associated with several host factors, and the other was a replicase complex consisting of only (viral) RNA, polymerase and nucleocapsid protein. This seems to point to a switch mediated by host factors, which may be similar for the segmented viruses.

By analogy to the nucleoproteins of other negative strand viruses, TSWV N protein may also function as antiterminator during replication. The low replicational activity of purified virions (Chapter 2) may thus be explained by the absence of a pool of free soluble N protein. The high replicational activity of purified cytoplasmic RNPs (Chapter 2), in this view, seems to imply the presence of free soluble N protein. The RNP purification method used may allow for co-purification of N protein, especially if N forms RNP-like structures devoid of RNA as demonstrated for *Influenza virus* NP (Ruigrok & Baudin, 1995).

Transcription initiation: cap snatching

All segmented negative sense RNA viruses studied thus far, with the exception of Varicosaviruses, initiate transcription by the mechanism of cap snatching. The current model for TSWV cap snatching (Duijsings *et al*., 2001; see Chapter 1) includes a single basepairing interaction between cap donor and viral template. This model is based on *in vivo* observations using mixed infection of tobacco plants with both TSWV and *Alfalfa mosaic virus* (AMV), the latter providing the cap donors for TSWV transcription initiation. Although the transgenic "P12" plant-system (plants transgenically expressing functional AMV replicase; Taschner *et al.*, 1991) that was used allowed some manipulation of cap donor sequences, this was limited to *in vivo*-viable mutants as demonstrated by the occurrence of revertants of several mutant sequences to either wild type or other mutant sequences (Duijsings *et al.*, 2001).

In this thesis cap snatching was studied *in vitro*, allowing further manipulation of cap donor sequences. The observation that globin mRNAs with a dinucleotide sequence complementary to the viral template and even capped transcripts resembling the viral N mRNA, with a 14-nt complementarity, were used as cap donors (Chapter 2) indicated that the interaction between donor and template may not be restricted to a single basepair. It is not known, however, to what extent actual basepairing between these complementary residues physically occurs.

The finding that the preferred substrates for cap snatching are donors with a longer stretch of complementarity suggests that the extent of complementarity plays a role in selection of cap donors. *In vitro* analyses of cap donor requirements for *Influenza* endonuclease revealed that the site of endonuclease cleavage is determined solely by the cap donor sequence and does not involve basepairing interactions with the viral template. Only after cleavage are basepairing interactions required for correct positioning of the cleaved leader on the viral template for proper priming of transcription (Hagen *et al*., 1995). Since elongation is highly inefficient on a tetranucleotide complementary to the template 3'-end, the extent of complementarity between a cleaved primer and the viral template appears to be restricted to 3 basepairs (Honda *et al*., 1986). Moreover, cap donors with a tri-nucleotide (AGC) complementarity to the viral template are cleaved exclusively after the second complementary residue (Chung *et al*., 1994; Hagen *et al*., 1995; Rao *et al*., 2003). The observation that a cap donor with a long (14 nt) stretch of complementarity to the TSWV template appears to be cleaved exclusively after the first and maybe second basepairing residue (Chapter 5) is in agreement with these findings. However, if cleavage site selection for TSWV is based on nucleotide identity alone it remains enigmatic why donors with a long stretch of complementarity are preferred to those with only one or two complementary residues. These observations seem to point toward a selection mechanism for TSWV in which, in contrast to what has been reported

for *Influenza virus*, basepairing interactions with the template are involved at the stage of cleavage site selection, since this would be the only distinction on which the observed preference could be based. Therefore, the model as proposed by Duijsings *et al*. (2001) can be extended by incorporating a separate leader-selection step prior to the endonuclease cleavage reaction, as depicted in Fig. 1.

