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Purified *Tomato spotted wilt virus* Particles Support Both Genome Replication and Transcription *in Vitro*

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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 synthesized that hybridized to strand-specific probes for the N and NSs genes. Cloning of these transcripts demonstrated the presence of nonviral 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 mRNA, present in the reticulocyte lysate, as well as added *Alfalfa mosaic virus* (AMV) RNA sequences, were utilized as cap donors. Moreover, an artificially produced N mRNA containing an AMV-derived leader was shown to be used as cap donor, indicating that resnatching of viral mRNAs takes place *in vitro*.

Key Words: TSWV; *Bunyaviridae*; cap snatching; transcription *in vitro.*

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the plant viruses belonging to the genus *Tospovirus* within the arthropod-borne *Bunyaviridae* (Van Regenmortel *et al.,* 2000). Similar to 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 in the viral complementary (vc) sense and a nonstructural protein (NSs), of as yet unknown function, in the viral (v) sense (De Haan *et al.,* 1990). The M RNA (4.8 kb) codes for the glycoprotein precursor (GP) in the vc-sense and the cell-to-cell movement protein (NSm) in the v-sense (Kormelink *et al.,* 1992a, 1994). The L RNA (8.9 kb) codes for the viral RdRp in the 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 pseudocircular 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 nonviral leader sequences (Kormelink *et al.,* 1992b,c). 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 utilized 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 the 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), *LaCrosse virus* (Bellocq *et al.,* 1987b), and *Germiston virus* (Vialat and Bouloy, 1992), purified TSWV particles show only limited *in vitro* RdRp activity (Adkins *et al.,* 1995; Van Poelwijk, 1996). The 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

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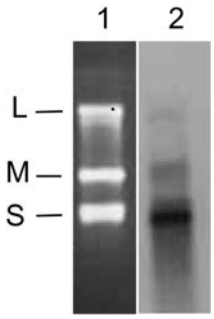

FIG. 1. Northern blot analysis using *in vitro* synthesized TSWV RNA as a probe. Ethidium bromide stained RNA profile of purified TSWV RNA resolved on a 1.5% agarose gel (Lane 1) and Northern blot hybridization of *in vitro* synthesized radiolabeled products to TSWV RNA (Lane 2). The positions of the L, M, and S RNA segments are indicated.

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 article 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, TSWV particles support either genome replication or genome transcription. Evidence for genuine viral transcription is provided by the synthesis of RNA molecules containing 5′ nonviral leader sequences. These nonviral leader sequences originated not only from endogenous RNA present in the reticulocyte lysate, but also from exogenously added cap donor RNAs.

RESULTS

RNA synthesis in the absence of reticulocyte lysate

For a first product analysis of *in vitro* synthesized RNA, purified TSWV was incubated in the presence of NTPs (including radiolabeled 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 synthesized (Fig. 1, Lane 2).

To identify the nature of the RNA molecules synthesized, *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 about 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 hybridized 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 synthesized as a result of replication. Occasionally, an additional distinct RNA product of 5 kb was observed (Fig. 2A, Lane 3), matching in size 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).

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 seemed more prevalent in the profile of RNA products (Fig. 2A, Lane 3), in general the profile did not differ from that of reactions that were performed in the absence of a cap donor during repeated analyses (Fig. 2A, Lane 2). Furthermore, no potential N or NSs transcripts could be observed after Northern blot hybridization (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 carrying out genome replication, as demonstrated by the

