Short Communication

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

Ingeborg van Knippenberg, Rob Goldbach and Richard Kormelink

Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

Correspondence
Richard Kormelink
richard.kormelink@wur.nl

Received 29 October 2003
Accepted 7 January 2004

Ongoing transcription *in vitro* of *Tomato spotted wilt virus* (TSWV) has previously been 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, an inhibitor that still allows scanning of nascent mRNAs by the 40S ribosomal subunit, and cycloheximide, an inhibitor that completely blocks translation including ribosome scanning. Neither of these inhibitors blocked TSWV transcription initiation or elongation *in vitro*, as demonstrated by *de novo*-synthesized 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.

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 virus 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 utilized 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 virus transcription or replication, depending on the presence or absence of rabbit reticulocyte lysate, respectively (Van Knippenberg *et al.*, 2002; see also Fig. 1A). In its presence, virus transcription was observed, as demonstrated by *de novo* synthesis of subgenomic RNA molecules (Fig. 1A, lane 3) that hybridized to strand-specific probes for the N and NSs genes and co-migrated with these mRNAs as found in total RNA from infected plants (Van Knippenberg *et al.*, 2002). Moreover, evidence for genuine virus 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 the reticulocyte lysate or from exogenously added AMV RNA.

The requirement for reticulocyte lysate for ongoing virus 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 the lysate was required for stabilization of the nascent transcript by scanning ribosomes. The observation that TSWV *in vitro* transcription also requires the presence of reticulocyte lysate (Van Knippenberg *et al.*, 2002) prompted the question of whether the underlying mechanism for this dependence was similar to that suggested for La Crosse and Germiston viruses. To investigate this requirement for reticulocyte lysate, the effect of translation inhibitors on *in vitro* TSWV transcription and transcription initiation, i.e. cap snatching, was analysed.

As a first step, TSWV transcription *in vitro* (i.e. in the presence of rabbit reticulocyte lysate) was performed as described previously (Van Knippenberg *et al.*, 2002) in the absence or presence of edeine and cycloheximide. In brief, 10–15 μg purified virus was incubated for 1·5 h at 30 °C in 25 μl reactions containing 4 mM magnesium acetate, 1 mM each NTP, 0·1 % NP-40, 0·8 U RNasin μl^{-1}, 2·5 μl AP-Biotech translation mix and 10 μl AP-Biotech rabbit reticulocyte lysate. For visualization of the RNA products, 2 μl [α-^{32}P]CTP (800 Ci $mmol^{-1}$) was added instead of CTP. No exogenous (AMV RNA) cap donor was added to the transcription assays, since the endogenous globin mRNAs present in the reticulocyte lysate have previously been shown to be used as cap donors (Van Knippenberg *et al.*, 2002). After extraction and precipitation, radiolabelled 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 profile on this Northern blot was analysed by autoradiography.

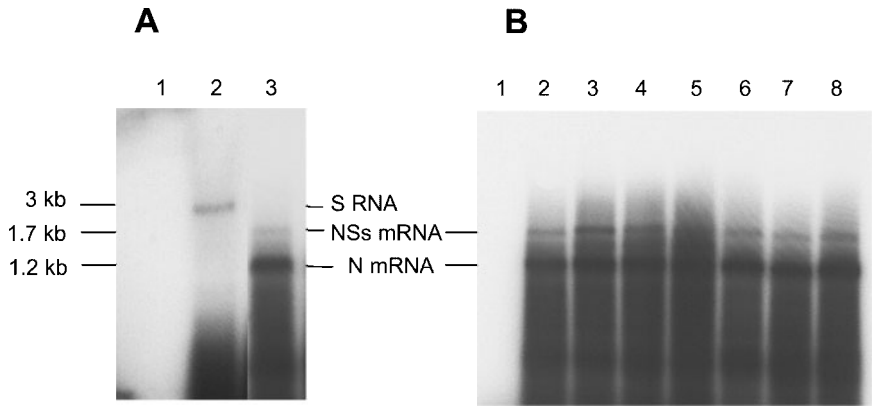

Fig. 1. Northern blot of radiolabelled TSWV *in vitro* transcription products. (A) RNA-dependent RNA polymerase (RdRp) activity of TSWV in the presence or absence of reticulocyte lysate. Lane 1, negative control reaction with heat-inactivated virus; lane 2, RdRp activity in the absence of lysate; lane 3, RdRp activity in the presence of lysate. (B) TSWV transcriptional activity in the presence of translation inhibitors. Lane 1, control reaction with heat-inactivated virus; lane 2, standard transcription reaction in the absence of inhibitors; lanes 3–5, transcription in the presence of cycloheximide at concentrations of 50, 100 and 250 μg ml^{-1}, respectively; lanes 6–8, transcription in the presence of edeine at concentrations of 10, 50 and 80 μM, 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 with 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, 7 and 8). These findings indicated that virus transcription does not depend on reticulocyte lysate for translation of a viral protein, nor for ribosome scanning of the nascent transcript.

To verify that cycloheximide and edeine were indeed functional in blocking *in vitro* translation, protein synthesis from control RNA templates (supplied with the rabbit reticulocyte lysate kit and performed according to the manufacturer's recommendations) was analysed by 12 % SDS-PAGE. 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 (Fig. 2, lane 1), no detectable protein synthesis was observed in the ^{35}S-labelled TSWV transcription reaction (Fig. 2, lane 3). In the presence of translation inhibitors, translation of control RNA was already effectively blocked at the lowest concentration used (results not shown). These data indicated that cycloheximide and edeine were indeed fully effective in blocking translation.