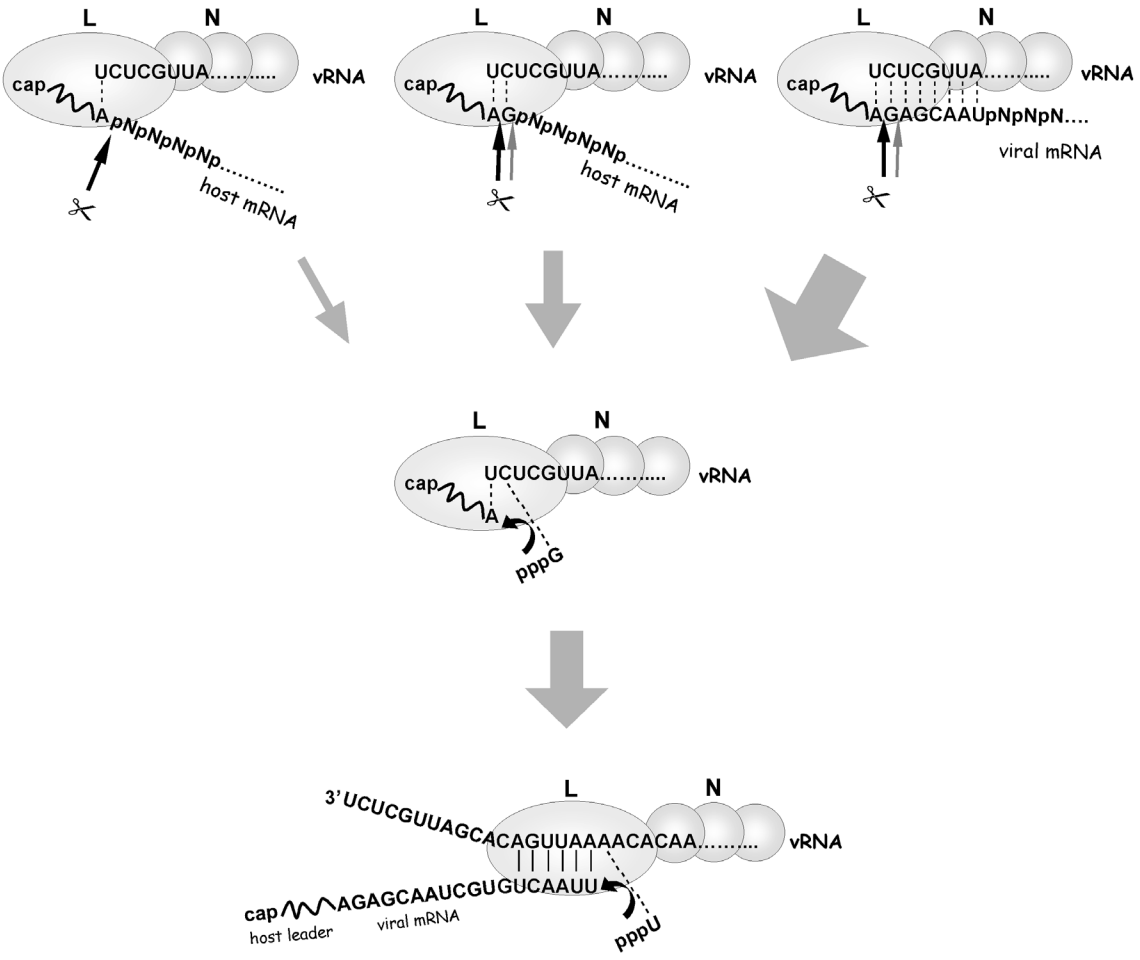

Figure 1: A model in which cap snatching by TSWV is initiated with a cap donor selection-step.
Selection of a cap donor, either a host mRNA or a viral mRNA, is based on the extent of complementarity to the viral template. As indicated by the respective sizes of the top set of down-pointing arrows, viral mRNAs are the preferred substrates for the production and subsequent usage of primers for transcription initiation. Selection of cap donors by basepairing to the viral RNA template may occur either at the selection or at the cleavage step, or both.

Resnatching of viral mRNAs *in vitro* has previously been observed for *Influenza virus* (Peng *et al*., 1996). The occurrence of resnatching *in vivo* was deemed unlikely,

since it would result in no net synthesis of viral mRNAs which is incompatible with efficient virus multiplication. For *Influenza virus* the viral polymerase was found to selectively protect viral mRNAs from being re-snatched *in vitro* (Shih & Krug, 1996). It was demonstrated that a pool of free, non-RNP-bound polymerase exists in the nuclei of infected cells (Detjen *et al*., 1987), which could serve the purpose of mRNA protection since they only need protection until they are transported out of the nucleus. The observations that TSWV mRNAs are not only re-snatched but are in fact the preferred target of the viral endonuclease *in vitro* (Chapters 2 and 5), emphasised that for TSWV too, a protection mechanism seems likely to exist. A mechanism involving protection by the viral polymerase does not seem feasible since TSWV transcribes in the cytoplasm and such protection would involve synthesis of an extra polymerase protein for every single mRNA synthesised. Viral mRNAs can be distinguished from cellular mRNAs by the presence of the conserved 8 viral residues immediately downstream of the capped leader sequence, and the 3'-end hairpin structure. Neither of these features, however, can prevent re-snatching *in vitro* (Chapters 2 and 5). Intriguingly, this seems to imply that a nascent viral mRNA needs to be protected from re-snatching as soon as its capped 5'-end emerges from the transcribing polymerase.

TSWV transcription termination

The fact that transcription termination of TSWV S-segment mRNAs occurs at the end of the intergenic hairpin suggests that the mechanism of termination of these mRNAs is based on secondary structure formation in the nascent transcript (Chapter 4). Messenger RNAs of *Tacaribe arenavirus* also possess such 3'-end hairpin structures and it is thought that these structures constitute the actual termination signal, in the same manner as prokaryotic transcription termination occurs (Iapalucci *et al*., 1991). Such a mechanism of termination is highly compatible with the model for the role of N (or NP) as anti-terminator. A nascent mRNA transcript is not encapsidated by N and as a result the hairpin structure can be formed as soon as this sequence is transcribed. This could subsequently lead to transcription termination. A nascent genome copy, on the other hand, is encapsidated by N, presumably cooperatively from its 5' end. As a result, the hairpin sequence will be encapsidated upon transcription, preventing formation of the hairpin structure. Hence, replicative transcription is not terminated and ensuing read-through of the hairpin sequence allows synthesis of a full-length genome copy.

