J. Biosci., Vol. 21, Number 6, December 1996, pp 743–753. © Printed in India.

Interactions of 3' terminal and 5' terminal regions of physalis mottle virus genomic RNA with its replication complex

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Ms received 7 August 1996; revised 14 October 1996

Abstract. Physalis mottle virus (PhMV) belongs to the tymogroup of positive-strand RNA viruses with a genome size of 6 kb. Crude membrane preparations from PhMV-infected *Nicotiana glutinosa* plants catalyzed the synthesis of PhMV genomic RNA from endogenously bound template. Addition of exogenous gnomic RNA enhanced the synthesis which was specifically inhibited by the addition of sense and antisense transcripts corresponding to 3' terminal 242 nucleotides as well as the 5' terminal 458 nucleotides of PhMV genomic RNA while yeast tRNA or ribosomal RNA failed to inhibit the synthesis. This specific inhibition suggested that the 5' and 3' non-coding regions of PhMV RNA might play an important role in viral replication.

Keywords. Physalis mottle virus; replication; inhibition; SSRNA plant virus.

1. Introduction

Replication of (+) strand RNA viruses is catalysed by an RNA-dependent RNA polymerase (RdRp), usually referred to as the replicase. Replication is belived to begin with the binding of the RdRp to a promoter region at the 3' end of the genomic (+) strand RNA. Using the four NTPs as substrates and genomic RNA as template, a complementary (–) strand is synthesized. The RdRp then recognizes and binds to a promoter sequence at the 3' end of the (–) strand which serves as the preferred template for the asymmetric synthesis of progeny (+) strands (David *et al* 1992).

Viral RdRp is a complex of host- and viral-coded polypeptides; its membrane-bound nature makes its purification difficult. In most *in vitro* replication systems of plant viruses such as turnip yellow mosaic virus (TYMV) (Mouches *et al* 1974), cowpea chlorotic mottle virus (CCMV) (Miller and Hall 1984), alfalfa mosaic virus (AMV) (Houwing and Jaspars 1986) and brome mosaic virus (BMV) (Miller and Hall 1983; Quadt *et al* 1988), viral RNA synthesis on endogenously-bound RNA templates has been demonstrated using crude or partially pure preparations, while in cucumber mosaic virus (CMV) (Hayes and Buck 1990), complete replication has been reported using purified preparations. In TYMV, the type member of the tymovirus group to which physalis mottle virus (PhMV) belongs, a 115 K viral-coded polypeptide and a presumably host-coded factor have been identified in partially pure preparations (Mouches *et al* 1984). Replication presumably requires the coordinated action of the polymerase, the helicase and the methyl transferase functions of the RdRp complex. Motifs characteristic of these activities have been located through sequence

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comparisons of the regions corresponding to the nonstructural proteins of most (+) strand RNA viruses (Gorbalenya and Koonin 1989; Habili and Symons 1989). However, the roles of individual host- and viral-coded factors of the RdRp preparations in the various steps of replications have not been delineated.

PhMV belongs to the tymo virus group of plant viruses. This virus, isolated by Moline and Fries (1974) in Iowa, USA was originally called the physalis strain of belladonna mottle virus [BDMV(I)] based on serlogical relationships with the European strain BDMV(E). A comparison of the coat protein sequences of these two viruses with other tymoviruses showed that these are two distinct tymoviruses (Ding *et al* 1990; Jacob *et al* 1992; Peter et *al* 1989) and therefore BDMV(I) was renamed PhMV (Jacob *et al* 1992). Here we report the isolation and characterization of an RdRp capable of specifically synthesizing PhMV RNA *in vitro*. We demonstrate the inhibition of PhMV RNA synthesis by transcripts encompassing the 3' and 5' terminal region of PhMV genomic RNA.

2. Materials and methods

2.1 *Chemicals and enzymes*

[α-P^{32}] UTP (3000 Ci/mmol) and Hyperfilm TM - MP for autoradiography were from Amersham. Restriction enzymes, T3, T7 RNA polymerases and NTPs were from Pharmacia and New England Biolabs. All other chemicals used were of the highest purity available.

