Coat protein blocks the in vitro transcription of the virion RNAs of alfalfa mosaic virus

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Using a method described by Miller and Hall [(1983) Virology 125, 236–241] for brome mosaic virus, we succeeded in obtaining preparations of an alfalfa mosaic virus specified RNA polymerase that is totally dependent on added RNA. The enzyme makes full-size transcripts on each of the four virion RNAs. Transcription is inhibited by small amounts of coat protein subunits. This is in accordance with a model proposed by Nassuth and Bol [(1983) Virology 124, 75–85] which says that in a late stage of the infection cycle the coat protein is a factor that regulates the minus-strand RNA synthesis in a negative sense.

Alfalfa mosaic virus *RNA dependence* *RNA polymerase* *Coat protein regulation*

1. INTRODUCTION

In alfalfa mosaic virus and in ilarviruses in general the coat protein plays a decisive role at the start of the infection process. If the three genome RNAs of these viruses are inoculated onto leaves of host plants or on leaf cell protoplasts, no infection occurs, unless a few copies of coat protein are added. It has been shown that in order to be effective the coat protein must bind to all three genome RNA species (review [1]). Since the coat protein binds preferentially to the homologous 3′-terminal regions of the virion RNAs it has been postulated that it is an essential factor in the initiation of the transcription of plus-strand RNAs by the virus induced RNA polymerase [2]. In order to test this hypothesis in vitro a viral RNA polymerase must be available that is able to synthesize full-size transcripts on added virion RNAs. Here we report on such an enzyme preparation and on the fact that small amounts of coat protein, rather than stimulating the transcription of virion RNAs, had an inhibiting effect.

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Abbreviations: YSMV, yellow spot mosaic virus; TYMV, turnip yellow mosaic virus; BMV, brome mosaic virus; SSC, 0.15 M NaCl, 0.015 M Na citrate

2. MATERIALS AND METHODS

French bean (*Phaseolus vulgaris* L. cv. Berna) plants were grown at 20–22°C under fluorescent lighting for 15 h per day. After a dark period of 1 day, 9-day-old plants were inoculated on both sides of the primary leaves with a suspension of 1 mg/ml of purified virus of a strain of alfalfa mosaic virus which is systemic in this host, viz. YSMV [3].

YSMV, its RNAs and coat protein, and TYMV-RNA were prepared as reported ([4], and references therein). BMV-RNAs were extracted as were YSMV-RNAs, from virus prepared according to [5]. MS2-RNA was kindly provided by Mr G.P. Overbeek of our department.

A crude particulate fraction (PF) able to synthesize RNA in vitro on endogenous viral templates in the presence of actinomycin D was

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prepared from infected leaves 3 days after inoculation as described in [6] up to the stage of the first 35000 × *g* pellet, except that the volume of buffer used to homogenize the leaves was doubled. The pellet was resuspended in 4*x* ml of buffer A from [7] containing 10% (w/v) sucrose instead of 15% (v/v) glycerol, *x* being equal to the weight of the leaves. Volumes up to 20 ml were layered on top of 10 ml of 20% (w/v) sucrose in the same buffer, and were centrifuged for 30 min at 35000 × *g*. The pellet was then treated as described in [7] with a final concentration of 2% (w/v) of the detergent dodecyl-β-D-maltoside (Calbiochem-Behring) and centrifuged twice at high speed, the last time through a deep pad of 40% (w/v) sucrose. In all steps the pellets were resuspended very carefully using a Potter-Elvehjem homogenizer. The white-greenish pellet of the last run was resuspended in 1/20*x* of the assay buffer from [8] which, however, in this case contained 1 rather than 10 mM Mg acetate. The suspension was mixed with an equal volume of glycerol for storage at −20°C (PFp). Just before the RNA synthesis was performed the PFp was treated with micrococcal nuclease as described in [8] using 1.25 units of nuclease per μl of PFp. This preparation is designated PFpn.

