Tomato RNA polymerase II interacts with the rod-like conformation of the left terminal domain of the potato spindle tuber viroid positive RNA genome

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INTRODUCTION

Viroids are small (approx. 300–400 nt), single-stranded, circular RNA pathogens infecting higher plants and inducing diseases (Flores et al., 2005, 2011; Tsagris et al., 2008; Ding, 2010). Unlike RNA viruses, viroids do not have a protein coat or protein-encoding capacity; they rely on specific RNA features to attract host proteins for their replication. Viroids are classified into two separate families, the Pospiviroidae and the Avsunviroidae (Flores et al., 2005). Most viroids fall into the family Pospiviroidae, fold into a rod-like secondary structure (Flores et al., 2005) and replicate in infected cells by an asymmetrical rolling-circle mechanism involving only RNA intermediates (Branch & Robertson, 1984). Circular monomeric RNAs of positive (+) polarity are used as templates to produce linear, multimeric, head-to-tail RNAs of negative (−) polarity. These (−) multimers then serve as templates for the synthesis of linear (+) multimers, which are cleaved and ligated into monomeric (+) circles.

Because the replication of viroids belonging to the family Pospiviroidae is inhibited by low concentrations of z-amanitin, a mycotoxin that inhibits DNA-dependent RNA synthesis by RNA polymerase II (RNAP II) (de Mercuyrol et al., 1989), it is accepted that host RNAPs II are involved in their replication. Specifically, a low concentration of z-amanitin was shown to inhibit the accumulation of cucumber pale fruit viroid (Mühlbach & Sänger, 1979), hop stunt viroid (Yoshikawa & Takahashi, 1986), citrus exocortis viroid (CEVd) (Flores & Semancík, 1982; Semancík & Harper, 1984; Flores, 1989; Rivera-Bustamante & Semancík, 1989; Rakowski & Symons, 1994) and potato spindle tuber viroid (PSTVd) (Rackwitz et al., 1981; Schindler & Mühlbach, 1992; Kolonko et al., 2006). Furthermore, CEVd, coconut cadang-cadang viroid (CCCVd) and PSTVd localize in the nucleoplasm (Bonfiglioli et al., 1994, 1996; Qi & Ding, 2003), consistent with a role of the nucleoplasm-localized RNAP II in their replication. Both polarities of CEVd RNA were reported to associate with the largest subunit of RNAP II in tomato (Warrilow & Symons, 1999), wheatgerm RNAP II binds PSTVd RNA (Goodman et al., 1984), and partially purified RNAP II complexes from both tomato tissue and wheatgerm were reported to transcribe from PSTVd RNA of (+) polarity (Rackwitz et al., 1981). On the other hand, both CCCVd and PSTVd also accumulate in the nucleolus of infected cells (Schumacher et al., 1983; Harders et al., 1989; Bonfiglioli et al., 1994; Qi & Ding, 2003), giving rise to speculation on the role of the nucleolus-localized RNAP II in the replication of these viroids (Bonfiglioli et al., 1996). However, no experimental data support a role for another RNAP in the replication of these viroids.

Five structural domains have been proposed for these viroids: terminal left (TL), pathogenicity (P), central (C), variable (V) and terminal right (TR) (Fig. 1) (Keese & Symons, 1985). Previous studies have implicated both the TL and C domains in replication (Baumnstark et al., 1997; Schrader et al., 2003; Kolonko et al., 2006; Zhong et al., 2008). Using an in vitro transcription assay on circular PSTVd (+) RNA and a potato cellular extract, a putative transcription start site located in the TL loop was proposed (Kolonko et al., 2006). However, the method used did not
allow characterization of the 5’ end of the transcript in order to discriminate between either an authentic transcription initiation site or an extremity generated by RNA cleavage/degradation. Additionally, a genome-wide mutational analysis of the PSTVd RNA genome revealed that the loop/bulge motifs located within the TL and C domains affect PSTVd accumulation in Nicotiana benthamiana protoplasts (Zhong et al., 2008). However, it was not possible to conclude whether these motifs are needed for the polymerization activity or for later events in the replication cycle (i.e. cleavage and ligation) (Baumstark et al., 1997; Schrader et al., 2003; Zhong et al., 2008).