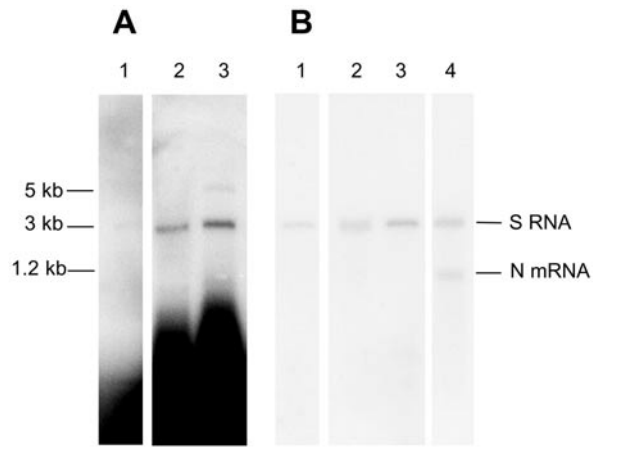

FIG. 2. *In vitro* RNA synthesis directed by TSWV virions in the absence of reticulocyte lysate. TSWV RNA synthesized *de novo* in the presence of ^{32}P-CTP was resolved on a 1.5% agarose gel and subsequently blotted to Hybond-N. (A) Northern blot of radiolabeled RNA synthesized *in vitro* by heat-inactivated virus (Lane 1) and untreated virus in the absence (Lane 2) and presence (Lane 3) of exogenous AMV RNA 4. (B) Northern blot hybridization of (A) with a strand-specific probe to detect vc-sense S RNA and N mRNA. Lanes 1–3 as in (A). Total RNA of TSWV-infected *Nicotiana rustica* (Lane 4). Hybridization was performed only after full decay of the radioactive signal from (A).

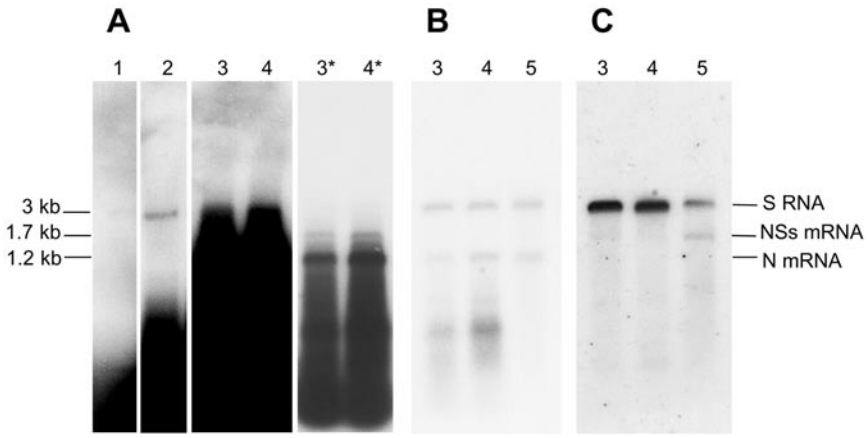

FIG. 3. Effect of reticulocyte lysate on *in vitro* RNA synthesis directed by TSWV virions. TSWV RNA synthesized *de novo* in the presence of ^{32}P-CTP was resolved on a 1.5% agarose gel and subsequently blotted to Hybond-N. (A) Northern blot of radiolabeled RNA synthesized *in vitro* by heat-inactivated virus (Lane 1) and untreated virus in the absence (Lane 2) and presence (Lane 3) of lysate, and in the presence of both lysate and AMV RNA 4 (Lane 4). Lanes marked with * indicate a shorter exposure. (B and C) Hybridization signal of the samples in (A) using strand-specific probes to detect vc-sense S RNA and N mRNA (B) and v-sense S RNA and NSs mRNA (C). Lanes 3–4 as in (A). Total RNA from TSWV-infected *Nicotiana rustica* (Lane 5).

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 *LaCrosse virus*, both representing animal-infecting members of the *Bunyaviridae*, addition of reticulocyte lysate 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 and Bouloy, 1992). To test whether the presence of reticulocyte lysate would also stimulate TSWV transcription *in vitro*, RdRp assays containing reticulocyte lysate were performed as described under Materials and Methods.

In the presence of lysate, the total amount 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 and 1.7 kb, respectively (Kormelink *et al.,* 1992b). After decay of the radioactive signal, Northern blot hybridization 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 hybridization 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 hybridization signal was most likely due to the low amount of *de novo* synthesized 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 synthesized in the presence of reticulocyte lysate (Fig. 3A, Lanes 4 and 4*).

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

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

To verify whether RNA synthesis in the presence of reticulocyte lysate was indeed genuine viral transcription, the RNA products were analyzed for the presence of nonviral 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 reticulocyte lysate as fragmented (micrococcal endonuclease cleaved) globin mRNAs.