RNA synthesis in vitro was performed essentially according to [8] in 50 μl reaction mixture containing 15% (v/v) glycerol, 5–40 μl of particulate fraction and 0.5 nmol [α-^{32}P]UTP (10 Ci/mmol, Amersham). When PF was used the detergent was omitted from the reaction mixture. After the reaction the volume was adjusted to 200 μl with distilled water. Products were extracted, precipitated with isopropanol, treated with nuclease S_1 (Sigma), denatured with glyoxal and subjected to electrophoresis in 1% agarose minigels as described in [6] except that the isopropanol precipitation step was repeated once. The nuclease S_1 treatment was followed by two phenol/chloroform extractions which were carried out as described for the crude product [6]. Material to be used for annealing experiments was not treated with nuclease S_1 but instead with 10 μg ribonuclease A (Boehringer) and 10 units ribonuclease T_1 (Sankyo) per ml for 30 min at 30°C in order to degrade more carefully any single-stranded RNA.

Annealing experiments were performed at 37°C in 22 μl of 60% (v/v) formamide [9]. After the annealing the solution was diluted to 200 μl with 2 × SSC and treated with ribonucleases A and T_1 as decsribed above, omitting the phenol/chloroform extraction step. The ribonuclease-resistant material was precipitated with isopropanol, using 5 μg *Escherichia coli* tRNA as carrier, and Čerenkov counted.

To degrade the viral coat protein in PFpn preparations and in RNA/coat protein complexes, treatments with trypsin (TPCK treated, Worthington) were carried out. The conditions were: 10 μg per ml of incubation mixture for 10 min at 20°C in the former case, and 1/50 weight ratio of trypsin (related to coat protein) for 15 min at 20°C in the latter case. The reactions were terminated by addition of a 10-fold weight excess of trypsin inhibitor from chicken egg (Boehringer).

Electrophoresis of enzyme preparations and of RNA/coat protein complexes was in 10% polyacrylamide gels with 3% stacking gels [10]. The gels were stained with Coomassie blue and silver according to [11], but the time of development was diminished in order to avoid overstaining.

3. RESULTS

PF preparations from virus-infected leaves synthesized products on endogenous templates. These products after denaturation had the mobility of virion RNAs (fig.1, lane 3). Addition of YSMV-RNAs did not cause any significant increase in the incorporation of radioactive label (not shown). Comparable preparations from mock-inoculated leaves did not synthesize any product (fig.1, lane 1), unless RNA was added (fig.1, lane 2). However, this product was small in size. Apparently, this is material synthesized by a host-coded RNA-dependent RNA polymerase (review [12]).

On the other hand, PFpn preparations did not synthesize any product (fig.1, lane 6), unless template RNA was added. With 10 μg of a total mixture of the four YSMV-RNAs a higher incorporation was obtained than with the same enzyme preparation in a crude state with endogenous RNAs as templates (cf. fig.1, lanes 3 and 7). The denatured products had an electrophoretic mobility similar to that of denatured virion RNAs. No product was found after addition of 20 μg RNA of bacteriophage MS2 (not shown), whereas 10 μg of a mixture of the four RNAs of BMV, a non-ilar

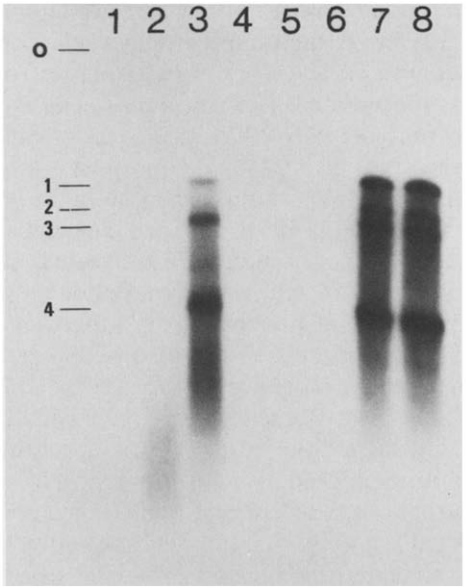

Fig.1. PAGE of nuclease S_1 and glyoxal-treated products of RNA polymerase in a crude (PF) and in a template-dependent stage (PFpn). Lanes: 1, PF (mock), no RNA; 2, PF (mock), 20 μg YSMV-RNAs; 3, PF, no RNA; 4, PFpn (mock), no RNA; 5, PFpn (mock), 20 μg YSMV-RNAs; 6, PFpn, no RNA; 7, PFpn, 10 μg YSMV-RNAs; 8, PFpn, 20 μg YSMV-RNAs. In all cases the reaction mixtures contained enzyme aliquots derived from about 100 mg of leaf tissue. O, origin. The position of the 4 virion RNAs of YSMV (1–4 in order of increasing mobility) is indicated.

tricornavirus, gave only 45% of the incorporation of the YSMV-RNAs. In the latter case the products comigrated with the virion RNAs of BMV (not shown). PFpn preparations from mock-inoculated leaves did not respond to addition of RNA (fig.1, lanes 4 and 5) showing that the host enzyme had been removed.

