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In vitro evidence that the coat protein is the programming factor in alfalfa mosaic virus-induced RNA synthesis

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Viral RNA-dependent RNA polymerase preparations were isolated from cowpea mesophyll protoplasts 26 h after inoculation with the complete genome (RNAs 1, 2 and 3) and the incomplete genome (RNAs 1 and 2) of alfalfa mosaic virus. Although in incompletely inoculated protoplasts the ratio minus-/plus-strand RNA synthesis is much higher than in completely inoculated ones [(1983) Virology 124, 75–85)], both enzyme preparations produced largely plus-strand RNA on their endogenous templates. When made template-dependent both were strongly inhibited by small amounts of coat protein. The experiments show that among the two proteins encoded by RNA 3 only the (parental) coat protein can be responsible for the programming of the viral RNA polymerase to plus-strand synthesis.

Alfalfa mosaic virus; RNA-dependent RNA polymerase; Coat protein regulation; Protoplast infection

1. INTRODUCTION

Two of the three parts of the RNA genome of alfalfa mosaic virus, viz. RNAs 1 and 2, are sufficient for viral RNA synthesis to take place in cowpea mesophyll protoplasts. The gene products of these RNAs with molecular masses of 126000 and 90000 kDa, respectively, are supposed to form part of an RNA synthesizing complex. Remarkably, the ratio of minus- to plus-strand RNA in incompletely infected protoplasts is considerably higher than that in completely infected protoplasts. Nassuth and Bol [1], who discovered this phenomenon, suppose that RNA synthesis in incompletely infected protoplasts is symmetrical and that in the completely infected protoplasts, and in infected cells in general, one of the gene products of RNA 3, either the non-structural 32 kDa protein or the sole coat protein of the virus, make the RNA synthesis become strongly asymmetrical, in the sense that plus-strand RNA is produced in excess. The authors suggest that the coat protein has a dual regulatory effect on RNA synthesis. An early effect would be that a few coat protein molecules in binding to sites close to the 3′-end of the plus-strand RNA molecules would make recognition by the viral RNA polymerase possible, so that initiation of minus-strand synthesis could take place. This would explain why naked plus-strand RNAs are incapable of starting an infection. Some coat protein must be added to make the genome RNAs infectious and the coat protein molecules must bind to each of them in order to have this effect [2]. A late regulatory effect of the coat protein would result from its accumulation in the cell causing the polymerase to associate with coat protein. This would block the recognition of the 3′-ends of the plus-strand RNAs, which are already occupied by coat protein. In that way only minus-strand RNAs which lack high-affinity binding sites for coat protein (Huis in 't Veld, M.A., Zuidema, D. and Jaspars, E.M.J., to be published) would be transcribed and the majority of the progeny RNA molecules would have

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the plus polarity.

The hypothesis of Nassuth and Bol, attractive as it is in its simplicity and testability, invites further experimentation. The interaction of coat protein with viral RNA is well-documented (review [3]). Recently we obtained preparations from bean seedlings of a template-dependent RNA polymerase induced by alfalfa mosaic virus and showed that coat protein has a strongly negative effect on plus-strand transcription in vitro [4]. The enzyme was isolated 3 days after inoculation and contained about 0.1 μg endogenous coat protein per μl. Removal of the endogenous coat protein by trypsin treatment of the preparation did not change the direction of its response toward the addition of small amounts of coat protein. However, we could not exclude the possibility that after the trypsin treatment a small amount of undegraded coat protein was still attached to the enzyme. Also, it is quite possible that the 32 kDa protein, either alone or in combination with coat protein, programmes the RNA polymerase to asymmetric RNA synthesis. Therefore, our rationale was that if we were to detect the early regulatory effect of the coat protein we had to prepare the RNA polymerase in an unprogrammed state. Preferably, this would be the enzyme from incompletely infected protoplasts where the genes for the 32 kDa protein and the coat protein are lacking and where RNA plus-strands are frequently transcribed.