2.2 *Isolation of PhMV RdRp*

Nicotiana glutinosa plants were inoculated with PhMV and leaves were harvested 2–4 weeks post-inoculation. Leaves from healthy plants served as control. About 20 g of leaves were homogenized in 80 ml of TMDPG buffer [50 mM Tris-HCl, pH 8.2, 20 mM $MgCl_2$ 10 mM dithiothreitol (DTT), 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), 18% glycerol] all steps being carried out at 4°C (Hayes and Buck 1993). The homogenate was centrifuged at 3000 *g* for 30 min. The supernatant was then centrifuged at 35, 000 *g* for 30 min. The pellet was resuspended in 10 ml of TMDPG buffer, yielding the crude membrane fraction. This fraction could be stored at – 80°C for at least one month without significant loss of RdRp activity.

2.3 *Assay of RdRp activity*

RdRp activity was assayed in 20 µl reactions by mixing 10 µl of the enzyme preparation (125 µg of protein) with 10 µl of 100 mM Tris-HCl (pH 8.2) containing 20 mM $MgCl_2$, 20 mM DTT, 2 mM each of CTP, GTP and ATP, [α-P^{32}] UTP 5 μCi and 3 pmol of template RNA. The reaction mixture was incubated at 30°C for 1 h.

2.4 *Analysis of RdRp reaction products*

Incorporation of radioactivity into RNA was determined by spotting 2 µl aliquots onto GF/C discs (Whatman) and washing the discs with chilled 5% (v/v) trichloroacetic

acid (TCA) containing 0.05 M sodium pyrophosphate (5 times, 15 min each wash), followed by ehanol, ethanol: ether (1:1) and ether washes. The filters were dried and the TCA-precipitable radioactivity was measured in a liquid scintillation counter.

The remainder of the reaction mixture (18 µl) containing RdRp reaction products was extracted using phenol/chloroform (1:1, v/v) and precipitated by addition of 2.5 vol of ethanol in the presence of 1/10 vol 3 M sodium acetate, pH 5.5. The resulting RNA was washed twice with 70% (v/v) ethanol and electrophoresed in 1% agarose gels; the gels were then dried and autoradiographed.

2.5 *Preparation of viral RNA template*

PhMV bottom component was purified from infected *N. glutinosa* leaves (Savithri *et al* 1987). Genomic RNA was extracted (Jacob *et al* 1992) after disruption of the capsids in buffer containing 100 mM Tris HCl (pH 7.5), 10 mM EDTA and 2% SDS for 15 Ain at 37°C, chilling on ice for 3 min, followed by phenol/chloroform extraction. The viral RNA was precipitated with 2.5 vol of ethanol in the presence of l/10 vol 3 M sodium acetate (pH 5.5) and stored at –20°C until use.

2.6 *In vitro transcriptions*

Run-off transcripts derived only from the inserts, were produced by linearizing the plasmids with the appropriate restriction endonuclease, followed by purification of the linearized plasmid by electroelution (Sambrook *et al* 1989).

Clone pBS242 encompassed the 3' terminal 242 nucleotides of PhMV genomic RNA inclusive of 68 nucleotides of the coat protein coding sequence, 149 nucleotides of the 3' noncoding region. Since this cDNA clone was obtained after Poly A tailing of the genomic RNA by Poly A polymerase reaction it had an additional 25 nucleotides of poly(A) sequence at its 3' end (Jacob 1992). It was linearized with *Hind*III and *Bam*HI and used as templates in T3 and T7 transcription systems to yield the sense and antisense transcripts, respectively. Clone pBS 458 (Srividhya 1995) encompassed the 5' terminal 147 nt of non coding sequence and 311 of the coding sequence of the PhMV replicase protein. This clone was linearized with *Hind*III or *Pst*I and used as templates in T3 and T7 transcription system to yield sense and antisense transcripts respectively. Transcriptions were carried out as described in Sambrook *et al* (1989). In parallel, the transcription reactions were performed in the presence of [α-P^{32}] UTP instead of unlabelled UTP for quantitation of the transcripts.

2.7 *Competition experiments with transcripts*

RdRp was preincubated for 10 min at 4°C with the competing transcripts (pBS242 sense or antisense transcripts, pBS458 sense or antisense transcripts or yeast tRNA) in 2-fold (6 pmol) and 10-fold (30 pmol) molar excess over the genomic RNA template (3 pmol) before the addition of other reaction components. After the reaction, the RdRp reaction products were extracted and analysed as mentioned above.