Although several experiments have linked RNAP II with PSTVd replication (Rackwitz et al., 1981; Goodman et al., 1984; Schindler & Muhlbach, 1992; Kolonko et al., 2006), it is still unknown where RNAP II interacts with the PSTVd RNA genome. The objectives of this work were: (i) to determine whether RNAP II from a known natural host (i.e. tomato) interacts with PSTVd RNA; (ii) to identify the region(s) of the PSTVd genome interacting with RNAP II; and (iii) to investigate the RNA features needed for the interaction. The approaches used include co-immunoprecipitation experiments with in vitro-synthesized PSTVd-derived RNA and RNAP II from a tomato nuclear extract (NE), combined with deletion analyses and site-directed mutagenesis. Overall our results indicate that RNAP II interacts, either directly or indirectly, with the rod-like conformation of the TL domain of PSTVd (+) RNA.

RESULTS

Tomato RNAP II interacts with the TL domain of PSTVd (+) RNA

To establish the interaction between RNAP II and the PSTVd RNA genome, we performed co-immunoprecipitation experiments using in vitro-transcribed radiolabelled PSTVd RNA and tomato NE, as a source of RNAP II. For this study, we used RNAs derived from the KF5 strain of PSTVd, a mild isolate replicating efficiently in Rutgers tomato plant (Lakshman & Tavantzis, 1993). To immunoprecipitate RNAP II, we used the mouse mAb H14, which recognizes the highly conserved heptapeptide (i.e. YSPTSPS) located at the carboxy-terminal domain of the largest subunit of RNAP II (Bregman et al., 1995). As shown in Fig. 2(a), this antibody recognizes a protein in tomato NE with the same migration as the RNAP II found in HeLa NE, suggesting that it also recognizes the carboxy-terminal domain of the largest subunit of tomato RNAP II. Identical results were obtained using the 8WG16 mAb (data not shown), which recognizes the same epitope on the carboxy-terminal domain of RNAP II (Greco-Stewart et al., 2007).

We conducted the first set of co-immunoprecipitation experiments using full-length PSTVd (+) RNA. In vitro transcription was performed on a PCR fragment in order to generate a full-length PSTVd (+) RNA, which was open in the TL loop between positions 1 and 360. Following incubation of RNA with Rutgers tomato NE, which allowed for the formation of RNA–protein complexes, we added the H14 mAb. The samples were immunoprecipitated using protein-G–agarose beads, resolved by denaturing PAGE, and the radiolabelled RNA was visualized by phosphorimaging. We detected a band corresponding to radiolabelled PSTVd (+) RNA in the sample that was co-immunoprecipitated using the H14 mAb in the presence of NE (Fig. 2b). This band was not detected in the absence of NE or when using an anti-IgG antibody for the immunoprecipitation (Fig. 2b), indicating that the signal observed was not the result of non-specific binding of the RNA to either the H14 mAb or the beads. To ensure the specificity of the interaction, we also challenged the interaction with various RNA competitors. When we used commercial total tRNA from Escherichia coli as an unrelated RNA competitor, we observed no decrease in intensity of the co-immunoprecipitated radiolabelled RNA. We also competed the interaction with P11.60 (Pelchat et al., 2002), a small RNA derived from the peach latent mosaic viroid and folding into a hairpin structure, thus providing an excellent competitive RNA to test for non-specific binding of RNAP II to dsRNA molecules or stem-loop secondary structures. Upon the addition of a 50-fold molecular excess of P11.60, no decrease in the co-immunoprecipitation of the radiolabelled RNA was observed. However, when we added a 50-fold molecular excess of unlabelled homologous RNA, the band corresponding to the radiolabelled RNA was no longer detected. These observations indicate that tomato RNAP II interacts specifically with full-length PSTVd (+) RNA and that, because the RNA was open in the TL loop between

![Fig. 1. Sequence and secondary structure of the PSTVd (+) RNA genome. The five structural domains of pospiviruses are indicated: terminal left (TL), pathogenicity (P), central (C), variable (V) and terminal right (TR). The L70 region indicates the 70 nt PSTVd (+) RNA fragment located in the TL domain used as model in this study.](image-url)
positions 1 and 360, RNAP II interaction does not require an intact TL loop. Interestingly, we observed an increase in the band intensity for the lanes containing the two unrelated RNA competitors (i.e. tRNA and P11.60) compared with the lane with NE alone. Our interpretation for this observation is that there are many RNA-binding proteins present in the NE. By saturating these proteins with these RNA species, more PSTVd (+) RNA becomes available for interaction with RNAP II. However, this observation should be taken with caution, as the co-immunoprecipitation experiments were not quantitative.