Here, we report upon RNA polymerase preparations from completely and incompletely infected protoplasts. We were unable to detect any significant difference in the polarity of the RNA synthesized by the two kinds of enzyme preparations on endogenous templates. Moreover, addition of coat protein had a negative effect on the transcription of added plus-strands in both cases. Some residual parental coat protein was still found endogenously in the enzyme preparations. It is argued that the coat protein rather than the 32 kDa protein is responsible for the programming of the RNA synthesis during the infection cycle of alfalfa mosaic virus.

2. MATERIALS AND METHODS

Preparation of leaf mesophyll protoplasts from cowpea [*Vigna unguiculata* (L.) Walp cv. California Blackeye] was essentially according to [5]. Batches of 30×10^6 protoplasts were inoculated in the presence of polyethylene glycol (pharmaceutical grade, Polysciences, Warrington, PA) [6,7] and incubated in Aoki medium with 0.2 mg/ml chloramphenicol for 26 h at 25°C and 2000 lx in two 250 ml Erlenmeyer flasks, each containing 15 ml protoplast suspension. About 40% of the protoplasts was lost during the washing procedure after the inoculation. Inocula contained either 300 μg total virus or 600 μg bottom plus 600 μg middle nucleoprotein component of strain 425 of alfalfa mosaic virus [8], purified and measured as reported ([9] and references therein). Bottom and middle nucleoproteins are the virions that contain RNA 1 and RNA 2, respectively.

At the end of the incubation period 0.5 ml protoplast suspension was used for assaying the amount of infectious virus and the remainder for preparing enzyme extract. The infectivity assay was as described [5]. Crude enzyme preparations with endogenous templates (PF) as well as detergent- (dodecyl-β-D-maltoside) and nuclease-treated, template-dependent enzyme preparations (PFpn) were obtained from completely and incompletely infected protoplasts essentially as described previously for infected bean leaves [4]. In the first step of the procedure the protoplasts had to be treated differently from leaves. The pellets from the culture medium were resuspended and incubated for 10 min at 0°C in 4.5 ml buffer A from [10] without sucrose. They were homogenized by 20 strokes with a Potter-Elvehjem homogenizer. The homogenate was then made 10% (w/v) in sucrose and centrifuged for 15 min at 35000 $\times$ g. The pellet was then processed as a comparable pellet from leaves. The resuspension volumes for detergent treatment and storage were 1.8 ml and 180 μl (90 μl assay buffer + 90 μl glycerol), respectively. If PF was to be used the crude pellet was resuspended with 540 μl assay buffer without detergent. The PF suspension was diluted with an equal volume of glycerol for storage at -20°C.

In vitro RNA synthesis, extraction and gel-electrophoretic characterization of products, and annealing experiments were performed as reported ([4] and references therein). Double-stranded RNA isolated according to [11] was a gift from Mr Ad J.M. van der Geest.

Electrophoresis of enzyme preparations and of

coat protein was in 10% polyacrylamide gels [12], which were electroblotted onto Immobilon PVDF transfer membranes (Millipore, Bedford, MA) [13] and immunoprobed with rabbit antiserum raised against the viral coat followed by horseradish peroxidase conjugated anti-rabbit IgGs (ICN ImmunoBiologicals, Lisle, IL).

3. RESULTS

PF preparations obtained from completely (ciPF) and incompletely infected protoplasts (iiPF) synthesized products on endogenous templates which after extraction are in a nuclease-resistant form. After denaturation the electrophoretic mobilities of these products equalled those of the virion RNAs (fig.1). In annealing experiments the ribonuclease-treated products of both PF preparations were shown to have also the polarity of virion RNAs (table 1). The only qualitative difference between the two types of enzyme preparations was that the iiPF preparations in contrast to the ciPF preparations did not synthesize RNAs 3 and 4, the latter RNA being the subgenomic coat protein messenger. This is a good check for the incompleteness of infection. Also, the incompletely infected protoplasts did not contain any infectious virus, whereas the completely infected protoplasts yielded about 50 pg virus per cell on the basis of infectivity.

