RNA duplex unwinding activity of alfalfa mosaic virus RNA-dependent RNA polymerase

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Abstract An RNA-dependent RNA polymerase (RdRp) purified from alfalfa mosaic virus-infected tobacco is capable of synthesizing in vitro full-size RNAs of minus and plus polarities. However, the enzyme is not able to perform a complete replication cycle in vitro. The products were found to be completely base-paired to their templates. The enzyme was able to use double-stranded RNA as a template for RNA synthesis if it could initiate from a single-stranded promoter. The inability (of most) of our enzyme preparations to create a single-stranded initiation site could explain why they could not perform a complete replication cycle in vitro. This is the first report on duplex RNA unwinding activities by a plant viral RdRp.

Key words: Alfalfa mosaic virus; RNA-dependent RNA polymerase; RNA duplex unwinding

1. Introduction

The genome of many RNA plant viruses consists of single-stranded plus-polarity, meaning messenger-sense, RNA molecules. After infection of the host cell the virion RNAs will be translated and the viral RNA-dependent RNA polymerase (RdRp) will be assembled (for a recent review on plant viral RdRps see [1]). The replication cycle starts with the synthesis of complementary minus strands on the parental plus strands, followed by the synthesis of new plus-stranded virion RNAs using the minus RNAs as templates. The new plus strands will be released into the cytoplasm of the infected cell, where they can be used as messengers for viral protein synthesis, or are encapsidated into virions. This implies that the RdRp might have duplex RNA unwinding activity in order to prevent an early stop of RNA synthesis if complete base-pairing of the RNA products would occur.

In vitro viral RNA synthesizing systems using purified viral RdRps are useful tools to study the mechanisms of viral RNA synthesis on a molecular level. Thusfar, only for cucumber mosaic virus (CMV) a system is described where the complete replication cycle of viral genomic RNAs and of satellite RNA takes place in vitro [2–4]. This indicates that the RdRp is capable of preventing double-stranded RNA structures to be formed, or if formed of unwinding them. The enzyme prepared by Hayes and Buck [2] produced in vitro, in addition to double-stranded products also single-stranded genomic RNAs. However, their CMV RdRp was not able to use full-length double-stranded CMV RNAs as templates for in vitro RNA synthesis [5]. This suggests that the duplex formation did only occur after phenol extraction, or that RNA duplex unwinding could only be executed by an enzyme which was continuously elongating and did not have to re-initiate.

In our laboratory we developed a rapid purification method for plant viral RdRps based on high-salt, high-detergent treatment, which freed the viral RNA polymerase from the membranes and the endogenous templates to which it was bound. This protocol showed to be applicable to purify the RdRps of brome mosaic virus (BMV, [6]), alfalfa mosaic virus (AlMV, [7]), and CMV [8]. Moreover, these enzyme preparations proved to be very stable when stored at –80°C. However, they did not show the production of single-stranded RNAs in vitro. Apparently, RNA products synthesized remain attached to the template RNAs, suggesting that RNA duplex unwinding activity is lacking.

In this paper we describe for the first time the ability of a plant viral RdRp, viz. that of AlMV, to unwind RNA duplexes in vitro.