Alternatively, termination has been suggested to be the result of hairpin formation in the viral template RNA. However, the template is encapsidated by N protein, in a very strong protein-RNA binding interaction which prevents secondary structure formation. Binding experiments with N protein from *Bunyamwera* orthobunyavirus, *Sin nombre* hantavirus and TSWV indicate this binding can only be disrupted in NaCl concentrations

of (much) more than 300 mM (Osborne & Elliott, 2000; Richmond *et al*., 1998; Mir & Panganiban, 2004). In addition, while polymerase is lost from RNP complexes by CsCl gradient centrifugation, binding of N protein to RNA remains intact in these conditions. Moreover, in this hypothesis the presence of a hairpin in the viral template is assumed to constitute an insurmountable block for the viral polymerase, which would lead to termination of transcription. Termination by this mechanism would thus occur *before* the hairpin sequence, while the results presented in Chapter 4 indicate termination occurs at the end of the hairpin sequence.

The M RNA segment of TSWV, like the S RNA segment, possesses an intergenic hairpin sequence and therefore it is most likely that M-segment transcription is terminated in the same fashion as that of the S-segment. The L RNA segment is entirely negative sense coding and lacks an intergenic region. Folding predictions of the untranslated region (UTR) following the L-gene stop codon indicate potential formation of a hairpin structure (Fig. 5 in Chapter 4). Although several folding patterns are possible, a particular stem-loop structure is present in all predictions. Therefore, it is possible that termination of the L mRNA is also triggered by hairpin formation in the nascent transcript. Alternatively, it cannot be ruled out that termination for L occurs in a different fashion, or that L mRNAs are no shorter than L genome segments.

Translation initiation of viral mRNAs

Like many viral mRNAs, TSWV mRNAs are not polyadenylated but instead posses a particular 3'-end structure. This 3'-end hairpin may functionally replace the poly(A) tail in circularisation of the mRNA for efficient translation. Functional replacement of the poly(A) tail-PABP complex has been reported for *Tobacco mosaic virus* (TMV), *Alfalfa mosaic virus* (AMV), *Turnip mosaic virus* (TuMV), *Tomato bushy stunt virus* (TBSV), *Turnip yellow mosaic virus* (TYMV) and *Tobacco necrosis virus* (TNV), all plant-infecting RNA viruses (Gallie, 1998; Gallie & Kobayashi, 1994; Neeleman *et al.,* 2001; Leonard *et al*., 2004; Fabian & White, 2004; Matsuda & Dreher, 2004; Meulewaeter *et al*., 2004).

For segmented negative stranded RNA viruses not much is known about translation of viral mRNAs. *Influenza virus* mRNAs are both capped and polyadenylated, yet viral mRNAs are preferentially translated while cellular protein synthesis is down-regulated. The *Influenza virus* NS1 protein is involved in counteracting the host's anti-viral interferon (IFN) response and is a suppressor of RNA silencing (Bergmann *et al*., 2000; Talon *et al*., 2000; Wang *et al*., 2000; Krug *et al*., 2003; Delgadillo *et al*., 2004; Bucher *et al.,* 2004; Li *et al*., 2004;). In addition, NS1 acts as a viral mRNA translation enhancer through binding both PABP and eIF4G (De La Luna *et al*., 1995; Marion *et al*., 1997;

Salvatore *et al*., 2002; Qiu & Krug 1994; Chen *et al*., 1999; Aragon *et al*., 2000; Burgui *et al*., 2003), indicating NS1 may have a 3'-5' bridging function similar to PABP.

The 3'-end hairpin or conserved sequence motif of TSWV mRNAs, as a poly(A) tail replacement, may be the recognition site for a putative viral translation enhancer. Like *Influenza* NS1 protein, NSs proteins of the animal-infecting *Bunyaviridae* are involved in counteracting the host's anti-viral interferon (IFN) response (Bouloy *et al.,* 2001; Weber *et al.,* 2002; Kohl *et al.,* 2003). In addition, TSWV NSs has been shown to be a suppressor of RNA silencing (Bucher *et al*., 2003; Takeda *et al*., 2002). Moreover, several bunyaviral NSs proteins have been found associated with polysome fractions or the 40S ribosomal subunit (Di Bonito *et al*., 1999; Simons *et al*., 1992; Watkins & Jones 1993), implying some involvement of NSs in translation. Therefore, like *Influenza virus* NS1 and in addition to suppression of gene silencing, the TSWV NSs protein may also have a role in translation, possibly by acting as a viral functional homologue of PABP.

Concluding remarks

The TSWV *in vitro* transcription assay described in this thesis has proved a valuable asset to the study of the mechanism of TSWV transcription. The results of this research have not only led to a better understanding of transcription initiation, resulting in fine-tuning of the model for cap snatching, but have also pointed towards the involvement of a hairpin structure in termination of TSWV transcription. Whether this structure and one or more viral proteins interact during translation initiation, possibly thereby masking viral mRNAs to avoid re-snatching, remains a challenging question to be tackled in the near future. In conclusion, TSWV may be used as a model system to further elucidate the processes of replication, transcription and translation of segmented negative strand RNA viruses in general.