In order to identify the region of PSTVd (+) RNA where RNAP II interacts, we divided the PSTVd RNA genome into two segments: the right half (R), which corresponds to the region between nt 92 and 269, and the left half (L), which corresponds to the region between nt 268 and 91. We designed appropriate primers for PCR amplification of the cDNA fragments corresponding to these regions, and we used the resulting DNA products as templates for in vitro transcription. The resulting PSTVd-derived RNAs were radiolabelled and used in co-immunoprecipitation experiments, as above. No radioactive band was detected when the co-immunoprecipitation assays were performed with the R half of the PSTVd (+) RNA genome [R(+); Fig. 2b]. However, when we performed the experiment using an RNA that corresponds to the L half of PSTVd (+) RNA, we observed a band corresponding to the radiolabelled RNA [L(+); Fig. 2b]. The immunoprecipitated L(+) RNA was also detected following the addition of 50-fold excess of the two unrelated competitors (i.e. tRNA and P11.60). Upon the addition of a 50-fold excess of non-radiolabelled L(+) RNA, the band intensity was greatly diminished. The band corresponding to radiolabelled L(+) RNA was also absent in the negative control, to which no NE was added.

Using the same experimental approach, we tested the interaction of tomato RNAP II with PSTVd (−) RNA. As before, the full-length PSTVd (−) RNA was open in the TL loop between positions 1 and 360. We detected a band corresponding to radiolabelled PSTVd (−) RNA when tomato NE was added to the samples (Fig. 2b). However, this band was very faint and could only be detected when the gel was overexposed. Surprisingly, the band corresponding to radiolabelled PSTVd (−) RNA was not only absent when either tomato NE was excluded or in the presence of homologous RNA, but also when a 50-fold excess of the two unrelated RNA competitors was used (Fig. 2b). Because the PSTVd (−) RNA molecule tested was open in the TL loop, it is possible that a nick at this location prevented recognition by RNAP II. To test this possibility, the co-immunoprecipitations were performed with either half of PSTVd (−) RNA (i.e. R(−) and L(−)). As shown in Fig. 2(b), no radioactive bands were detected when the co-immunoprecipitations were performed with any of the PSTVd (−) RNA tested.

To corroborate our findings, the association of RNAP II with the various PSTVd RNAs was tested using RNA-affinity chromatography followed by Western blotting to detect RNAP II. Each RNA species was oxidized and bound covalently to adipic acid dihydrazide agarose beads and incubated with tomato NE in the presence of an excess of E. coli tRNA. After washing of the beads, the proteins bound were eluted, subjected to SDS-PAGE and Western blotting with anti-RNAP II antibody. PSTVd(+), positions 1–360; PSTVd(−), positions 360–1; L(+), positions 268–91; L(−), positions 91–268; R(+), positions 92–269; R(−), positions 269–92.
Features of the RNAP II-interaction site on the PSTVd RNA genome

As only the L half of PSTVd (+) RNA exhibits a specific interaction with tomato RNAP II, we focused on this area in an attempt to investigate the RNA features needed for the interaction. Truncation of this region revealed that a TL segment consisting of a 70 nt hairpin (Fig. 1; L70) is still capable of specific interaction with RNAP II. Under our experimental conditions, we detected a band corresponding to radiolabelled L70 in the sample that was co-immunoprecipitated using the H14 mAb in the presence of tomato NE with or without an excess of commercial E. coli tRNA (Fig. 3). As above, the interaction between RNAP II and L70 was specific, as we did not detect the band in the absence of tomato NE and its intensity diminished greatly when we added a 50-fold molecular excess of unlabelled homologous RNA.

Using L70 as a model, various RNA mutants were constructed and tested for RNAP II interaction by co-immunoprecipitation with the H14 mAb, as above. First, we investigated the requirement for the predicted rod-like structure of this region. L70-1a to L70-1f are L70-derivative RNAs in which one or both of the two major double-stranded regions near the terminal loop are destabilized (Fig. 3, mutations indicated in white). No significant co-immunoprecipitation with any of these mutants was detected. Because these mutations also result in the disruption of their predicted rod-like structures, and also that unrelated secondary structures could be predicted for L70-1c to L70-1f (Fig. 3; see free energy values and shapes for the most stable conformations next to the autoradiograms), the absence of RNAP II interaction with these RNA molecules could be the result of the loss of their rod-like conformation. We therefore synthesized the ‘flip’ mutants L70-2a and L70-2b, which contain compensatory mutations restoring the predicted secondary structure of these hairpins, and tested them for association with RNAP II. In addition, we also tested L70-2c, which is a ‘flip’ mutant of the 2 nt double-stranded region. Bands corresponding to these ‘flip’ mutants were detected in the samples that were co-immunoprecipitated using the H14 mAb in the presence of tomato NE with or without an excess of commercial E. coli tRNA.