2. Materials and methods

2.1 Preparation of AlMV RdRp and RNA templates

Tobacco plants (*Nicotiana tabacum* L. cv Samsun NN) were grown and inoculated, and AlMV RdRp was prepared essentially as described earlier [7]. The continuous glycerol gradients were centrifuged for 45 h instead of 20 h. AlMV virion RNAs were prepared as described ([9] and references therein). AlMV has a tripartite genome (RNAs 1, 2 and 3) and one subgenomic RNA (RNA4) which is coded for by the 3′-terminal half of RNA3. Transcript AlMV RNA3 was prepared by run off transcription with T7 RNA polymerase of *Pst*I-linearized cDNA clones; minus-RNA3 (2167 nt) was prepared from cDNA clone sg1 [10] resulting in a transcript with a correct 3′ end, but containing 25 non-viral nucleotides at the 5′ end; plus-polarity RNA3 (2142 nt) was prepared from cDNA clone pAL3 [11] resulting in a transcript with correct 5′ and 3′ ends. After transcription RNA was treated with DNase, phenol extracted, isopropanol precipitated and purified from 1% (w/v) agarose gels using the RNaid-kit (bio 101), according to the manufacturer. Hybrid minus-RNA3/plus-RNA4 molecules (Fig. 1) were obtained by annealing T7 transcript minus RNA3 to virion plus RNA4 (881 nt) in 60% (v/v) formamide, 2 × SSC (1 × SSC: 0.15 M NaCl, 0 015 M sodium citrate) at 37°C for 2 h. Hybrid-RNA template was purified by preparative gel electrophoresis [12]

2.2. RNA-dependent RNA polymerase assay

The standard reaction mixture of 50 μl contained: 10 μl purified RdRp in 50 mM Tris-HCl, pH 8.2, 8 mM $MgCl_2$, 8mM dithiotreitol, 0.8 mM each of ATP, CTP and GTP, 10 μM UTP, 5 μCi [α-^{32}P]UTP (400 Ci/mmol). The RdRp was programmed with 0.2–2 μg RNA template. Incubation was done at 28°C for 60 min. After synthesis the RNA products were phenol extracted, electrophoresed in 1% (w/v) agarose gels and autoradiographed as described [13]. Nuclease S1 diges-

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tion of RNA products was performed as described earlier [13]; RNase A (10 μg/ml final concentration) digestion was done in 2 × SSC by incubation for 30 min at 37°C.

2.3. RNA duplex unwinding assays

Unwinding assays were performed using the hybrid minus-RNA3/plus-RNA4 as a template. Antibody inhibition studies were done by preincubating about 0.2 μg of the template RNA with 1 μg mouse monoclonal IgG2a antibodies directed against double-stranded RNA (hybridoma line J2; [14]), or control mouse monoclonal IgG2a anti bodies (hybridoma line 5/14; [15]) for 15 min at 0°C in 5 mM Na-phosphate, pH 7.0, 15 mM NaCl prior to in vitro RNA synthesis. The double- stranded RNA used to titrate the J2 antibodies was made by annealing complementary T7 transcripts from plasmids pRH751 and pRH752 and then purifying double strands by Qiagen columns (Diagen, Hilden, Germany). No single-stranded transcripts or other contaminants were detected in the purified double-strand preparation by temperature- gradient gel electrophoresis [16], and silver staining [17].

2.4 Liquid RNA-RNA hybridizations

The polarity of RNA products synthesized in vitro on virion RNA3 was assessed by liquid RNA-RNA hybridizations in 60% (v/v) formamide, 2 × SSC to gel-purified T7 transcripts of RNA3 of either plus or minus polarities essentially as described earlier [7]. Annealing efficiencies were quantified by Cerenkov counting.

3. Results and discussion

3.1. AlMV RdRp synthesizes plus RNA in vitro

The RdRp of AlMV prepared by the high-salt, high-detergent procedure from infected tobacco plants is completely dependent on added RNA templates [7]. No enzyme activity is detectable in the absence of added template RNA (Fig. 2A, lane 1). Full-length minus-RNA products are synthesized after adding AlMV virion RNAs to the reaction mixtures (Fig. 2A, lane 2). After phenol extraction of the reaction mixtures the synthesized RNA products are found base-paired to their template RNAs resulting in nuclease S1-resistant, double-stranded structures.