REFERENCES

Aragón, T., De La Luna, S., Novoa, I., Carrasco, L., Ortín, J. and Nieto, A. (2000). Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of *Influenza virus*. *Mol Cell Biol* **20**, 6259-6268

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for *La Crosse virus* S-mRNA synthesis: A possible mechanism. *J Virol* **61**, 3960-3967

Bergmann, M., García-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P. and Muster, T. (2000). *Influenza virus* NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* **74**, 6203-6206

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. and Haller, O. (2001). Genetic evidence for an interferon-antagonistic function of *Rift Valley fever virus* nonstructural protein NSs. *J Virol* **75**, 1371-1377

Bucher, E., Sijen, T., De Haan, P., Goldbach, R. and Prins, M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J Virol* **77**, 1329-36

Bucher, E., Hemmes, H., De Haan, P., Goldbach, R. and Prins, M. (2004). The *Influenza A virus* NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* **85**, 983-991

Burgui, I., Aragon, T., Ortín, J. and Nieto, A. (2003). PABP1 and eIF4G associate with *Influenza virus* NS1 protein in viral mRNA translation intiation complexes. *J Gen Virol* **84**, 3263-3274

Chen, Z. Y., Li, Y. Z. and Krug, R. M. (1999). *Influenza A virus* NS1 protein targets poly(A)-binding protein II of the cellular 3 '-end processing machinery. *EMBO J* **18**, 2273-2283

Chung, T. D., Cianci, C., Hagen, M., Terry, B., Matthews, J. T., Krystal, M. and Colonno, R. J. (1994). Biochemical studies on capped RNA primers identify a class of oligonucleotide inhibitors of the *Influenza virus* RNA polymerase. *Proc Natl Acad Sci USA* **91**, 2372-2376

De La Luna, S., Fortes, P., Beloso, A. and Ortín, J. (1995). *Influenza virus* NS1 protein enhances the rate of translation initiation of viral mRNAs. *J Virol* **69**, 2427-2433

Delgadillo, M. O., Saenz, P., Salvador, B., Garcia, J. A. and Simon-Mateo, C. (2004). Human *Influenza virus* NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J Gen Virol* **85**, 993-999

Detjen, B. M., Angelo, C. S. T., Katze, M. G. and Krug, R. M. (1987). The three *Influenza virus* polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J Virol* **61**, 16-22

Di Bonito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. and Giorgi, C. (1999). Immunological characterization of *Toscana virus* proteins. *Arch Virol* **144**, 1947-1960

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 2545-2552

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D. H. (1985). Analyses of the mRNA transcription processes of *Snowshoe hare bunyavirus* S and M RNA species. *J Virol* **55**, 681-689

Fabian, m. R. and White, K. A. (2004). 5'-3' RNA-RNA interaction facilitates cap- and poly(A) tail-independent translation of *Tomato bushy stunt virus* mRNA: a potential common mechanism for *Tombusviridae*. *J Biol Chem* **279**, 28862-28872

Gallie, D. R. and Kobayashi, M. (1994). The role of the 3' untranslated region of non-polyadenylated plant viral mRNAs in regulating translational efficiency. *Gene* **142**, 159-165

Gallie, D. R. (1998). A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**, 1-11

Hagen, M., Tiley, L., Chung Thomas, D. Y. and Krystal, M. (1995). The role of template-primer interactions in cleavage and initiation by the *Influenza virus* polymerase. *J Gen Virol* **76**, 603-611

Honda, A., Mizumoto, K. and Ishihama, A. (1986). RNA polymerase of *Influenza virus* dinucleotide-primed initiation of transcription at specific positions on viral RNA. *J Biol Chem* **261**, 5987-5991

Iapalucci, S., Lopez, N. and Franze-Fernandez, M. T. (1991). The 3' end termini of the *Tacaribe Arenavirus* subgenomic RNAs. *Virology* **182**, 269-278

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. and Elliott, R. M. (2003). *Bunyamwera virus* nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death. *J Virol* **77**, 7999-8008

Krug, R. M., Yuan, W., Noah, D. L. and Latham, A. G. (2003). Intracellular warfare between human *Influenza* viruses and human cells: the roles of the NS1 protein. *Virology* **309**, 181-189

Leonard, S., Viel, C., Beauchemin, C., Daigneault, N., Fortin, M. G. and Laliberte, J. F. (2004). Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein in planta. *J Gen Virol* **85**, 1055-1063

Li, W.-X., Li, H., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E. W. A., Johnson, K. L., García-Sastre, A., Ball, L. A., Palese, P. and Ding, S.-W. (2004). Interferon antagonist proteins of *Influenza* and *Vaccinia* viruses are suppressors of RNA silencing. *Proc Natl Acad Sci USA* **101**, 1350-1355

Marión, R. M., Aragón, T., Beloso, A., Nieto, A. and Ortín, J. (1997). The N-terminal half of the *Influenza virus* NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. *Nucleic Acids Res* **25**, 4271-4277

Matsuda, D. and Dreher, T. W. (2004). The tRNA-like structure of *Turnip yellow mosaic virus* RNA is a 3'-translational enhancer. *Virology* **321**, 36-46

Meulewaeter, F., van Lipzig, R., Gultayev, A. P., Pleij, C. W., van Damme, D., Cornelissen, M. and van Eldik, G. (2004). Conservation of RNA structures enables TNV and BYDV 5' and 3' elements to cooperate synergistically in cap-independent translation. *Nucleic Acids Res* **32**, 1721-1730

Mir, M. A. and Panganiban, A. T. (2004). Trimeric *Hantavirus* nucleocapsid protein binds specifically to the viral RNA panhandle. *J Virol* **78**, 8281-8288