Previously an RT-PCR protocol was developed to selectively amplify viral mRNAs with a nonviral leader sequence and to 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 specific internal primers for the TSWV N, NSm, and GP genes, 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). Subsequent cloning and sequencing of the PCR products obtained from the N gene transcripts showed that they

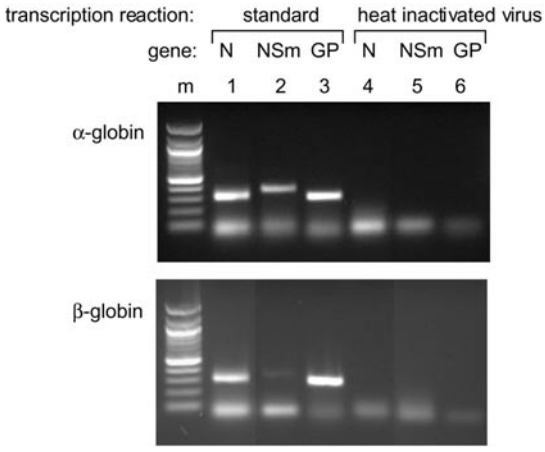

FIG. 4. RT-PCR analysis of *in vitro* synthesized TSWV RNA. RNA synthesized *in vitro* in the presence of reticulocyte lysate (using active virus in Lanes 1–3 and heat-inactivated virus in Lanes 4–6) was reverse transcribed using internal primers specific for the TSWV genes N, NSm, and the glycoprotein precursor 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 (top) or the β-globin (bottom) mRNA. The reaction and the TSWV genes are indicated above the panels. Lane m: 100-bp-size marker.

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*.

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 base-pairs 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 internal base-pairing 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 base-pairing (Fig. 5). This base-pairing is observed both to the ultimate UC dinucleotide and internally to the second UC dinucleotide of the template (Table 1 and Fig. 5). Since this α-globin mRNA was used by TSWV as a cap donor, the complementarity is apparently not restricted to single base-pairing. However, it still 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 base-pairing, though in this case with a dinucleotide GA at positions 13 and 14 (Fig. 5). With this cap donor both single base-pairing, of A14 with the ultimate U residue of the TSWV template, and double base-pairing, 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).

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 synthesized *in vitro* in the presence of an added mixture of AMV RNA 3 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 RNA 3 and 4, respectively, in combination with a nested internal primer for the N gene. Products of expected size were cloned and sequenced and indeed

TABLE 1

5′-Terminal Sequences of Viral N Gene Transcripts Synthesized *in Vitro*

Cap donor	5′-Terminal N mRNA 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 RNA 4	5′ *GUUUUUAUUUUUAA* GAGCAA...	2/9
	5′ *GUUUUUAUUUUUAA* GCAA...	5/9
	5′ *GUUUUUAUUUUUA* GAGAGCAA...	2/9
AMV RNA 3	5′ *GUAUUAAUACCAUUUUCA* GAGCAA...	5/5
Synthetic AMV RNA 3 (wild-type)	5′ *GUAUUAAUACCAUUUUCA* GAGCAA...	10/10
Synthetic AMV RNA 3 (mutant: C17G)	5′ *GUAUUAAUACCAUUUUGA* GAGCAA...	6/7
	5′ *GUAUUAAUACCAUUUUGA* GCAA...	1/7

Note. Sequence results showing *in vitro* synthesized TSWV N mRNA 5′ leader sequences derived from exogenous (AMV RNA 4, AMV RNA 3) or endogenous (α-globin, β-globin) mRNAs. Non-TSWV leader sequences are shown in italics. The donor residue that is assumed to base-pair with the viral template is represented in bold. Sequences underlined represent extra inserted residues.