Further prerequisites for complete replication in vitro are synthesis of plus RNA on minus-RNA templates and possibly RNA duplex unwinding activity. The AlMV RdRp is capable of accepting T7 transcript minus RNA3 as a template for in vitro RNA synthesis (Fig. 2B, lane 1). Products synthesized on the minus-RNA3 template are largely nuclease S1-resistant (Fig. 2B, lane 2). Denaturation of the RNA products by glyoxal treatment showed that predominantly RNA4 was synthesized from the subgenomic promoter, and to a lesser extent also full-length plus RNA3, initiated at the 3′ end of the minus RNA3 (Fig. 2B, lane 3). In earlier reports, using a less purified AlMV RdRp preparation from French beans, the synthesis of full-length plus RNA3 was never shown [10], indicating the improved quality of the present RdRp preparation. Besides RNA4 and RNA3 a smaller than full-length RNA3 product is visible, which is thought to be due to a cryptic initiation on the minus-RNA3 template (Fig. 2B, lane 3; [10]).

3.2. Reaction intermediates are mainly double-stranded

Despite the ability of the RdRp to synthesize both minus and plus-polarity RNA, the enzyme does not perform a complete replication cycle in vitro. After programming the RdRp with virion plus-RNA3, no plus-RNA products could be detected using liquid RNA-RNA hybridizations (not shown). A possible explanation could be that product RNAs completely base-pair to their template RNAs during elongation, resulting in the formation of dead-end RNA structures. Initiation of RNA synthesis is then no longer possible. Therefore, we investigated whether reaction intermediates after programming the RdRp with virion RNAs as templates are of single-stranded or double-stranded nature. As expected, the reaction products after phenol extraction of the reaction mixtures yielded RNase-resistant double-stranded structures (Fig. 3, lane 4), showing a similar pattern on the gel than RNA products that were not nuclease-treated (Fig. 3, lane 1). Surprisingly however, even when RNase A digestion was performed before RNA isolation, the products appeared to be fully resistant (Fig. 3, lane 7). RNA accumulation had not reached its maximum after 20 and 40 min of incubation. Nevertheless, RNA products already appeared to be fully base-paired (Fig. 3, lanes 2 and 5, where synthesis was done for 20 min; and lanes 3 and 6, where synthesis was done for 40 min). Apparently, the obtained double-stranded products were not 'dead-end' products simply due to the 60 min of incubation at 28°C. These results are in contrast with studies on bacteriophage Qβ RdRp, where it was shown that this enzyme produced minus-RNA products as single-stranded structures [18]. In that case phenol extraction of the reaction mixtures resulted in the isolation of double-stranded RNAs due to base-pairing during de-proteinization of the samples.

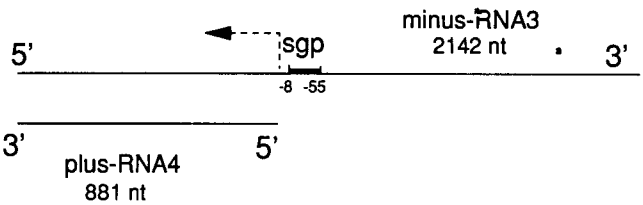

Fig. 1. Schematic representation of the synthetic minus-RNA3/plus-RNA4 hybrid. The position of the subgenomic promoter (sgp: positions −8 to −55, [10]) for RNA4 synthesis is indicated with a solid line. The transcription start (position +1) is indicated with an arrow.

3.3. AlMV RdRp has RNA duplex unwinding activity

In a previous report we showed that the AlMV RdRp was able to accept full-length double-stranded AlMV RNAs as templates in vitro [7]. However, we could only reproduce these results in incidental cases (not shown). Occasionally, enzyme preparations or fractions from a column may contain a factor necessary for initial unwinding.

To further investigate the ability of the AlMV RdRp to synthesize RNA products on double-stranded RNA molecules, we developed an RNA duplex unwinding assay, which may resemble the natural constellation for subgenomic RNA synthesis. As a template for the RdRp we used a gel-purified minus-RNA3/plus-RNA4 hybrid. This hybrid-RNA molecule was readily accepted as a template for in vitro RNA synthesis by the AlMV RdRp (Fig. 4, lane 4). Three major products are seen on the gel, which have the mobilities of double-stranded RNA3, the minus-RNA3/plus-RNA4 hybrid, and double-stranded RNA4. After denaturation they yield labelled molecules with the mobilities of RNA3, RNA4 and RNA4, respectively. The first and the third product are fully resistant to nuclease S1 at a moderate salt concentration (0.2 M NaCl). The second product upon S1 treatment changes into a product with the mobility of double-stranded RNA4, just as the hybrid does. These identification tests, which are not shown here, demon-