From the annealing experiments it is clear that nearly all the products of iiPF preparations like those of ciPF preparations and of preparations from bean plants [4] were synthesized on en-

Table 1

Annealing of in vitro products of crude viral RNA polymerase (PF) from completely and incompletely infected cowpea protoplasts with plus (+) and double-stranded (ds) RNA

Source of PF	RNA added to annealing mixture		Annealing (%)
	μg + RNA	μg ds RNA	
Complete infection	-	-	5
	5	-	3
	-	0.25	68
	0.1	0.25	27
	0.4	0.25	13
	0.9	0.25	8
	1.9	0.25	5
Incomplete infection	-	-	14
	5	-	7
	-	0.25	62
	0.1	0.25	20
	0.4	0.25	12
	0.9	0.25	9
	1.9	0.25	8

Input was 50 cpm

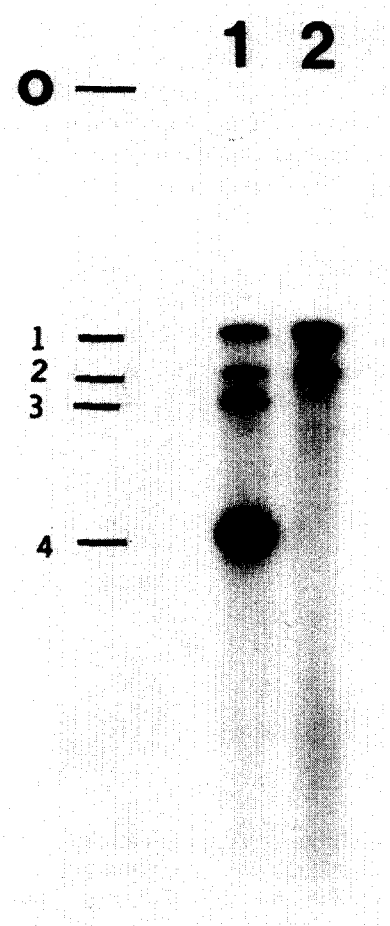

Fig.1. PAGE of glyoxal-treated products of crude RNA polymerase (PF) from completely (lane 1) and incompletely infected protoplasts (lane 2). The reaction mixtures contained enzyme aliquots from 7×10^5 protoplasts. O, origin. The position of the 4 virion RNAs (1–4 in order of increasing mobility) is indicated.

dogenous minus-strand templates, suggesting that the endogenous plus-strands were inaccessible for the enzyme. When purified, template-dependent enzyme preparations were prepared and plus-strands were given as templates the RNA synthesis was strongly inhibited if coat protein was complexed with these templates (fig.2). This phenomenon was similar for ci and ii preparations and resembled the coat protein inhibition of RNA synthesis by PFpn preparations from bean plants [4]. This led us to look for the presence of coat protein in the PFpn preparations from protoplasts. Although no coat protein could have been synthesized in incompletely infected protoplasts its presence in enzyme preparations from such protoplasts was evident upon gel electrophoresis and immunoblotting (fig.3). The concentrations of coat protein roughly estimated on the basis of the appearance on gels (not shown) were 0.05 and 0.25 μg per μl of ii and ci enzyme preparation, respectively. The coat protein present in ii preparations could only be derived from parental virus. This is not impossible if a part of the inoculated particles is strongly adsorbed onto the protoplasts and their coat protein is not degraded afterwards. 1 μl enzyme preparation is derived from 10^5 protoplasts and these were inoculated with 4 μg virus of which 84% consists of coat protein.

In order to be able to draw a conclusion about the necessity of coat protein for transcription any coat protein that is present in an RNA polymerase preparation should be removed or inactivated. Trypsin treatment of PFpn preparations from protoplasts was not as effective (not shown) as with PFpn preparations from bean leaves [4], and even if it could have been made so, there could remain doubts as to whether some traces of coat protein associated with the enzyme could have escaped degradation.

Prior to RNA synthesis we incubated an iiPFpn preparation with affinity-purified antibodies against the coat protein but found no effect (not shown). Again one could argue that the effective coat protein molecules in the enzyme preparation were not accessible for binding to antibodies.