3. Results

3.1 *In vitro synthesis of PhM V RNA by PhMV RdRp*

Crude membrane fractions prepared from healthy and PhMV-infected *N. glutinos* leaves were assayed in the presence and absence of PhMV genomic RNA. A distinct band corresponding in size to the genomic RNA was apparent when membrane extracts from infected leaves were used as source of RdRp even in the absence of added RNA (figure 1, lane 1). The mobility of PhMV genomic RNA (g) was ascertained by staining a parallel lane with ethidium bromide. No such incorporation was observed when equivalent extracts from uninfected leaves were used as control (lane 5). However the intensity of this band increased to some extent in the presence of 3 picomol of

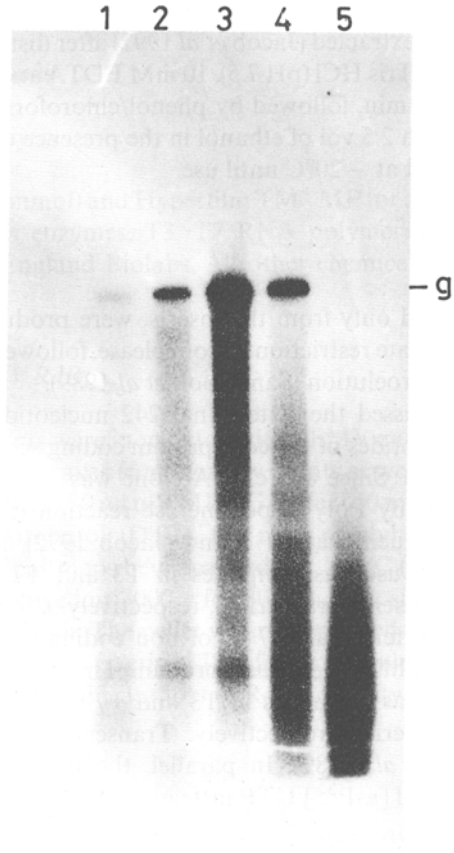

Figure 1. Time course of induction of PhMV RdRp activity.

Autoradiograph of PhMV RdRp reaction products electrophoresed through 1% agarose gels. Lane 1, reaction carried out without exogenously added template using RdRp isolated 3 weeks post-inoculation. Lanes 2-4, reactions carried out using RdRp isolated at 2, 3 and 5 weeks post inoculation respectively and assayed in the presence of exogenous PhMV RNA. Lane 5, reaction carried out using an equivalent preparation from 3 week-old uninfected leaves. g indicates the position of the PhMV genomic RNA (6 kb) ascertained by staining a parallel lane with ethidium bromide.