Neeleman, L., Olsthoorn, R. C. L., Linthorst, H. J. M. and Bol, J. F. (2001). Translation of a nonpolyadenylated viral RNA is enhanced by binding of viral coat protein or polyadenylation of the RNA. *Proc Natl Acad Sci USA* **98**, 14286-14291

Osborne, J. C. and Elliott, R. M. (2000). RNA binding properties of *Bunyamwera virus* nucleocapsid protein and selective binding to an element in the 5 ' terminus of the negative-sense S segment. *J Virol* **74**, 9946-9952

Peng, Q., Shi Jose, M. G. and Summers Donald, F. (1996). *Influenza A virus* RNA-dependent RNA polymerase cleaves influenza mRNA *in vitro*. *Virus Res* **42**, 149-158

Pinschewer, D. D., Perez, M. and de la Torre, J. C. (2003). Role of the virus nucleoprotein in the regulation of *Lymphocytic choriomeningitis virus* transcription and RNA replication. *J Virol* **77**, 3882-3887

Qanungo, K. R., Shaji, D., Mathur, M. and Banerjee, A. K. (2004). Two RNA polymerase complexes from *Vesicular stomatitis virus*-infected cells that carry out transcription and replication of genome RNA. *Proc Natl Acad Sci USA* **101**, 5952-5957

Qiu, Y. and Krug, R. M. (1994). The *Influenza virus* NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol* **68**, 2425-2432

Rao, P., Yuan, W. M. and Krug, R. M. (2003). Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis. *EMBO J* **22**, 1188-1198

Richmond, K. E., Chenault, K., Sherwood, J. L. and German, T. L. (1998). Characterization of the nucleic acid binding properties of *Tomato spotted wilt virus* nucleocapsid protein. *Virology* **248**, 6-11

Salvatore, M., Basler, C. F., Parisien, J. P., Horvath, C. M., Bourmakina, S., Zheng, H. Y., Muster, T., Palese, P. and García-Sastre, A. (2002). Effects of *Influenza A virus* NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. *J Virol* **76**, 1206-1212

Shih, S.-R. and Krug, R. M. (1996). Surprising function of the three *Influenza* viral polymerase proteins: Selective protection of viral mRNAs against the cap-snatching reaction catalyzed by the same polymerase proteins. *Virology* **226**, 430-435

Simons, J. F., Persson, R. and Pettersson, R. F. (1992). Association of the nonstructural protein NSs of *Uukuniemi virus* with the 40S ribosomal subunit. *J Virol* **66**, 4233-4241

Skorko, R., Summers, D. F. and Galarza, J. M. (1991). *Influenza A virus in vitro* transcription: roles of NS1 and NP proteins in regulating RNA synthesis. *Virology* **180**, 668-77

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. and Okuno, T. (2002). Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. *FEBS Letters* **532**, 75-79

Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P. and Garcia-Sastre, A. (2000). Activation of interferon regulatory factor 3 is inhibited by the *Influenza A virus* NS1 protein. *J Virol* **74**, 7989-7996

Taschner, P. E., van der Kuyl, A. C., Neeleman, L. and Bol, J. F. (1991). Replication of an incomplete *Alfalfa mosaic virus* genome in plants transformed with viral replicase genes. *Virology* 181, 445-450

Vialat, P. and Bouloy, M. (1992). *Germiston virus* transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66**, 685-693

Wang, X. Y., Li, M., Zheng, H. Y., Muster, T., Palese, P., Beg, A. A. and García-Sastre, A. (2000). *Influenza A virus* NS1 protein prevents activation of NF-kappa B and induction of alpha/beta interferon. *J Virol* **74**, 11566-11573

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. and Elliott, R. M. (2002). *Bunyamwera bunyavirus* nonstructural protein NSs counteracts the induction of alpha/beta interferon. *J Virol* **76**, 7949-7955

SUMMARY

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* within the *Bunyaviridae*, a family of segmented negative strand RNA viruses. Although much ground has been covered in the past two decades, many questions concerning the mechanism of replication and transcription of this important group of viruses have thus far remained unanswered. Elucidation of the molecular mechanisms of viral transcription and replication requires manipulable systems in which these processes can be studied and unravelled. At the onset of the research described in this thesis, an *in vitro* assay was available in which RdRp (RNA-dependent RNA polymerase) activity of purified TSWV virions was observed, although it was unclear what kind of RNA synthetic activity was observed: transcription or replication.

With the aim to unravel the molecular details of TSWV transcription, the *in vitro* assay was further developed into a well-defined transcription assay (Chapter 2). Transcription of TSWV, like that of all segmented negative strand RNA viruses, is initiated by cap snatching. In this process, a host mRNA (i.e. cap donor) is cleaved in its non-coding leader sequence by a virally encoded endonuclease, and the resulting capped leader is subsequently used to prime transcription of the viral genome. The existing *in vitro* system, in which only viral replication was observed, was modified by the addition of rabbit reticulocyte lysate (RRL) which allowed viral transcription to take place. In this way a system was established in which the mechanism of cap snatching could be studied. Not only globin mRNAs present in the lysate, but also exogenously added capped RNA molecules were used as cap donors.