Finally, we tested the interaction with L70-SW, which has the same sequence as L70, but completely inverted. No band was detected when L70-SW was co-immunoprecipitated with the H14 mAb. Although the predicted secondary structure of L70-SW is similar to that of L70 near the terminal loop, the bulges are ‘flipped’ in relation to the structure. To test the importance of the bulged regions, three L70 derivatives were constructed where either the bulging nucleotides were removed (i.e. L70-AC13 and L70-DA10) or the internal loop was closed (i.e. L70-DIL). None of these mutants were able to co-immunoprecipitate with RNAP II, indicating the requirement of these three bulges for RNAP II interaction.

RNAP II interacts with the rod-like conformation of the TL domain of PSTVd (+) RNA

The TL domain of PSTVd (+) RNA has been proposed to be capable of adopting two distinct conformations: an unbranched rod-like structure and a bifurcated (i.e. Y-like) structure (Gast et al., 1996; Dingley et al., 2003). This is due to a twofold-complementary sequence repeat present in this region of the RNA genome (Fig. 4a). We were particularly interested in the importance of the bifurcated structure, as it is proposed only for the region interacting with RNAP II. To determine the extent of the conservation of these alternative secondary structures, we collected and aligned the sequences corresponding to L70 from a total of 156 natural variants indexed in the Subviral RNA Database (Rocheleau & Pelchat, 2006). Sequence alignments, the location of the predicted base pairs, and the calculated free energies (∆G) for the two alternative secondary structures are presented in Fig. 4(b, c). For both motifs, predicted base-paired nucleotides are also indicated as masked: black for the most frequent base pairs and grey for a mutation maintaining base pairing (e.g. G-C mutated into G-U). To simplify the alignments, identical sequences were combined, and their frequencies are indicated. For example, 131 PSTVd sequences (84%) corresponding to L70 were found to be identical in all of the variants analysed. This conservation suggests enhanced in vivo selected sequence fitness. Based on this analysis, we identified several mutations in these sequences that support both the rod-like and the Y-shape conformations by maintaining the formation of the base pairs (nucleotides with a grey background). Hence, in accordance with previous reports (Gast et al., 1996; Dingley et al., 2003), the sequence of the TL domain might have been selected to allow the formation of base-pairing interactions in one of two ways, giving rise to two possible secondary structures. However, our analysis also indicates that, for all variants tested, the rod-like conformation is energetically favoured (i.e. lower ∆G). The ∆G values for both conformations were calculated for all of the variants with the RNAeval software (Hofacker, 2003) using either the rod-like or Y-shape conformation as structural restraints [∆G in Fig. 4(b, c), respectively]. The mean calculated ∆G values were −34.4 ± 2.2 and −27.9 ± 2.2 kcal mol⁻¹ for rod-like and Y-shape conformations, respectively.

To investigate which of the two possible secondary structures is bound by RNAP II, the co-immunoprecipitation procedure used previously was employed. As presented above, both L70-2a and L70-2b interacted with RNAP II. These two mutants not only have short stretches of dsRNA inverted in such a way as to preserve base pairing, but are also predicted to favour the formation of the rod-like structure by removing the twofold-complementary sequence repeat found in L70. In contrast, L70-3a and L70-3b, which are predicted to strongly favour the bifurcated form, did not co-immunoprecipitate with RNAP II. When compensatory mutations were made to restore the 2 nt double-stranded region, thus allowing for the possibility of forming both structures (i.e. L70-3c), co-immunoprecipitation with RNAP II was observed.
These experiments suggest that RNAP II interacts with this region when it adopts a rod-like conformation. However, the structures of the various L70 mutants were not analysed experimentally. To confirm that the rod-like conformation is required for RNAP II interaction, we made use of two PSTVd point mutants extensively characterized by Dingley et al. (2003). Using nuclear magnetic resonance spectroscopy, temperature-gradient gel electrophoresis and UV spectroscopy, it was shown that an RNA corresponding to L70 adopts predominantly a rod-like conformation under various physico-chemical conditions (Dingley et al., 2003). A rod-like stabilized mutant (i.e. L70-U18C,A344G) adopts the same structure as L70 (Dingley et al., 2003). However, the mutant L70-A5G,U18C, predicted to favour the bifurcated form, adopts a conformation that is markedly different from that of L70 (Dingley et al., 2003). We decided to test the effect of these two mutants on RNAP II interaction. Consistent with our previous results, the point mutant favouring formation of the rod shape (mutant L70-U18C,A344G) exhibited

