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The 3′ promoter region involved in RNA synthesis directed by the turnip yellow mosaic virus genome in vitro

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We have previously shown that the last 100 nucleotides from the 3′ end of turnip yellow mosaic virus (TYMV) RNA compete in vitro with genomic RNA for the TYMV-specific RNA-dependent RNA polymerase (RdRp). To further characterize the promoter on genomic RNA that produces complementary RNA strands, shorter fragments corresponding to the 3′ region of the viral RNA were generated and used in in vitro assays. Fragments as short as 38 nucleotides corresponding to the 3′ end of TYMV RNA compete with the viral RNA for the RdRp suggesting that the 3′ promoter on plus strand RNA is probably ≤ 38 nucleotides long. These transcripts are themselves used as templates in vitro.

Viral RNA-dependent RNA polymerase; Turnip yellow mosaic virus; Viral RNA synthesis in vitro; Turnip yellow mosaic virus (TYMV) RNA promoter

1. INTRODUCTION

Turnip yellow mosaic virus (TYMV), the type member of the tymovirus group possesses a monopartite single-stranded RNA genome of positive polarity. The virion also encapsidates a subgenomic RNA that corresponds to the 3′ region of the genome, and served as mRNA for the synthesis of the coat protein. Both RNAs possess a tRNA-like region of 86 nucleotides at their 3′ end that can be aminoacylated with valine by the valyl-tRNA synthetase and that is recognized by several other tRNA-specific enzymes [1–3]. The aminoacyl stem (the 3′ terminal 27 nucleotides) of the tRNA-like region can adopt a pseudoknotted structure [4].

The possibility of isolating from TYMV-infected Chinese cabbage leaves an RNA-dependent RNA polymerase (RdRp) that uses exogenous TYMV RNA as template to synthesize the complementary RNA strand [5,6], and the availability of cDNA clones corresponding to the 3′ region of the TYMV genome [7] had previously enabled us [8] to demonstrate that the 3′ terminal 100 nucleotides of TYMV RNA interact with the RdRp in vitro: they compete with TYMV RNA for the RdRp and are themselves used as templates. To further characterize the promoter on the TYMV genome involved in the synthesis of complementary RNA strands, we have investigated whether RNA fragments shorter than 100 nucleotides also inhibit viral RNA synthesis in vitro. To this end, short RNA transcripts designated tRNA-like fragments (tlfs) and corresponding to the 3′ end of TYMV RNA were produced; they were tested in competition experiments with TYMV RNA for the RdRp in an in vitro assay, and were assayed for their capacity to serve as template for the RdRp.

Abbreviations: TYMV, turnip yellow mosaic virus; BMV, brome mosaic virus; RdRp, RNA-dependent RNA polymerase; glf, genome-like fragment; tlf, tRNA-like fragment.

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2. MATERIALS AND METHODS

Escherichia coli strain DH5αF′ and the cloning vector pT7/T3α-18 were purchased from BRL. [α^{32}P]UTP and [α^{32}P]CTP, both at 14.8 TBq/mmol, were from Amersham. All enzymes were of the highest quality available. TYMV-infected Chinese cabbage leaves were kindly provided by S. Astier-Manifacier (INRA, Versailles). TYMV was purified [9] and the viral RNA was extracted [10] and stored at −70°C.

To transfer most of the TYMV cDNA fragment from the initial plasmid B26 [7] to the transcription vector pT7/T3α-18, B26 was linearized by *Eco*RI, made blunt end by fill in and digested by *Pst*I; the purified fragment was ligated into the transcription vector previously linearized by *Sal*I, made blunt end and digested by *Pst*I [11]. After transformation into DH5αF′, recombinant plasmids were screened on the basis of their size, and checked by digestion with appropriate restriction enzymes. Plasmid p242 containing a 912 base-pair fragment corresponding to the 3′ end of TYMV RNA was selected.