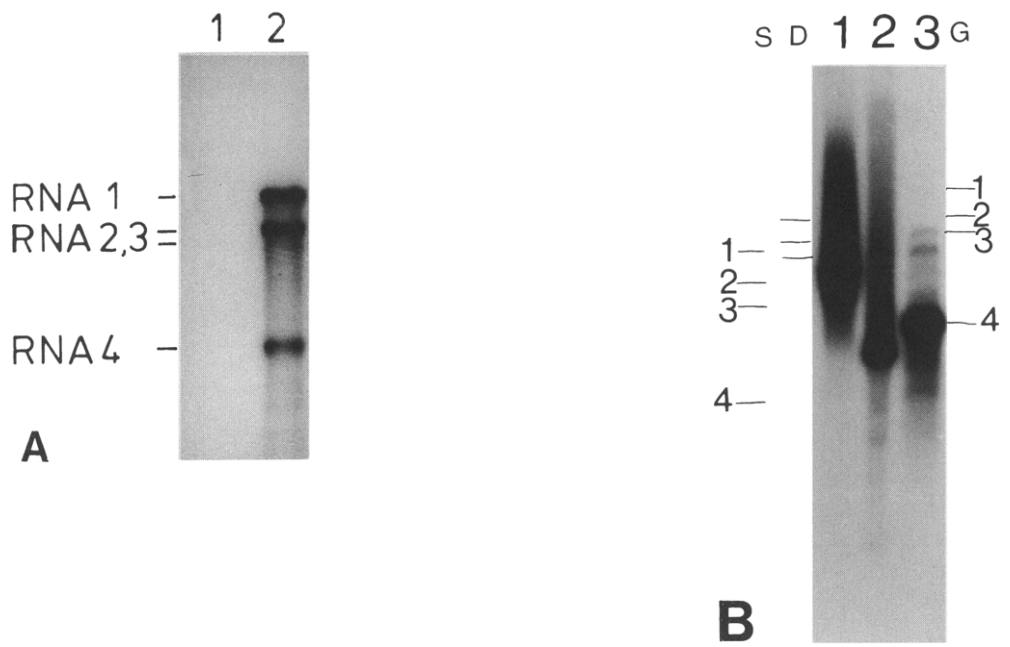

Fig. 2. RNA products synthesized in vitro by the AlMV RdRp. (A): RdRp programmed with 2 μg AlMV virion RNAs (lane 2), or no RNA templates (lane 1). On the left are indicated the positions of double-stranded AlMV RNAs as determined from control gels. (B): RdRp programmed with 0.5 μg minus RNA3 as a template. RNA products were not treated (lane 1), treated with nuclease S1 (lane 2), treated with nuclease S1 and denatured by glyoxal treatment (lane 3). The positions of the single-stranded AlMV virion RNAs (S), double-stranded genomic RNAs (D), and glyoxal-treated single-stranded virion RNAs (G) are indicated in the margins.

strate that the RdRp upon initiating and elongating an RNA3 strand or a new RNA4 strand was able to remove an RNA4 strand which was annealed to the template, and thus was able to unwind an RNA double strand. Labelled double-stranded RNA4 is probably produced by degradation of the single-stranded part of the template. Curiously enough the template as it is seen on the stained gel (not shown) was not degraded to a significant extent.