**Fig. 3. Interaction of RNAP II with various L70 mutants.** Co-immunoprecipitation experiments were performed using radiolabelled PSTVd L70 derivatives, tomato NE and the H14 mAb. Immunoprecipitates were run on denaturing polyacrylamide gel (10%) and visualized by autoradiography. A 50-fold excess of tRNA was used as a non-specific competitor, and a 50-fold excess of non-radiolabelled homologous RNA was used as a specific competitor. Input controls are indicated for each experiment. White font/crosses indicate changes made to L70. Also indicated are the free energy (ΔG) values and shapes for the most stable predicted conformations for each mutant.
specific interaction with RNAP II, whereas the RNA with the mutations favouring the branched structure (mutant L70-A5G,U18C) did not co-immunoprecipitate with RNAP II. Thus, the TL domain of PSTVd (+) RNA must adopt a rod-like secondary structure to interact specifically with RNAP II.

**DISCUSSION**

Based on the inhibition of PSTVd accumulation by low concentrations of α-amanitin, a role for host RNAP II in its replication was proposed (Rackwitz et al., 1981; Schindler & Muhlbach, 1992; Kolonko et al., 2006). Here, we have
investigated the first step of the transcription process (i.e. promoter recognition) by locating the interaction site of tomato RNAP II on the PSTVd RNA genome and the features needed for this interaction. Co-immunoprecipitation experiments using tomato NE and PSTVd-derived RNAs were performed using the H14 mAb, an antibody recognizing the serine 5-phosphorylated form of the carboxy-terminal domain of RNAP II, which is associated with the formation of a ‘closed complex’ at sites of transcription initiation (Bregman et al., 1995; Palancade & Bensaude, 2003; Smale & Kadonaga, 2003). Overall, our experiments indicate that RNAP II interacts specifically with the rod-like conformation of the TL domain of PSTVd (+) RNA, and support the idea that this enzyme is responsible for generation of the (−)-strand RNA. However, because NE was used, we cannot rule out the possibility that a protein partner mediates the RNAP II and PSTVd RNA interaction.

Surprisingly, we were not able to detect an interaction between RNAP II and the PSTVd (−) RNAs tested. This contrasts with a report showing that both polarities of CEVd RNA associate with RNAP II (Warrillow & Symons, 1999). In this study, co-immunoprecipitation of RNAP II with the CEVd RNAs was performed with a chromatin-enriched fraction of infected tomato leaf and the 8WG16 mAb. Although the different approach used could explain the divergence, because extracts of infected tomato leaf were used, it was impossible to differentiate between RNAP II interaction on the RNA template from that occurring in the course of nascent transcription during CEVd replication. RNAP II interacts and transcribes from PSTVd (+) templates (Kolonko et al., 2006); RNAP II interaction with PSTVd (−) RNAs could thus be expected to be associated as a complex with the RNA transcription products. The full-length PSTVd (−) RNA molecule that we tested was open in the TL loop; it is possible that such a nick might have prevented recognition by RNAP II. However, when we performed additional experiments using a PSTVd-derived RNA that corresponds to the L half of the genome without such a nick in the terminal loop (i.e. L(−)), no interaction with RNAP II was observed. Another possibility is that monomeric PSTVd (−) RNA might not be recognized by RNAP II. During the asymmetrical rolling-circle replication of PSTVd, the RNA template used to produce PSTVd (+) is a multimeric, head-to-tail RNA of (−) polarity. However, we could not detect an interaction with RNAP II when we used a trimeric PSTVd (−) RNA (data not shown). Alternatively, our results might also indicate that another host RNAP is involved in PSTVd replication, which is supported by the differential subnuclear accumulation of the (+) and (−) strands of PSTVd in both infected cultured cells and plant (Harders et al., 1989; Qi & Ding, 2003).