Annealing experiments (table 1) showed that the products made by PFpn preparations when YSMV-RNAs were added were of negative polarity, in contrast to the products made by the PF preparations on endogenous templates.

Having RNA polymerase (PFpn) preparations which were able to synthesize minus-strands on added plus-strand templates and which in some way were virus-specified since they copied homologous templates with the highest efficiency, we could now test our hypothesis that the coat protein of alfalfa mosaic virus is necessary for the initiation of minus-strand RNA synthesis of this virus. However, before doing so, we had to remove

Table 1

Annealing of viral RNA polymerase products with virion RNAs

Product synthesized on	RNA added to annealing mixture	Annealing (%)
Endogenous templates (PF prep.)	–	4
	20 μg YSMV virion RNAs	3
Added YSMV virion RNAs (PFpn prep.)	–	4
	10 μg YSMV virion RNAs	79
	10 μg TYMV virion RNAs	5

Input was 1000 cpm

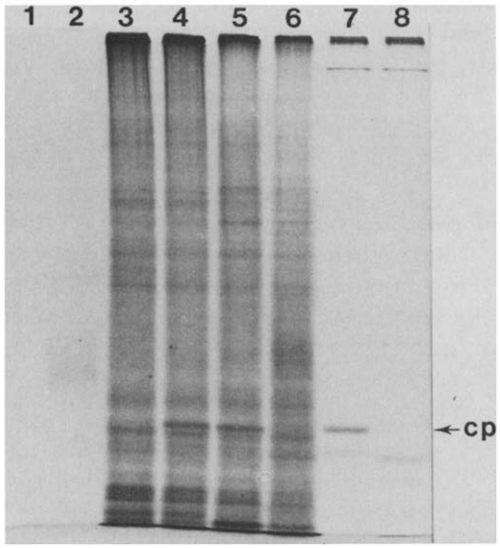

Fig.2. SDS-PAGE of RNA polymerase preparations (7 μl samples) and of viral RNA/coat protein complexes (1 μg RNA plus 0.5 μg coat protein; molar ratio 1:20). Effect of trypsin treatment. Lanes: 1, trypsin preparation (same amount as in 6); 2, trypsin inhibitor preparation (same amount as in 6); 3, PFp (mock); 4, PFp; 5, PFpn; 6, PFpn, trypsin-treated; 7, RNA/coat protein complex; 8, RNA/coat protein complex, trypsin-treated. In lanes 7 and 8 the RNAs are visible at the top. Also an unknown trypsin sensitive component is present.

the endogenous coat protein. As judged from the appearance on gels, 10 μl of a PFp(n) enzyme preparation contained about 1 μg of coat protein. We found that a mild trypsin treatment was sufficient to degrade the coat protein (cf. fig.2, lanes 5 and 6). It is known that such a treatment removes a stretch of 25 amino acids from the N-terminus of the coat protein and thereby makes it biologically inactive [13]. Also, the truncated coat protein has lost its ability to bind specifically to the 3'-end of the alfalfa mosaic virus RNAs [14].

Surprisingly, the degradation of the endogenous coat protein, rather than abolishing, increased the activity of a PFpn preparation by 33% (not shown), the RNA synthesis reaction mixture containing 12 μl of PFpn and 10 μg of template RNAs. Thus, it was likely that the coat protein had an inhibitory effect. Indeed it was found that addition of coat protein to a PFpn preparation caused inhibition of transcription, irrespective of whether the PFpn preparation was pretreated with trypsin or not. A detailed experiment is shown in fig.3. In the experiment we added to a series of RNA synthesis reaction mixtures, containing a trypsin treated PFpn preparation, template RNAs which were complexed with increasing amounts of coat protein subunits. Complex formation was brought about by incubating RNAs and coat protein for 5 min at 40°C in 0.01 M Na phosphate, 0.001 M EDTA, pH 8.0 [4]. It appeared that the coat protein present in a molar excess of 10 over RNA (taking the average molecular mass of the RNA molecules as 1 MDa) decreased the incorporation already by more than 50% (fig.3), despite the fact that the formation of RNA/protein complexes is highly cooperative [4] and that the amount of template is close to saturation (cf. fig.1, lanes 7 and 8, where even more PFpn, viz. 12 μl, was added per reaction mixture than in fig.3). Therefore, it is likely that association of RNA molecules with a few coat protein subunits already annihilates their being suitable templates for the RNA polymerase present in PFpn preparations. Trypsin degraded the coat protein in the nucleoprotein complexes (fig.2, lanes 7 and 8) and at the same time restored their template function for the greater part (fig.3).