The observed requirement for RRL seemed to imply a translational dependence of TSWV transcription. This possibility was further investigated in Chapter 3, using two inhibitors of protein synthesis. Surprisingly, in contrast to what has been observed for other Bunyaviruses, addition of these translation inhibitors had no effect on TSWV transcription *in vitro*. Moreover, no actual protein synthesis could be detected in the *in vitro* transcription assay, due to the assay conditions being incompatible with *in vitro* translation. These results indicated that TSWV transcription, unlike that of several other *Bunyaviridae*, is not coupled to translation.

In the absence of RRL, viral replication was observed in the *in vitro* system (Chapter 2), although at a much lower rate than transcription. Not only RNPs (ribonucleoprotein complexes) as present in the lysed virus particles, but also purified cytoplasmic RNPs were active in RNA synthesis in the *in vitro* assay. However, in contrast to virion-RNPs, cytoplasmic RNPs were found to be highly active in replication (in the absence of RRL)

but hardly active in transcription (in the presence of RRL). These findings imply the existence of RNPs in two different states: replication-mode and transcription-mode.

As a first step towards understanding transcription termination, a rough mapping of the 3'-ends of the S RNA-encoded mRNAs was undertaken (Chapter 4). Viral mRNAs synthesised in the *in vitro* system were amplified by RT-PCR using primers for the leader sequence snatched from α-globin mRNAs in combination with a range of primers spanning the entire intergenic region (IR) of the S RNA segment. In addition, the size of the N mRNAs produced in infected plants was compared with the sizes of synthetic transcripts representing mRNAs terminated at defined positions in the intergenic region. The results indicated that transcription terminates near the 3'-end of the intergenic hairpin, yielding mRNAs that contain this hairpin structure at their 3' end, including a conserved sequence motif. These findings point toward a transcription termination mechanism reminiscent of that used by prokaryotes, where formation of a hairpin structure in the nascent transcript induces termination of transcription.

Meanwhile, based on a concurrent PhD study, a model for TSWV cap snatching was proposed in which a cap donor (host mRNA leader) is required to have a single base complementarity to the viral template for transcription. The globin mRNAs in the RRL that are used as cap donor in the *in vitro* assay (Chapter 2) have 2 bases complementary to the viral template, which led to the question how far this complementarity could be extended. To investigate this, a capped transcript resembling a TSWV N mRNA, having a 15-nt stretch of complementarity, was tested and indeed found to be used as cap donor (Chapter 2), i.e. the 'viral mRNA' was re-snatched. The potential for re-snatching of viral mRNAs occurring *in vivo* was investigated in Chapter 5. Competition assays using single-, double- and multiple-basepairing cap donors indicated a preference for donors with a long extent of base complementarity to the viral template. This implies that viral mRNAs would preferentially be used as cap donors, in other words re-snatching of viral mRNAs would prevail over snatching of host mRNAs. In addition, primer extension analyses on the products of a re-snatching reaction were used to examine the exact endonuclease cleavage site of re-snatching, indicating that endonuclease cleavage takes place after the first or second nucleotide complementary to the viral template.

In chapter 6 the results presented in chapters 2-5 and their implications are discussed in relation to current knowledge on replication, transcription, and translation of negative and ambisense RNA viruses. The current model for the mechanism of cap snatching is fine-tuned further in view of the findings reported in Chapter 5.

SAMENVATTING

Het *Tomatenbronsvlekkenvirus* (Engelse naam: *Tomato spotted wilt virus,* afgekort TSWV), behoort tot het genus *Tospovirus* binnen de *Bunyaviridae,* een grote familie van virussen met een gesegmenteerd minstrengs RNA genoom. Hoewel er de afgelopen 20 jaren veel onderzoek is verricht naar de wijze van vermenigvuldiging van deze virussen, zijn vele vragen omtrent hun replicatie- en transcriptiestrategie onbeantwoord gebleven. Bestudering van de mechanismen van virale genoomreplicatie en -transcriptie vereist manipuleerbare systemen, waarin de verschillende stappen ontleed en onderzocht kunnen worden. Bij aanvang van het in dit proefschrift beschreven onderzoek was er voor TSWV een *in vitro* systeem voorhanden waarin virusdeeltjes, uit planten gezuiverd, RNA-synthetiserende activiteit vertoonden. Het was echter niet duidelijk welk soort activiteit dit was: replicatie of transcriptie.

Teneinde het mechanisme van transcriptie van TSWV nader te kunnen bestuderen, werd dit *in vitro* systeem verder ontwikkeld (Hoofdstuk 2). De RNA synthetiserende activiteit die werd waargenomen in het systeem bleek virale genoomreplicatie te zijn. Virale transcriptie, d.w.z. synthese van de virale mRNA's, kon bewerkstelligd worden door toevoeging van (konijnen-) reticulocyten-lysaat, een extract dat doorgaans gebruikt wordt voor *in vitro* vertaling (translatie) van mRNA naar eiwitten. Nadere analyse van de in aanwezigheid van het reticulocyten-lysaat gesynthetiseerde mRNA's leerde dat deze een niet-virale 5'-terminale nucleotidenvolgorde bezitten die het resultaat is van "cap snatching". Dit is het mechanisme van transcriptie-initiatie dat gebruikt wordt door alle gesegmenteerde minstrengs RNA virusen om hun mRNA's van de benodigde 5'-*cap* structuur (een ^{m7}Gppp-groep) te voorzien. Tijdens *cap snatching* wordt een *gecapte* cellulaire mRNA gekliefd door de endonuclease-activiteit van het virale polymerase. De zo ontstane korte, *gecapte* 5'-"leader" wordt vervolgens verlengd tot een virale mRNA, met een der genomische RNA's als matrijs. De in het *in vitro* systeem geproduceerde TSWV mRNA's bleken 5'-*leaders* te bezitten afkomstig van de globine-mRNA's in het reticulocyten-lysaat. Daarnaast konden ook andere toegevoegde mRNA's gebruikt worden als zogeheten *cap donor*. Zodoende bleek het *in vitro* systeem, gesupplementeerd met reticulocyten-lysaat, geschikt om het mechanisme van TSWV transcriptie nader te onderzoeken.