Starting from p242, clones with smaller inserts were generated, using the procedure of Hong [12] further improved by Lin et al. [13]. Limited double-strand digestion of p242 with DNase I in the presence of Mn^{2+} was followed by digestion with *Sph*I, fill in and religation. Transformation into DH5αF′ led to deleted clones that were screened on the basis of their size and with appropriate restriction enzymes.

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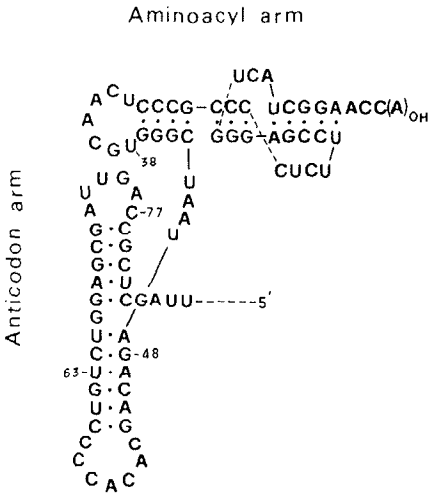

Fig. 1. Proposed structure for the tRNA-like region of TYMV RNA. The 3′ terminal A residue is in parentheses since it is absent from virion RNA. Numbering is from the 3′ end and includes the 3′ terminal A residue; the numbers refer to the 5′ nucleotide of the viral sequence contained in each tlf.

Four clones were produced whose viral sequences were verified by the method of Sanger et al. [14].

For transcription, each plasmid was linearized by *Xba*I essentially as previously described [8] except that the transcripts were additionally purified by Sephadex G50 column chromatography prior to phenol extraction. The transcripts obtained are designated tlf77, tlf63, tlf48 and tlf38; they contain 77, 63, 48 and 38 nucleotides, respectively, from the 3′ end of TYMV RNA. Fig. 1 illustrates the nucleotide sequence and a possible folding of the tRNA-like region of TYMV RNA and indicates the position of the 5′ boundary of each tlf. The tlfs are flanked at the 5′ end by 8 nucleotides and at the 3′ end by 13 nucleotides from the multiple cloning site. A transcript was also produced from *Xba*I-linearized pT7/T3α-18 devoid of insert.

The TYMV RdRp was purified from TYMV-infected Chinese cabbage leaves 14 days post-inoculation, up to and through the 'PEG' step [5,15], aliquoted and stored in liquid nitrogen. The standard conditions of the in vitro assay were essentially as indicated by Mouchès et al. [5] and Morch et al. [8] using either TYMV RNA, or one of the transcripts as template, and either [α^{32}P]UTP or [α^{32}P]CTP. Incorporation of radioactivity into 10% cold trichloroacetic acid was determined on duplicate or triplicate aliquots. For the analysis of the reaction products by 7 M urea/5–15% polyacrylamide gradient gel electrophoresis, the mixtures were ethanol-precipitated, resuspended in loading buffer and heated for 10 min at 65°C before loading. The gels were stained with silver nitrate [16], dried and autoradiographed. In the competition experiments, the mixtures were incubated for 5 min in ice in the presence of one of the transcripts prior to addition of the radioactive nucleoside triphosphate and TYMV RNA.

3. RESULTS AND DISCUSSION

RNA synthesis triggered using the TYMV genome (20 mM) as template was assayed in the absence or presence of increasing concentrations of each of the four tlfs, or of genome-like fragments (glfs; see below) as competitor (Fig. 2). In all cases the level of TYMV RNA synthesis decreased with increasing tlf concentration. Indeed, 50% inhibition of TYMV RNA synthesis was reached at tlf concentrations ranging from 2 to 10