In contrast to when single-stranded minus RNA3 was used as a template, also some production of single-stranded RNA4 could be observed (Fig. 4, compare lanes 4 and 1). Apparently, the enzyme is also capable of releasing newly synthesized RNA4 molecules from their template. Furthermore, RNA synthesis on the minus-RNA3/plus-RNA4 hybrid was inhibited by monoclonal antibodies directed against double-stranded RNA (J2 antibodies, Fig. 4, lane 6), but not appreciably by control antibodies (5/14 antibodies; Fig. 4, lane 5). The inhibiting activity of the J2 antibodies was titrated by preincubating them with increasing amounts of a double-stranded RNA molecule of 192 base-pairs. Per μg of J2 antibody 0.05 μg of double-stranded RNA was needed to neutralize the inhibiting activity, which is in accordance with earlier data [17]. Both antibodies hardly affected RNA synthesis when single-stranded minus RNA3 was used as a template (Fig. 4, lanes 2 and 3). Form observation of the stained gels (not shown) it is evident that the antibodies do not degrade the template, nor do they degrade an isolated product during incubation under the reaction conditions (result not shown).

This is a first report of a plant virus RdRp capable of unwinding double-stranded RNA molecules in vitro. An earlier report on poliovirus RdRp showed the ability of this enzyme to unwind RNA double-strands [19], but in that case the plus strand was annealed to a non-natural minus strand fragment and elongation was primed at a large distance from the duplex structure.

Clearly, the AlMV RdRp was not able to prevent base-pairing between RNA products and RNA templates during elongation in vitro, despite its ability to unwind double-stranded structures. The resulting full-length double strands are not templates for the RdRp and RNA synthesis comes to an early stop. In the minus-RNA3/plus-RNA4 hybrid used, the

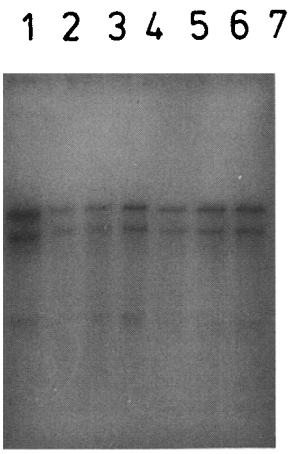

Fig. 3. RNA products synthesized in vitro by the AlMV RdRp programmed with 2 μg AlMV virion RNAs. Incubation was done for 20 min (lanes 2 and 5); 40 min (lanes 3 and 6); or 60 min (lanes 1, 4 and 7) RNA products were not treated with RNase A (lane 1); treated after phenol extraction of the reaction mixtures (lanes 2–4); treated prior to phenol extraction of the reaction mixtures (lanes 5–7). RNA products migrate as double-stranded AlMV RNAs as indicated in Fig. 2A.

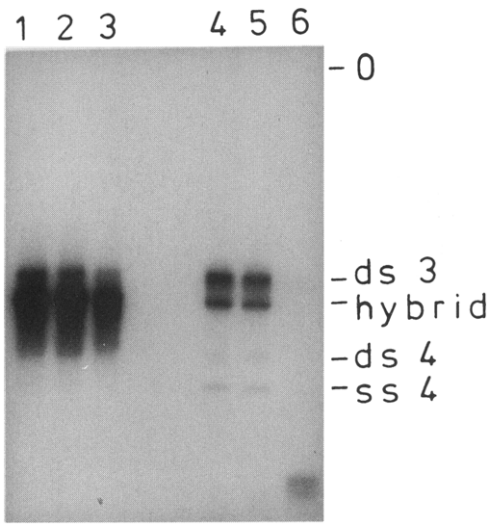

Fig. 4. RNA products synthesized in vitro by the AlMV RdRp after programming the enzyme with minus-RNA3 as a template (lanes 1, 2 and 3) or hybrid minus-RNA3/plus-RNA4 as a template (lanes 4,5 and 6). RNA templates were preincubated with antibodies directed against double-stranded RNA (J2 antibodies; lanes 3 and 6) or control antibodies (5/14; lanes 2 and 5) prior to RNA synthesis.

subgenomic promoter is not in the duplex structure ([10]; Fig. 1). This could provide a favourable condition for RNA synthesis and concomitant template unwinding to occur.

Possibly, an enzyme factor is inhibited or lacking in general in our in vitro system to prevent base-pairing during elongation. We will concentrate our efforts on this problem in order to find such a factor.

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