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 salt 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 reduced translation efficiency of the control RNA by approximately 80 and 95 %, respectively (results not shown), indicating that the circumstances used for virus transcription indeed hampered translation. Moreover, it was observed that translation of a control RNA in the presence of purified TSWV was severely reduced (Fig. 2, lane 2), although not

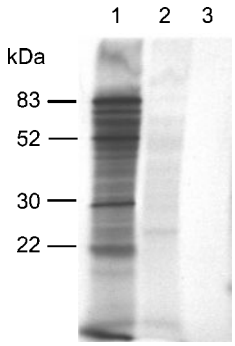

Fig. 2. SDS-PAGE of *in vitro*-synthesized ^{35}S-labelled products. Lane 1, standard translation of control RNA; lane 2, standard translation of control RNA in the presence of purified TSWV; lane 3, TSWV *in vitro* transcription reaction.

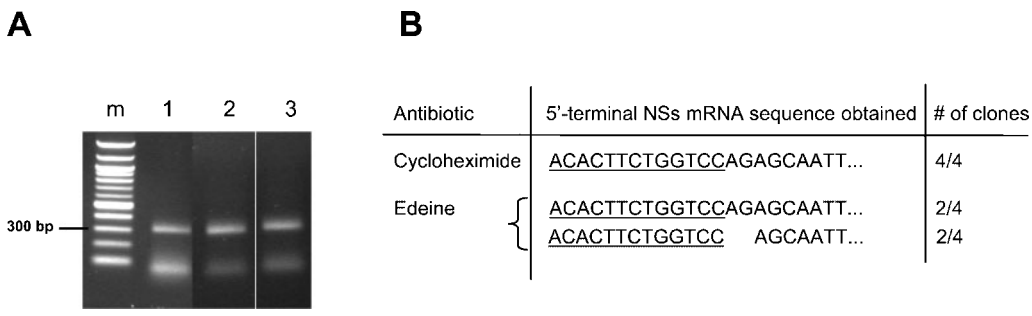

Fig. 3. The occurrence of cap snatching in the presence of translation inhibitors. (A) RT-PCR amplification of N gene transcripts with α-globin leader sequences synthesized in the TSWV *in vitro* transcription assay in the absence of inhibitors (lane 1), in the presence of 100 μg cycloheximide ml^{-1} (lane 2) and in the presence of 10 μM edeine (lane 3). Lane m, 100 bp molecular mass markers. (B) Sequence data. The PCR products of lanes 2 and 3 of (A) were cloned and sequenced, revealing the presence of α-globin leader sequences (underlined) at the 5′ end of N mRNA transcripts.

completely abolished. This decrease in translation activity was not due to the addition of NP-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 (results not shown).

To substantiate further the evidence that translation inhibitors did not affect TSWV transcription *in vitro*, viral RNA synthesized *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 globin mRNAs. 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 Van Knippenberg *et al.* (2002). 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 globin mRNA leader sequences. These results indicated that TSWV transcription initiation was not affected by inhibition of translation.

Stimulation of *in vitro* transcription by the addition of reticulocyte lysate has previously been reported for La Crosse virus (Bellocq & Kolakofsky, 1987; Bellocq *et al.*, 1987) and Germiston virus (Vialat & Bouloy, 1992), for which *in vitro* transcription in the absence of lysate always resulted in prematurely terminated transcripts. It was demonstrated that the lysate 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 base pair with C residues, was sufficient to compensate for the absence of lysate. For Germiston virus, the scanning ribosomes hypothesis was supported by the observation that ongoing transcription was still observed in the presence of the translation inhibitor edeine, where 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 reticulocyte lysate (Van Knippenberg *et al.*, 2002). The experiments described here have 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 lysate-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 which factor of the lysate confers stimulation of transcription.

The only other segmented ambisense RNA plant virus for which an *in vitro* transcription assay has been 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 reticulocyte lysate 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 (Moyer *et al.*, 1986; Hill *et al.*, 1986; De *et al.*, 1990; Ray & Fujinami, 1987). The identity of the factor required for TSWV transcription remains to be determined.

ACKNOWLEDGEMENTS

This work was supported in part by the Netherlands Foundation for Chemical Sciences (C.W.) with financial aid from the Netherlands Organization for Scientific Research (N.W.O.).

REFERENCES

Bellocq, C. & 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. & 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. & 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. & 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. & 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. & Herbert, E. (1967). Effects of cycloheximide on polyribosome function in reticulocytes. *J Mol Biol* **27**, 57–72.

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

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

Kozak, M. & 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. & Lessard, J. L. (1986). Tubulin: a factor necessary for the synthesis of both Sendai and vesicular stomatitis virus RNAs. *Proc Natl Acad Sci U S A* **83**, 5405–5409.

Nguyen, M., Ramirez, B. C., Goldbach, R. & 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. & Fujinami, R. S. (1987). Characterization of in vitro transcriptional products of measles virus. *J Virol* **61**, 3381–3387.

Van Knippenberg, I., Goldbach, R. & Kormelink, R. (2002). Purified *Tomato spotted wilt virus* particles support both genome replication and transcription *in vitro*. *Virology* **303**, 278–286.

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