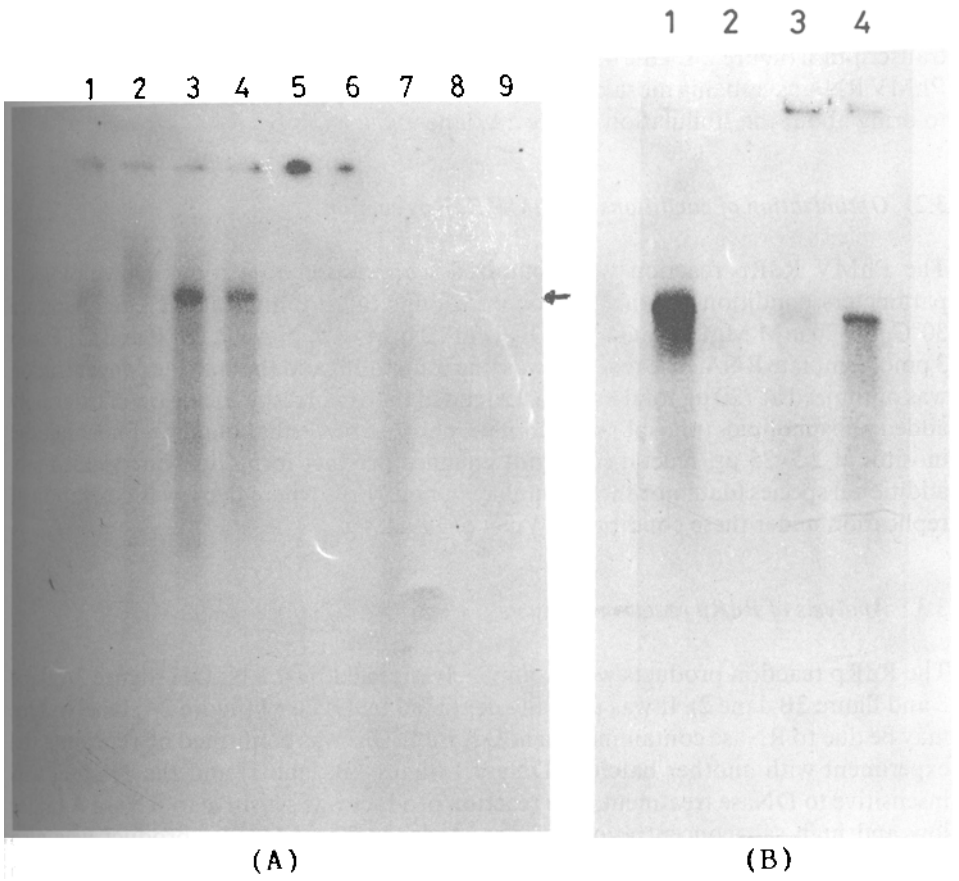

Figure 2. Analysis of PhMV RdRp reaction products.

Autoradiograph of RdRp reaction products subjected to various treatments and analysed by electrophoresis through 1% agarose gels. (A) Lane 1, 0 h blank immediately after the addition of the enzyme the products were extracted with phenol/chloroform. Lane 2, reaction carried out without exogenously added template. Lanes 3, reaction carried out in the presence of PhMV genomic RNA (3 pm ol). Lane 4, reaction carried out after preincubating the enzyme with 10 mg/m1 actinomycin for 15 min. Lanes 5 and 6, reaction products as in lane 3 treated with 0·1 µg/ml RNasc A at low salt (0·1 × SSC) and high salt (1 × SSC), respectively for 30min at 37"C. Lane 7, reaction products treated with 0·1 mg/mI DNase I for 30 min at 37°C. Lane 8, reaction products treated with 0·1 NaOH for 30 min at 25°C. Lane 9, reaction carried out in sesbania mosaic virus RNA 3 pmol as template instead of PhMV RNA. Arrow indicates position of genomic RNA. (B) All reactions were carried out in the presence of PhMV genomic RNA (3 pmol) and analysed on 1% agarose gels. Lane 1, treatment with 0·1 mg/mI DNase I for 30min at 37°C. Lane 2, treatment with 0·1 N NaOH. Lanes 3 and 4, treatment with 200 U and 20 U of S1 nuclease respectively in buffer containing 0·28 M NaCl, 0·05 M sodium acetate, pH 4·5, 4·5 mM $ZnSO_4$ and 0·5% glycerol for 30 min at 25°C.

exogenously added RNA (lane 3). The RdRp activity peaked at 3 weeks post-incublation (compare lanes 2, 3, 4), coinciding with the peak in virus titre. These results demonstrate the presence of a RdRp specific to infected leaves.

The replication was not due to terminal transferase activity, as no incorporation was seen upon omission of one of the substrates, CTP (data not shown). The reaction was

also insensitive to actinomycin D, ruling out the involvement of DNA-dependent transcription (figure 2A, lane 4). The stimulation in the RNA synthesis was specific to PhMV RNA as sesbania mosaic sebemo virus RNA added in equivalent amounts failed to bring about the stimulation (figure 2A, lane 9).

3.2 *Optimization of conditions of PhMV RdRp reaction*

The PhMV RdRp reaction was optimized with respect to various enzymological parameters: conditions for the reaction were found to be optimal at pH 8, temperature 30°C, 10-20 mM $MgCl_2$ 5 μCi [α-^{32}P] UTP, 2 mM each of ATP, GTP and CTP and 3 pmol template RNA. The reaction was linear up to 1 h and the enzyme concentration was optimized at 120 µg total protein. Extended time courses or inclusion of externally added phospholipids (phosphatidyl chorine, phosphatidyl ethanolamine, phosphatidyl inositol at 2.5–25 µg/reaction) did not enhance product formation nor yielded any additional species (data not shown), unlike in nodavirus where there was activation of replication under these conditions (Wu *et al* 1992).