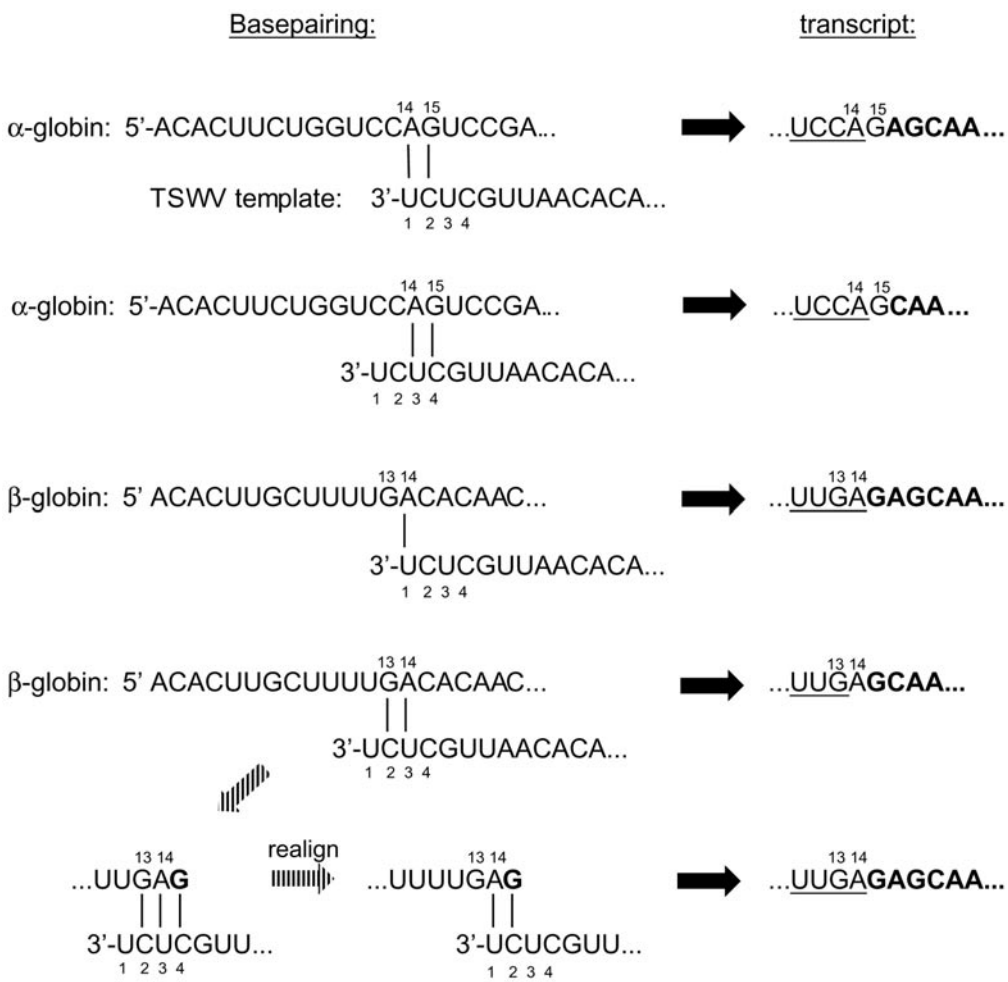

FIG. 5. Possible base-pairing interactions between globin mRNA cap donors and the TSWV template, and the transcript sequences obtained. The leader sequence derived from the cap donor is underlined; the nucleotides in bold represent viral sequence. Striped arrows indicate a possible prime-and-realign mechanism.

were shown to consist of 5′ terminal sequences of AMV RNA 3 and 4 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 RNA 3 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 was verified in RdRp assays without reticulocyte lysate. To this end, the RT-PCR reactions to detect AMV RNA 4-primed N mRNA were also performed on RNA synthesized *in vitro* in the presence of exogenous AMV RNA 4 but in the absence of lysate. No PCR products were obtained (data not shown), confirming that viral transcription *in vitro* required the presence of reticulocyte lysate.

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 *in vitro* transcripts of wild-type AMV RNA 3 or mutant RNA 3-C17G were provided as cap donor in an *in vitro* TSWV transcription reaction. RT-PCR and cloning analysis demonstrated that both transcripts were indeed used as cap donor (Table 1). Wild-type AMV RNA 3 transcript, similar to purified AMV RNA 3, consistently showed base-pairing to the ultimate template residue (Table 1). However, the C17G mutant also showed internal double base-pairing analogous to the situation described for the β-globin cap donor (see previous paragraph). These results indicate that the *in vitro* assay could be used as a reliable system to further study the process of cap snatching by testing mutable cap donors.