De bevinding dat reticulocyten-lysaat, d.w.z. een eiwit-synthetiserend systeem, moest worden toegevoegd aan het TSWV *in vitro* transcriptiesysteem leek erop te wijzen dat bij TSWV de transcriptie gekoppeld is aan translatie. Dit werd in Hoofdstuk 3 nader onderzocht met behulp van specifieke remmers van de eiwtisynthese. Toevoeging van deze remmers aan de *in vitro* transcriptie reacties bleek echter geen effect te hebben,

noch op het profiel van de RNA producten noch op initiatie van transcriptie d.m.v. *cap snatching*. Verder onderzoek bevestigde dat er in de transcriptie-reactie geen eiwitsynthese plaatsvindt, ondanks de aanwezigheid van het translatiesysteem. Deze resultaten wezen er op dat transcriptie van TSWV *in vitro* niet gekoppeld is aan translatie, en dat de noodzaak van toevoeging van het lysaat berust op een andere stimulerende factor.

In afwezigheid van reticulocyten-lysaat vond in het TSWV *in vitro* systeem uitsluitend replicatie plaats, hoewel in lagere mate dan transcriptie in aanwezigheid van het lysaat (Hoofdstuk 2). Virale ribonucleoproteïne-complexen (RNP's), bestaande uit viraal genomisch RNA, nucleoproteïne (N eiwit) en polymerase, zijn de actieve eenheden die transcriptie en replicatie uitvoeren. Wanneer in het *in vitro* systeem gezuiverde cytoplasmatische RNP's gebruikt werden in plaats van (RNP's uit) gezuiverde virus-deeltjes, bleek de mate van activiteit precies andersom te zijn (Hoofdstuk 2): cytoplasmatische RNPs waren bijzonder actief in replicatie (in afwezigheid van het lysaat) en nauwelijks actief in transcriptie (in aanwezigheid van het lysaat). Deze bevindingen lijken te wijzen op het bestaan van RNP's die op verschillende manieren geactiveerd zijn.

Teneinde het mechanisme van transcriptie-terminatie op te helderen werden in Hoofdstuk 4 de 3'-uiteinden bepaald van twee virale mRNA's. Hiertoe werden de *in vitro* van het S-segment overgeschreven mRNAs vermenigvuldigd d.m.v. RT-PCR. Bovendien werd d.m.v. "Northern blot"-analyse de grootte van twee transcripten met bekende lengte vergeleken met de grootte van het N mRNA zoals deze *in vivo* tijdens het infectieproces gemaakt wordt. Deze analyses toonden aan dat terminatie van transcriptie plaatsvindt aan het 3'-eind van een nucleotidenvolgorde die een stabiele haarspeld-structuur ofwel *hairpin* kan vormen. Hieruit volgt dat beide mRNA's van het S-segment een 3'-terminale hairpin bevatten, hetgeen zou kunnen wijzen op een mechanisme van transcriptie-terminatie zoals dat bij prokaryoten aangetroffen wordt: de vorming van een *hairpin* in het groeiende transcript veroorzaakt beëindiging van transcriptie.

Tijdens de uitvoering van het in dit proefschrift beschreven onderzoek, leidden de resultaten van een parallel onderzoeksproject tot een model voor het mechanisme van *cap snatching*. In dit model is sprake van een enkelvoudige complementariteit tussen het 3'-uiteinde van de virale matrijs en een geschikte *cap donor*. De in Hoofdstuk 2 beschreven resultaten toonden aan dat ook *cap donoren* met de mogelijkheid tot tweevoudige baseparing gebruikt worden voor transcriptie-initiatie. Dit riep de vraag op of er een limiet zou zijn aan de mate van complementariteit tussen de *cap donor* en de virale matrijs. De bevinding dat zelfs synthetische transcripten die identiek waren aan virale mRNA's, inclusief een niet-virale 5'-*leader*, gebruikt werden als *cap donor* (Hoofdstuk 2), wees erop dat een dergelijke begrenzing kennelijk niet bestaat. Aangezien

het gebruik van virale mRNA's als *cap donor*, ofwel "re-snatching", *in vivo* onwaarschijnlijk leek, werd in Hoofdstuk 5 onderzocht of het virale polymerase een voorkeur had voor *cap donoren* met een hogere of juist een lagere mate van complementariteit. Verrassend genoeg werd *in vitro* een zeer duidelijke voorkeur waargenomen voor *cap donoren* met een hoge mate van complementariteit, m.a.w. het zou bij voorkeur zijn eigen mRNAs gebruiken als *cap donoren*. Tevens werd in Hoofstuk 5 de exacte positie van klieving in een *cap donor* met een lang stuk (14-nt) complementariteit onderzocht. Klieving van deze donor vond slechts plaats na de eerste en mogelijk ook na de tweede complementaire nucleotide, maar niet verderop.