3.3 *Analysis of RdRp reaction products*

The RdRp reaction products were completely digested by 0.1 NaOH (figure 2A, lane 8 and figure 2B, lane 2). It was partially degraded by DNase I (figure 2A, lane 7). This may be due to RNase contamination in DNase I. This was confirmed by repeating the experiment with another batch of DNase I (figure 2B, lane 1) and the product was insensitive to DNase treatment. The reaction product was sensitive to RNase A under low and high salt concentrations (figure 2A, lanes 5 and 6). The product was also sensitive to S1 nuclease digestion (figure 2B, lanes 3 and 4). This showed that the RdRp reaction product is predominantly single-stranded.

3.4 *Inhibition of viral RNA synthesis in vitro*

The 3' terminal non-coding region of PhMV RNA is capable of folding into a distinct tRNA-like structure and is aminoacylatable by valine (Jacob *et al* 1992). In order to examine the probable interaction of this region with RdRp as reported in other systems like TYMV (Morch *et al* 1987; Zaccorner *et al* 1993), sense and antisense transcripts were produced from clone pBS242 that encompassed the 3' terminal 242 nucleotides of the PhMV RNA. These transcripts were used in competition experiments at 2-fold and 10-fold molar excess over the genomic RNA template in RdRp assays. As apparent from figure 3 (compare lanes 1–5), both sense and antisense transcripts inhibited *in vitro* PhMV RNA synthesis to similar extents. This inhibition was highly specific as yeast total tRNA, when added in a 10-fold molar excess under equivalent conditions, failed to compete with the synthesis of PhMV RNA (lane 6).

To examine the possibility of similar interactions of the PhMV RdRp with the 5' terminal region of the virus, sense and antisense transcripts representative of the 5'-terminal 458 nucleotides of PhMV RNA inclusive of the 147 nucleotide long 5'-noncoding region, were generated from clone pBS458 by *in vitro* transcription and used in competition experiments at 2-fold and 10-fold molar excess over the genomic

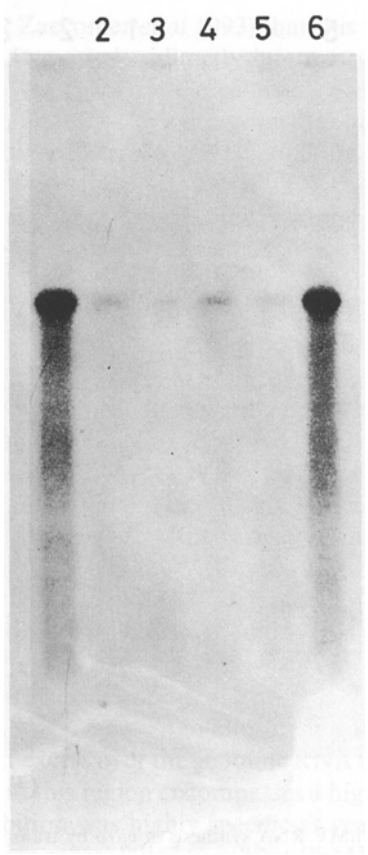

Figure 3. Inhibition of PhMV RNA synthesis by transcripts encompassing the 3' terminal region of PhMV RNA.

Competition experiments were carried out as described, in the methods section. The products were electrophoresed through 1% agarose gels and detected by autoradiography. All reactions were carried out in the presence of 3 pmol of PhMV RNA. Lane 1, control reaction without pretreatment. Lanes 2 and 4, reactions carried out in the presence of 2-fold molar excess of competing T3 (sense) and T7 (antisense) pBS242 transcripts, respectively. Lanes 3 and 5, reactions carried out in the presence of a 10-fold molar excess of the competing sense and antisense transcripts. Lane 6, reaction in the presence of 10-fold molar excess of yeast total tRNA.

RNA template in RdRp assays. Both sense (figure 4A, lanes 3 and 4) and antisense (figure 4B, lanes 2 and 3) transcripts inhibited PhMV RNA synthesis *in vitro* at concentrations as low as a 2-fold molar excess. This inhibition was also highly specific, as yeast tRNA (figure 4A, lane 5) or rat liver rRNA (figure 4B, lane 4) when added in a 10-fold molar excess under equivalent conditions failed to compete with the genomic RNA template.