Resnatching of TSWV mRNA

The possibility that the viral transcription complex was capable of reusing (*de novo* synthesized) viral mRNAs as cap donors was examined. To this end, capped *in vitro* transcripts consisting of the AMV RNA 3 leader sequence fused to the N-gene sequence were synthesized and tested as cap donor. It was anticipated that if viral mRNAs were reused as cap donors *in vitro*, the AMV

TABLE 2

5′-Terminal Sequences of Viral NSs Gene Transcripts Synthesized *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* GAGAGCAA . . .	1/3

Note. Sequence results showing *in vitro* synthesized TSWV NSs mRNA 5′ leader sequences derived from a capped T7 transcript of AMV3-N mRNAs. AMV RNA 3 leader sequences are shown in italics. The donor residue that is assumed to base-pair with the viral template is represented in bold.

RNA 3 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 AMV RNA 3-N gene transcripts were added indeed revealed the synthesis of NSs transcripts preceded by the AMV RNA 3 leader sequence, while control reactions using heat-inactivated virus yielded no RT-PCR products (data not shown). Resnatching of viral mRNAs was confirmed by subsequent cloning and sequence analysis (Table 2). Two of the retrieved sequences confirmed that base-pairing took place as expected at A18 of the donor, followed by cleavage and elongation of the mRNA. However, one of the sequences resulted from base-pairing at A12 and incorporation of an extra G residue.

DISCUSSION

The results presented in this article demonstrate 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 reticulocyte lysate. 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 synthesized, whereas subgenomic-length RNA molecules remained (visually) absent from autoradiographic analyses of radiolabeled RNA molecules. Additional Northern blot hybridization and RT-PCR cloning analyses confirmed these observations. These results give the impression that replication occurs independently, without concurrent assembly of the nascent genome chain. However, only low amounts of genome-length products are observed, which may be the result of an abortive replication cycle due to the absence of a pool of free soluble N protein. This still remains to be investigated.

In vitro transcription in the presence of reticulocyte lysate has also been reported for *Germiston virus* and *LaCrosse virus* (Vialat and Bouloy, 1992; Bellocq *et al.,* 1987; Bellocq and Kolakofsky, 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 stabilization of the nascent transcript by scanning ribosomes, inhibiting base-pairing interactions of the nascent strand, presumably with its template, and thereby preventing premature termination of transcription that normally took place in the absence of lysate. This was further confirmed by assays in the absence of reticulocyte lysate, but with GTP replaced by inosine triphosphate (ITP), which weakens RNA–RNA interactions. This abolished premature termination at the typical termination sites that were commonly observed in the absence of lysate (Bellocq *et al.,* 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 virus* (RHBV), also an ambisense RNA plant virus, was found to be independent of translation (Nguyen *et al.,* 1997). Apparently, processes such as transcription and replication seem more conserved within families than between plant-infecting ambisense RNA viruses.

The occurrence of transcription in the presence of reticulocyte lysate was not only supported by Northern blot analyses, but also by cloning of molecules harboring non-TSWV leader sequences from products of an *in vitro* transcription reaction. Nonviral leader sequences present at the 5′-end of TSWV RNA molecules are the result of cap snatching, a process by which an endonuclease activity of 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 utilized as cap donor (Duijsings *et al.,* 1999) and that the 5′-leader sequence of the AMV RNA requires the presence of a residue around 16 nt from the 5′-end that allows base-pairing 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 RNA 3 and 4 leaders *in vitro* (Table 1) yields similar cleavage profiles as obtained from *in vivo* studies (Duijsings *et al.,* 1999, 2001).

The appearance of similar RNA profiles in the absence or presence of additional AMV cap donor was not sur-

prising as the reticulocyte lysate is likely to contain sufficient amounts of (fragmented) endogenous cap donors despite being micrococcal nuclease treated. Previously, 5′-RACE cloning of *Germiston virus* mRNAs synthesized *in vitro* in the presence of untreated lysate revealed 5′ leader sequences derived from α- and β-globin mRNAs (Vialat and Bouloy, 1992). When primers for these leader sequences were used in RT-PCR analyses of TSWV mRNAs synthesized *in vitro*, cap snatching of both globin mRNAs indeed was demonstrated.