Once we established specific interaction between RNAP II and PSTVd (+) RNA, we used deletion analyses to determine that RNAP II interacts with the tip of the L half of the PSTVd (+)-strand RNA. As we only tested for interaction, but not for the polymerization activity, we could not conclude that the observed interaction leads to initiation of RNA transcription. Nonetheless, our data are consistent with various studies reporting (i) the presence of a 5′ end of PSTVd (−) RNA located in the TL loop (Kolonko et al., 2006), (ii) that the loop/bulge motifs located within the TL stem–loop are important for PSTVd accumulation in N. benthamiana protoplasts (Zhong et al., 2008), and (iii) that wheatgerm RNAP II preferentially binds one of the two extremities of the PSTVd RNA genome (Goodman et al., 1984).

To identify the sequence/structural requirement for interaction with RNAP II, we designed a series of RNA mutants. Interaction occurred when short stretches of sequence were flipped and was abolished when base pairs were disrupted, indicating that RNAP II recognizes a specific secondary structure. This is also consistent with the loss of infectivity of mutations eliminating the G-C base pairs at positions 343 and 344 (i.e. the segment disrupted by L70-1a); reversion was required for PSTVd accumulation (Kolonko et al., 2006). Also, disruption of base pairs adjacent to the terminal loop of the PSTVd genome resulted in both abolition of infectivity in tomato (Hammond & Owens, 1987; Hammond, 1994; Hu et al., 1997) and reduced accumulation in inoculated protoplasts of N. benthamiana (Zhong et al., 2008). However, these double-stranded segments do not seem to be sufficient as several other mutants, predicted to contain only minor changes in the overall secondary structure, did not demonstrate interaction. Mutants where either of the two bulging nucleotides is removed (i.e. L70-ΔA10 and L70-ΔC13) or the internal loop was closed (L70-ΔIL) did not exhibit interaction with RNAP II. The same mutants were reported to have significantly reduced accumulation in protoplasts of N. benthamiana (Zhong et al., 2008). This suggests that the overall secondary structure, together with some very specific features such as internal loops and bulges, is needed for the interaction. Surprisingly, the presence of the terminal loop was not required for RNAP II interaction, as indicated by the co-immunoprecipitation of RNAP II with the full-length PSTVd (+) RNA used [Fig. 2; PSTVd(+)], which was open in the TL loop. This indicates that, even if transcription initiates within the terminal loop (Kolonko et al., 2006), initial interaction of RNAP II might not require an intact loop.

The TL domain of PSTVd (+) RNA has the possibility of adopting one of two conformations, a rod-like and a bifurcated form (Gast et al., 1996; Dingley et al., 2003; Gast, 2003). Sequences of several variants in this region showed mutations supporting both conformations, although the rod-like conformation appears energetically favoured. To investigate the importance of the two possible conformations for RNAP II recognition, we designed mutants eliminating the possibility of forming one of the two conformations. Our experiments consistently showed that, in addition to the wild-type sequence, the mutants favouring the formation of the elongated rod-like structure
interacted with RNAP II, whereas those favouring the bifurcated form did not. This is in agreement with several mutational approaches of this region, indicating that the branched conformation does not appear to be significant for PSTVd replication (Hu et al., 1997; Owens & Thompson, 2005; Wiesyk et al., 2011). This also correlates with studies on the recognition of the hepatitis D virus (HDV) RNA genome by the human RNAP II. HDV shares several key features with viroids and is also replicated by RNAP II in human cells (Taylor & Pelchat, 2010; Flores et al., 2011). As observed for PSTVd, the HDV RNA domains used by RNAP II show a strong preference for maintaining rod-like conformations (Filipovska & Konarska, 2000; Greco-Stewart et al., 2007; Abrahem & Pelchat, 2008). Together, this suggests that an elongated rod-like conformation plays a role in mediating the interaction of RNA templates with RNAP II.

The present study indicates that specific features of the rod-like secondary structure of the TL region of PSTVd (+) RNA are recognized by RNAP II. Although the bifurcated conformation does not seem to be relevant for the initial interaction of RNAP II, it might serve an important biological purpose for another step in the viroid’s replication cycle, as this feature appears to be conserved among PSTVd variants. Further studies are necessary to determine the possible role of the conformation switching in this region and to investigate the role of both the bulges and the internal loop in the RNA tertiary structure needed for RNAP II interaction.