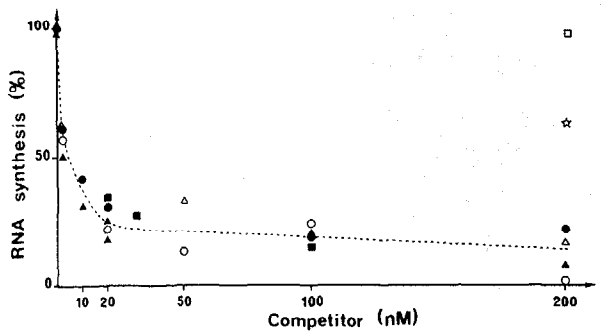

Fig. 2. Effect of increasing concentrations of competitors on the synthesis of RNA directed by TYMV RNA (20 nM) in vitro in the presence of TYMV RdRp. RNA synthesis is expressed as the percentage of the value obtained in the absence of competitor. 100% = cpm of [α-^{32}P]UMP or [α-^{32}P]CMP incorporated per (duplicate or triplicate) 3 μl aliquots in the absence of competitor. ●, tlf77; ○, tlf63; △, tlf48; ▲, tlf38; ■, glf20-E; □, glf20-D; ☆, pT7/T3α-18-derived transcript.

nM which suggests that both the viral RNA and the various tlfs have comparable affinities for the RdRp. With all tlfs, including tlf38 that contains only the 3′ terminal 38 nucleotides of TYMV RNA, a 10-fold molar excess of tlf over viral RNA led to about 90% inhibition of RNA synthesis. As expected [8], the effect of increasing concentrations of a larger transcript such as glf20-E that contains the last 334 nucleotides of the 3′ terminal region of TYMV RNA was comparable to that of the tlfs. As negative control, glf20-D [8], which corresponds to glf20-E except for the deletion of the 3′ terminal 101 nucleotides, was used: RNA synthesis was not affected by the presence of this transcript, even in 10-fold molar excess. The transcript produced from vector devoid of viral sequence and used in a 10-fold molar excess over TYMV RNA led to a 37% inhibition.

It was further shown that the small transcripts could serve as template for the TYMV RdRp in vitro. The products synthesized with tlf77, tlf63 or tlf38 as templates were analyzed on denaturing gels: all the tlfs analyzed are efficient templates in vitro (Fig. 3). The omission of one of the four nucleoside triphosphates from the incubation mixture totally abolished RNA synthesis (not shown), indicating that the RdRp has no terminal transferase-like activity as observed previously [6]. In these conditions, the ^{32}P-labelled RNA strand produced in vitro is of negative polarity [5].

The data presented suggest that tlfs containing as few as 38 nucleotides from the 3′ end of TYMV RNA are efficiently recognized by the RdRp in vitro: they block viral RNA synthesis by competing with the viral RNA for the enzyme. Even though the tlfs serve as template for the RdRp, they show an inhibitory effect of TYMV RNA synthesis. This is most likely due to the fact that they cannot be amplified since they lack the promoter sequence at their 5′ end that would be required to initiate the next round of RNA synthesis from the newly synthesized RNA of negative polarity.

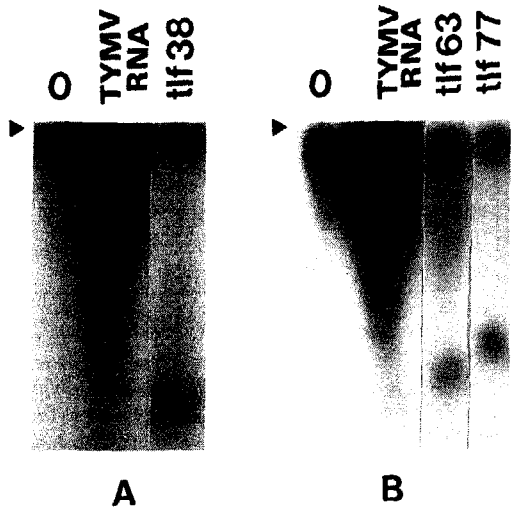

Fig. 3. Analysis of ^{32}P-labeled products on 7 M urea/5–15% polyacrylamide gels. (A and B) Autoradiograms of two parallel experiments performed using tlf38, tlf63 or tlf77. Aliquots (16 μl) of the RdRp assays were analyzed. For each panel, the samples loaded correspond to incubations performed without RNA (0), or with either 1.6 μg of TYMV RNA or 300 ng of tlf38, tlf63 or tlf77. The origin is indicated by an arrowhead.