Moreover, the results obtained with α- and β-globin mRNAs and with a mutant AMV RNA 3 (C17G) as cap donor indicate that the presence of two base-pairing residues does not exclude these RNAs from being used as cap donor. However, the effect of two base-pairing 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 base-pairing, cleavage is expected to occur after the base-paired A residue (Duijsings *et al.,* 2001). In the case of double base-pairing, two possibilities exist: cleavage after the first or cleavage after the second base-paired residue. Although it is tempting to assume that cleavage will occur after the most downstream positioned (second) base-paired residue, this scenario does not seem likely for the observed resnatching of viral mRNAs, which can have up to 100% complementarity. The resnatching *in vitro* favors the hypothesis that cleavage takes place after one of the first base-paired residues. This would be analogous to, and in agreement with, observations for *Influenza virus*, where elongation of the transcription-initiation complex has been demonstrated to occur from the first, second, and third viral residue, although with decreasing efficiencies, but not from the fourth viral residue (Honda *et al.,* 1986). It will therefore be important to determine the actual cleavage site of multiple base-pairing cap donors, to further elucidate the mechanism of cap snatching.

Interestingly, base-pairing of the β-globin mRNA to the TSWV template was found to occur exclusively on A14 (with or without concurrent base-pairing of G13), whereas A16, which was previously found to be the optimal base-paired nucleotide, was not used (Table 1). It is tempting to conclude that the presence of G13 influences the site of base-pairing, leading to internal double base-pairing of G13A14. However, half of the clones obtained seemed to indicate single base-pairing 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 base-pairing, resulting in exclusively internal (double) base-pairing, which in combination with a "prime-and-realign" mechanism would result in transcripts indiscernible from mRNAs resulting from single base-pairing 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 resnatching 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 recleavage of functional viral mRNAs. This may require the presence of an additional viral protein, not present in the virion, and NSs could be a potential candidate for this function.

MATERIALS AND METHODS

Virus purification

TSWV isolate BR-01 was purified from systemically infected leaves of *N. 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.

In vitro TSWV RdRp activity assay

In vitro RdRp assays were performed using 10–15 μg of purified TSWV in a final volume of 25 μl. Assays without reticulocyte lysate were done as described by Adkins *et al.* (1995), with slight modifications, and contained 20 mM HEPES pH 7.4, 0.5 mM Mg acetate, 5 mM $MnCl_2$, 2.5 mM DTT, 1 mM of each NTP, 0.1% NP-40, and 0.8 U/μl RNasin. Assays with lysate were done according to Nguyen *et al.* (1997). These assays contained 4 mM Mg acetate, 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°C for 1.5 h. For visualization of the RNA products, 2 μl 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 synthesized were phenol-chloroform extracted, ethanol precipitated, and resuspended in sterile ddH_2O. Radiolabeled RNA products were resolved by electrophoresis on a 1.5% agarose gel, followed by downward Northern blotting in 10× 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 hybridization

Synthesis of strand-specific riboprobes and hybridization 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. Hybridization of Northern blots was performed only

after full decay of the radioactive signal from the *in vitro* synthesized ^{32}P-labeled RNA present on these blots.

RT-PCR and cloning analyses

To amplify *in vitro* synthesized 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′-CCCTTCT-GACTCTGTGATC-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 transcription *in vitro* of (mutant) AMV RNA3 constructs

AMV RNA 3 transcripts were obtained by run-off transcription with T7 RNA polymerase of pXO32NcoP3, containing a full-length cDNA clone of AMV RNA 3 (Neeleman *et al.,* 1993). Transcription was performed in the presence of cap-analog m7G(5′)ppp(5′)G according to the manufacturer's procedures (Ambion mMESSAGE mMACHINE). Prior to transcription the DNA template was linearized 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 RNA 3 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 AMV RNA 3-N transcripts, T7 promoter sequences were introduced upstream of 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. (1987a). 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. (1987b). 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 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.

Duijsings, D., Kormelink, R., and Goldbach, R. (1999). Alfalfa mosaic virus RNAs serve as cap donors for tomato spotted wilt virus transcription during coinfection 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′ end 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.

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.

Van Poelwijk, F. (1996). On the expression strategy of the tospoviral genome. Ph.D. thesis, Wageningen Univ., pp. 1–100.

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.