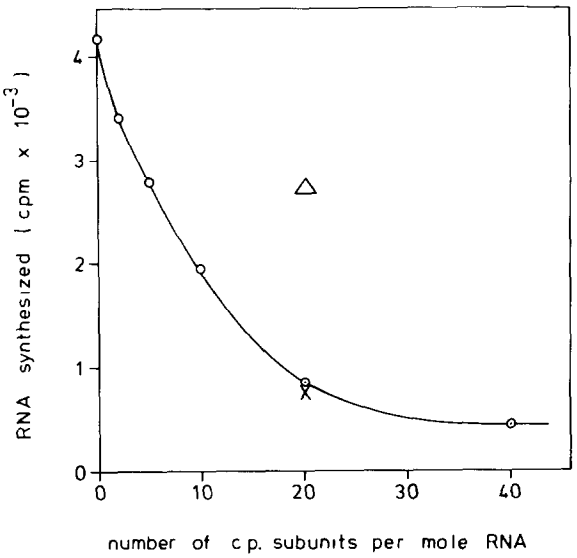

Fig.3. Inhibition of RNA-dependent viral RNA polymerase (6.3 μl of trypsin-treated PFpn preparation) by coat protein. Reaction mixtures contained 10 μg of YSMV-RNAs complexed with the indicated numbers of coat protein molecules; ⊙, untreated nucleoprotein complexes; △, nucleoprotein complex treated with trypsin and subsequently with trypsin inhibitor; X, control with only trypsin inhibitor.

4. DISCUSSION

The present experiments seem to disprove a positive regulatory role of the coat protein in the synthesis of minus-strand viral RNA. However, before rejecting our hypothesis, we would like to point out that the enzyme was prepared from leaves 3 days after inoculation. The infection cycle in the majority of the cells of such leaves is likely to be in a late stage. Probably, in that stage the enzyme is programmed to synthesize virion RNA. In the presence of coat protein such a programmed enzyme might be prevented from transcribing plus-strands into minus-strands.

An elegant model for the differential activity of the coat protein in early and late stages of the infection cycle was proposed by Nassuth and Bol [15]. They found that cowpea mesophyll protoplasts inoculated with alfalfa mosaic virions containing only RNAs 1 and 2 were able to synthesize viral RNAs, but that the ratio of plus- to minus-strands was markedly changed. Minus-strands were synthesized in much larger quantities and plus-strands in much lower quantities in the incomplete infection as compared with the produc-

tive infection. Probably, in a productive infection a product of RNA 3 programs the RNA polymerase so that it switches from minus- to plus-strand synthesis. The authors propose that the coat protein itself is the programming factor. In an early stage of the infection the coat protein level in the cell is low and the coat protein would reside mainly on the high-affinity binding sites for the coat protein at the 3′-ends of the RNA molecules. In a later stage the excess coat protein would also go to the active site on the enzyme molecules, and so the initiation of minus-strand synthesis on 3′-ends which are complexed with coat protein would become blocked. In our experiments the coat protein associated with the RNA polymerase could be protected against trypsin degradation in contrast to the coat protein associated with the template. This could explain why trypsin-treated enzyme preparations are able to synthesize minus-strands on protein-free plus-strands.

In our opinion the fate of the current hypotheses concerning the regulatory role(s) of the coat protein of alfalfa mosaic virus depends on the effects that will be found in vitro with RNA polymerase preparations to be isolated from protoplasts inoculated only with RNAs 1 and 2. Consequently, the next step in our investigations will be the isolation of such an unprogrammed enzyme.

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