4. Discussion

The results presented here clearly demonstrate that PhMV RdRp is capable of *in vitro* RNA synthesis and that it is highly specific to PhMV RNA. The replication product

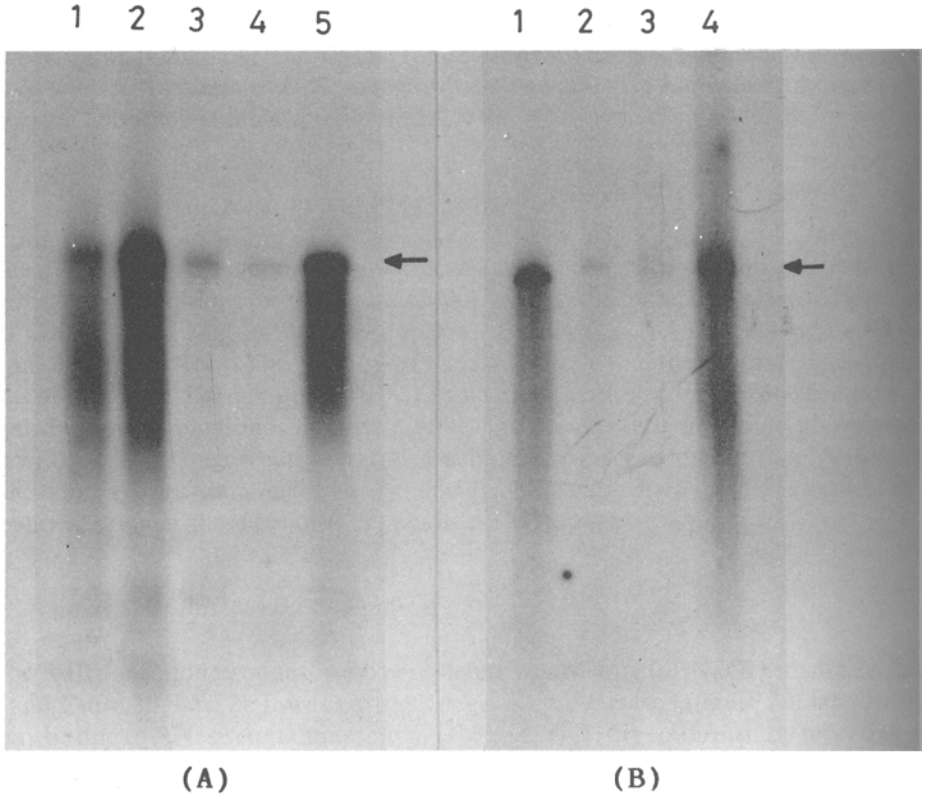

Figure 4. Inhibition of PhMV RNA synthesis *in vitro* by transcripts encompassing the 5' terminal 458 nucleotides of PhMV RNA.

Competition experiments were carried out as described, in §2. The products were electreophoresed through 1 % agarose gels and detected by autoradiography. (A) Lanes 1 and 2, no pretreatment and in the absence and presence of PhMV genomic RNA, respectively. Lanes 3 and 4, reactions carried out in the presence of PhMV RNA (3 pmol) after preincubation with a 2-fold and 10-fold molar excess of the pBS458 sense transcript, respectively. Lane 5, reaction carried out after preincubation with 10-fold molar excess of yeast total tRNA. (B): All reactions contained exogenously added PhMV RNA (3 pmol). Lane 1, no pretreatment. Lanes 2 and 3, reactions carried out after preincubation with a 2-fold and 10-fold molar excess of 5' terminal antisense transcript respectively. Lane 4, reaction carried out after preincubation with 10-fold molar excess of rat liver rRNA. Arrow indicates the position of the Ph MV genomic RNA (6 kb).

was single-standed RNA, corresponding in size to the genomic RNA and could arise from the elongation of endogenously-bound templates or by the initiation of fresh synthesis. The *in vitro* PhMV RNA synthesis was inhibited by sense and antisense transcripts (figure 3) encompassing the 3' terminal region of PhMV RNA. The yeast tRNA added in 10-fold molar excess over the genomic RNA failed to cause inhibition. Similarly the transcript from other parts of the genomic RNA such as coat protein was unable to inhibit replication (data not shown) suggesting that the inhibition by these transcripts was highly specific. The sense transcript encompassing the tRNA- like structure could inhibit replication by competing with the genomic RNA for binding to the RdRp complex, consistent with the observations made in other

systems Morch *et al* 1987; Zaccomer *et al* 1993) that this region may act as promoter for (–) strand synthesis. It may also directly interact with the polypeptides in the replication complex.