In Hoofdstuk 6 worden de resultaten van de Hoofdstukken 2 t/m 5 besproken in het licht van de huidige kennis over transcriptie, replicatie en translatie van gesegmenteerde minstrengs RNA virussen. Op basis van de bevindingen van Hoofdstuk 5 wordt in dit hoofdstuk het bestaande model voor het mechanisme van *cap snatching* bijgesteld.

NAWOORD

Nothing in life is to be feared. It is only to be understood
Marie Curie

Een dikke zeven maanden na De Grote Verhuizing lijkt Wageningen erg ver weg, en de tijd dat ik op Viro als AIO rondliep een grijs verleden. Een jaar geleden had ik nog het naïeve idee dat ik dit stukje veel eerder zou kunnen schrijven, maar ik had het natuurlijk kunnen weten. Wie haalt het toch ook in z'n hoofd om tijdens die cruciale fase van het schrijven van een proefschrift naar een ander land te verhuizen?! Ach, verandering van spijs doet eten, of in mijn geval: verandering van land doet schrijven. Zo is het na een bijzonder intensieve zes maanden dan uiteindelijk toch gelukt, het ei is gelegd. Dat ik deze mijlpaal heb weten te halen dank ik aan heel veel mensen.

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CURRICULUM VITAE

Op 6 oktober 1971 werd ik geboren in Leiden en kreeg de naam Ingeborg Christine van Knippenberg. In juni 1990 behaalde ik mijn VWO-diploma aan het Rijnlands Lyceum te Oegstgeest. De twee daarop volgende jaren vulde ik voornamelijk met werken in de Leidse horeca, afgewisseld met een maand muziek studeren in York, Groot-Brittannië (oktober 1990) en 5 maanden Geschiedenis studeren aan de (toen nog) Rijksuniversiteit Leiden (september 1991 - januari 1992). In september 1992 begon ik aan de studie Scheikunde aan de Rijksuniversiteit Leiden. Tijdens deze studie deed ik een stage aan het Imperial College of Science, Technology and Medicine te Londen onder begeleiding van dr. Peter Nixon. Terug in Leiden deed ik een stage op het gebied van DNA-reparatie in E. Coli in de groep van dr. Nora Goosen. In september 1998 behaalde ik mijn doctorandus-titel in de Chemie. In april 1999 begon ik aan mijn promotie-onderzoek aan de vakgroep Virologie van de (toen nog) Landbouw Universiteit Wageningen, onder begeleiding van prof. dr. R.W. Goldbach en dr. R.J.W. Kormelink. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Sinds maart 2004 ben ik werkzaam als postdoc aan de University of Aberdeen, Schotland, waar ik onderzoek hoe de replicatie machinerie van E.Coli blokkades zoals DNA-gebonden transcriptiefactoren weet te omzeilen.

TSP (training and supervision plan)
Congressen en symposia: IUBMB Symposium on Genomic Replication of RNA viruses, Juni 2002, Helsinki, Finland (mondelinge presentatie); XIIth International Congress of Virology (ICV), Juli - Augustus 2002, Parijs, Frankrijk (poster presentatie); Keulen-Wageningen Meeting, April 2001, Keulen, Duitsland; Dutch Annual Virology Symposium (DAVS), Maart 2002, Utrecht (mondelinge presentatie), Maart 2003, Amsterdam; NWO-CW, werkgroep nucleïnezuren, December 2001, Lunteren, December 2002, Lunteren (mondelinge presentatie); Symposium van de Nederlandse Kring voor Plantevirologie (NKP), November 2001, Wageningen (mondelinge presentatie), December 2002, Wageningen
Cursussen: Cursus 'Techniques for Writing and Presenting a Scientific Paper', 2002, georganiseerd door de onderzoekschool Wageningen Institute of Animal Sciences (WIAS), Wageningen; Winterschool Bioinformatics, 2001, georganiseerd door de onderzoekschool Experimentele Plantwetenschappen (EPW), Wageningen.
Organisatie: Vertegenwoordiger van de vakgroep Virologie in de EPW AIO-raad 2001-2002
Publicaties: van Knippenberg, I., Goldbach, R. and Kormelink, R. (2002). Purified Tomato spotted wilt virus particles support both genome replication and transcription *in vitro*. *Virology* **303**, 278-286; van Knippenberg, I., Goldbach, R. and Kormelink, R. (2004). *Tomato spotted wilt virus* transcription *in vitro* is independent of translation. *Journal of General Virology* **85**, 1335-1338; van Knippenberg, I., Goldbach, R. and Kormelink, R. *Tomato spotted wilt virus* S-segment mRNAs have overlapping 3'-ends containing a predicted stem-loop structure and conserved sequence motif, *submitted;* van Knippenberg, I., Lamine, M., Goldbach, R. and Kormelink, R. *Tomato spotted wilt virus in vitro* displays a preference for cap donors with multiple base complementarity to the viral template, *submitted.*