**METHODS**

**Synthesis of PSTVd-derived RNAs.** PCR using Vent polymerase (New England Biolabs) on a pBluescript II KS derivative containing a trimeric PSTVd cDNA [KF5 strain; (Lakshman & Tavantzis, 1993)] was employed for the amplification of PSTVd cDNA fragments. The T7 promoter sequence was incorporated into the appropriate primers used in the PCR in order to allow for subsequent synthesis of PSTVd RNA fragments of either (+) or (−) polarity. P11.60 and the L70 derivatives were synthesized using pairs of oligonucleotides as described previously (Pelchat & Perreault, 2004). The various RNA molecules were synthesized by in vitro run-off transcription using T7 RNA polymerase as described previously (Greco-Stewart et al., 2007). When needed, 10 pmol in vitro-synthesized RNA was 5′-end-radiolabelled with [γ-32P]ATP, as described previously (Greco-Stewart et al., 2007).

**Preparation of tomato NE.** Healthy young tomato leaves (Lycopersicon esculentum cv. Rutgers) were ground to a fine powder in liquid nitrogen, and NE was prepared using a Celllytic PN Plant Nuclei Isolation/Extraction kit (Sigma-Aldrich) in accordance with the manufacturer’s protocol. PMSF (1 mM) was added to inhibit endogenous proteases. Protein concentration was determined by Bradford assay (Bio-Rad). NE was flash-frozen and stored at −80 °C. The presence of RNAP II was verified by Western blot analysis [One-Step Western kit (GenScript Corporation)] with the mouse mAb H14 (Abcam), and NE from HeLa cells (Accurate Chemical and Scientific) as a control.

**Co-immunoprecipitations.** A Protein-G Immunoprecipitation kit (Sigma-Aldrich) was used for the co-immunoprecipitations as specified by the manufacturer, with minor modifications. Radiolabelled RNA (1 pmol) was incubated with 30 μg tomato NE in RIPA buffer (50 mM Tris/HCl, pH 7.5, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.05 % SDS, 1 mM EDTA, 150 mM NaCl) for 30 min at 4 °C. A 50-fold molecular excess of total E. coli tRNA (Sigma-Aldrich), P11.60 or non-radiolabelled homologous RNA was added to some reactions. Five micrograms of the mouse mAb H14 (Abcam) was then added to each reaction and incubated for 2 h at 4 °C. Pre-washed protein-G–agarose beads (50 μl) were added to the reactions and incubated for 4 h at 4 °C. Samples were then washed four times with RIPA buffer and eluted by incubation at 90 °C for 10 min with acrylamide loading buffer [0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 80 % formamide, 10 mM EDTA (pH 8.0)]. RNA was electrophoresed on denaturing 5 % polyacrylamide gels containing 7 M urea, and detected either by autoradiography or phosphorimaging.

**RNA-affinity chromatography.** RNA (200 pmol) was oxidized in 20 mM Tris/HCl pH 7.5 and 10 mM sodium metaperiodate at 4 °C for 1 h in the dark, ethanol-precipitated, resuspended in 0.1 M sodium acetate, pH 5, and coupled to pre-washed adipic acid dihydrazide agarose beads (Sigma-Aldrich) overnight at 4 °C. Unbound RNA was removed by three washes with binding buffer (10 mM Tris/HCl, pH 7.4, 10 mM KCl, 150 mM NaCl, 1 mM MgCl2, 5 % glycerol, RNA guard). Pre-cleared tomato NE (30 μg) containing 200 pmol total E. coli tRNA was then added to each reaction and incubated for 30 min at room temperature. Unbound proteins were removed by four washes with binding buffer. Proteins were eluted with SDS loading buffer at 90 °C, electrophoresed on SDS–polyacrylamide gels (10 % polyacrylamide) and detected by Western blot analysis with the mouse mAb H14 (Abcam).

**Computer analysis of the L70 region.** The L70 region was extracted from PSTVd sequences taken from the Subviral RNA Database (http://subviral.med.uottawa.ca/; Rocheleau & Pelchat, 2006). Alignments of the sequences were performed with CLUSTAL W (Thompson et al., 1994). Thermodynamic parameters for each secondary structure were calculated using RNAeval (Hofacker, 2003) using either the rod-like or Y-shape conformation as structural restraints. The predicted secondary structures and the calculated free energies (ΔG) were obtained from Mfold (Zuker, 2003).

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