On the other hand, the antisense transcripts may hybridize to the 3' terminus of the (+) sense RNA and inhibit further replication by making the authentic genomic RNA template unavailable for binding to the replicase. Alternatively, the complementary sequence may itself directly bind the replicase, thereby reducing the chance for replication of viral RNA. An analysis of the nucleotide sequence of the 5' terminus of the (–) strand (i.e., the 3' antisense) reveals the presence of internal control region (ICR2)-Iike sequence repeats characteristic of polymerase III promoters in tRNA genes, in the noncoding region between nucleotides 30-37 and 112-121 on the (–) strand. The motif between 30-37 is identical to that observed in TYMV (Marsh *et al* 1989) and it occurs in the bases complementary to those in the (+) strand which probably correspond to the PψC loop of the tRNA-like structures (table 1). The motif at 112-121 showed maximum similarity (73%) with the tRNA consensus and was present only in PhMV among tymoviruses (table 1). The antisense-mediated inhibition may be brought about by the interaction of the replicase with the ICR-like motif. It has been demonstrated in the case of BMV (Pogue *et al* 1990) that point mutations in the ICR 2-like motif of the BMV RNAs debilitate *in vitro* replication. Such an antisense-mediated inhibition of replication could be part of a feedback regulation of (–) stand synthesis.

Similar to the 3' terminal 242 nucIeotides of PhMV RNA transcripts, the 5' terminal 458 nucleotide transcripts were also able to inhibit viral RNA synthesis at concentrations as low as 2-fold molar excess over the genomic RNA template, in an *in vitro* RdRp reaction demonstrating that this region encompasses a high affinity binding site for the RdRp (figure 4). The inhibition was highly specific as yeast tRNA or rat liver rRNA when added at 10-fold molar excess failed to inhibit PhMV RNA synthesis. This is the first report of inhibition of viral RNA synthesis *in vitro* by transcripts encompassing the 5' terminal region of the genome in tymoviruses.

Table I. Identification of internal control region (ICR2) like motif in 5' terminal region of (–) strand of PhMV RNA.

tRNA ICR consensus[a]	5′ GGUUCGANUCC 3′
TYMV[b]	A⌒UUGCAC.CC
PhMV[c]	AGUUGCAC.CC
	AGUAGGAUUCC[d]

[a] The ICR-2 consensus sequence present in the promoter region of tRNA genes (Marsh *et al* 1989).

[b] 5' terminal region of (–) strand of TYMV RNA showing similarity to the ICR2 consensus sequence (Marsh *et al* 1989).

[c] This region is between 30–39 on (–) strand and is complementary to the TψC loop of tRNA-like structure of the (+) strand of PhMV RNA.

[d] This alignment between nucleotides 112–122 shows 73% similarity to the tRNA consensus and all other alignments in this table show 64% similarity.

(.) Spaces are inserted to maximize apparent homology.

The inhibition by the 5' terminal sense transcripts may be brought about by hybridization to the 3' terminus of the (–) strand, making the authentic (–) strand intermediate unavailable for binding to the replicase. On the other hand, it may also inhibit by direct interaction with the polypeptides in the RdRp complex. It was shown earlier that the 5' and 3' ends of several plant viral RNAs could base pair and the interaction of tRNA synthetase with tRNA like structure at the 3' end may assist in the efficient translation of the viral genome (Floretz *et al* 1984). It is possible that the inhibition by the 5' and 3' transcripts arise from the similarity in their structures.

The inhibition of PhMV RdRp activity by the 5' terminal antisense transcript [i.e., the 3' end of the (–) strand], is consistent with the idea that this region acts as a promoter for the initiation of (+) strand synthesis. The highly specific nature of the inhibiton observed with the 5' and 3' terminal transcripts in PhMV implicates the probable importance of these regions in interaction with PhMV RdRp. Generation of shorter transcripts of the 5' and 3' terminal region would help in finding out the minimal sequence necessary for binding to the RdRp.

Acknowledgements

We thank Dr A L Haenni and Dr N Appaji Rao for valuable discussions and Dr A N K Jacob for providing the clone pB242. We thank Shyamala and Ilango at the Bioinformatics Centre, Department of Physics, Indian Institute of Science, Bangalore for their help in carrying database search and sequence analysis. VS is a recipient of Senior Research Fellowship of the Council for Scientific and Industrial Research, New Delhi. This work was supported by the Department of Science and Technology, New Delhi, and Indo-French Centre for Promotion of Advanced Research.

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Corresponding editor: M S SHAILA