It is interesting to consider the structural elements that may be important for RNA replication. In brome mosaic virus (BMV) RNA, a 3′ terminal fragment corresponding to the last 134 nucleotides has a high template activity for the BMV RdRp, whereas a 3′ terminal fragment of 85 nucleotides is inactive [17]. The last 134 nucleotides, which correspond to the entire tRNA-like region of BMV RNA and encompass its pseudoknot structure, thus seem to participate in RNA synthesis in vitro, as they do also in adenylation and aminoacylation in vitro [18]. Furthermore, it has been demonstrated [19] that substitution of UCU by AGA (positions 113–115 from the 3′ end of the BMV RNA genome) thereby destroying the pseudoknot, reduces the template activity of the modified RNA to 8% of the wild-type in vitro, and abolishes adenylation and tyrosylation in vitro.

Transcript tlf38 encompasses the last 38 nucleotides from the 3′ end of TYMV RNA; it corresponds to most of the aminoacyl arm including the pseudoknot contained in the tRNA-like region of the viral genome (Fig. 1), and it appears to serve as efficient template in vitro for the RdRp. Based on a model 19-mer oligonucleotide with the potential of forming a pseudoknot, Puglisi et al. [20] demonstrated that such an oligonucleotide indeed adopts a pseudoknotted structure. Therefore, it is reasonable to propose that the TYMV 38-mer oligonucleotide used here also retains the capacity of forming a pseudoknot and that this pseudoknot might play a role in the synthesis of complementary RNA molecules. This hypothesis should be investigated using viral transcripts shorter than 38 nucleotides. However, attempts to investigate the template activity of a synthetic 19-mer RNA molecule corresponding to the 3′ end of the TYMV genome yielded inconclusive results.

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REFERENCES

[1] Joshi, S., Chapeville, F. and Haenni, A.L. (1982) Nucleic Acids Res. 10, 1947–1962.
[2] Haenni, A.L., Joshi, S. and Chapeville, F. (1982) Prog. Nucleic Acid Res. Mol. Biol. 27, 85–104.
[3] Hirth, L. and Givord, L. (1988) in: The Plant Viruses, vol 3 (Koenig, R. ed.) pp. 163–212, Plenum Press, New York.
[4] Rietveld, K., van Poelgeest, R., Pleij, C.W.A., van Boom, J.H. and Bosch, L. (1982) Nucleic Acids Res. 10, 1929–1946.
[5] Mouches, C., Bove, C. and Bove, J.M. (1974) Virology 58, 409–423.
[6] Joshi, R.L., Ravel, J.M. and Haenni, A.L. (1986) EMBO J. 5, 1143–1148.
[7] Morch, M.D., Boyer, J.C. and Haenni, A.L. (1988) Nucleic Acids Res. 16, 6157–6173.
[8] Morch, M.D., Joshi, R.L., Denial, T.M. and Haenni, A.L. (1987) Nucleic Acids Res. 15, 4123–4130.
[9] Leberman, R. (1966) Virology 30, 341–347.
[10] Porter, A., Carey, N. and Fellner, P. (1974) Nature 248, 675–678.
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[12] Hong, G.F. (1982) J. Mol. Biol. 158, 539–549.
[13] Lin, H.C., Lei, S.P. and Wilcox, G. (1985) Anal. Biochem. 147, 114–119.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Mouches, C., Candresse, T. and Bove, J.M. (1984) Virology 134, 78–90.
[16] Goldman, D. and Merril, C.R. (1982) Electrophoresis 3, 24–26.
[17] Miller, W.A., Bujarski, J.J., Dreher, T.W. and Hall, T.C. (1986) J. Mol. Biol. 187, 537–546.
[18] Joshi, R.L., Joshi, S., Chapeville, F. and Haenni, A.L. (1983) EMBO J. 2, 1123–1127.
[19] Dreher, T.W., Rao, A.L.N. and Hall, T.C. (1989) J. Mol. Biol. 206, 425–438.
[20] Puglisi, J.D., Wyatt, J.R. and Tinoco, I. (1990) J. Mol. Biol. 214, 437–453.