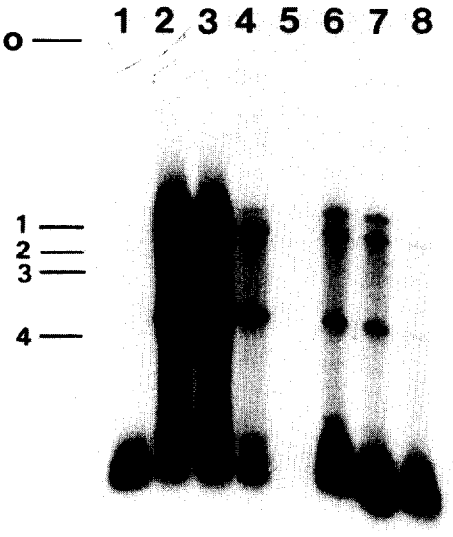

Fig.2. PAGE of non-denatured nuclease S_1-treated products synthesized by template-dependent RNA polymerase (PFpn) in reaction mixtures with enzyme aliquots from 10^6 protoplasts which were mock-inoculated (lane 1), completely infected (lanes 2–4) and incompletely infected (lanes 5–8). All reaction mixtures except 5, which was left without template, contained 20 μg virion RNAs as templates, to which 0 (lanes 1,2,6), 4 (lanes 3,7) or 20 coat protein subunits (lanes 4,8) were added per RNA molecule. Gel positions O, 1–4, as in legend to fig.1.

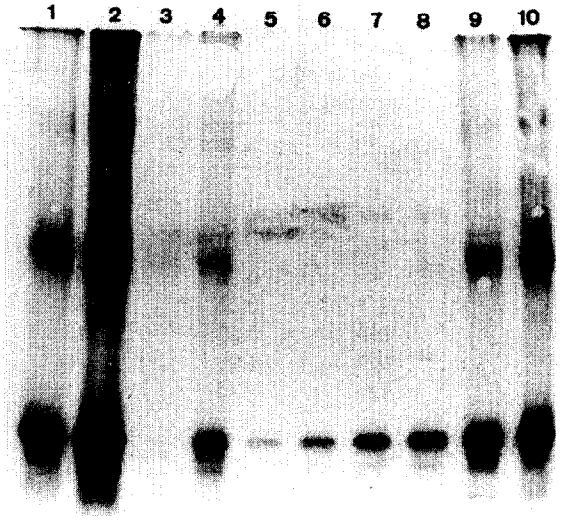

Fig.3. Immunoblot of SDS-PAGE of RNA polymerase preparations (PFpn) and viral coat protein. Lanes: 1, 2 μl, ci; 2, 6 μl, ci; 3, 6 μl, mock; 4, 6 μl, ii; 5–8, 25, 50, 100 and 150 μg coat protein, respectively; 9, 6 μl, ii; 10, 2 μl, ci. Preparations in 1, 2 and 4 are different from those in 9 and 10.

4. DISCUSSION

A viral RNA polymerase from incompletely infected protoplasts would offer an excellent possibility for studying in vitro the effect of the 32 kDa protein and of the coat protein on RNA synthesis. After being made template-dependent such an enzyme would show whether trace amounts of coat protein are necessary for plus-strand transcription and whether increasing amounts of coat protein would subsequently block the same, as predicted by the model of Nassuth and Bol [1]. In this way in vitro experiments with viral RNA synthesis might finally enable us to understand two important features of the life cycle of alfalfa mosaic virus and, possibly, of all ilarviruses, viz. the necessity of coat protein for starting the cycle and the excess of plus-strand over minus-strand RNA synthesis in a late stage of the cycle.

In this report we have shown that we were able to obtain the desired RNA polymerase preparation, but that it still contained parental coat protein, which, apparently, could not be inactivated with antibodies. The preparation did not show a dependence on coat protein for plus-strand transcription, but was rather inhibited by coat protein. Therefore, it seems likely to us that the enzyme was already programmed to plus-strand synthesis. It behaved exactly as enzyme preparations from bean plants and from completely infected cowpea protoplasts obtained 3 days and 26 h after infection, respectively. We could demonstrate parental coat protein to be present in incompletely infected protoplasts 26 h after inoculation. In the literature it is mentioned that in the presence of polyethylene glycol virions of cauliflower mosaic virus associate in large quantities with turnip protoplasts and are not removed by several washings but disappear by 24 h after inoculation [14]. It is possible that the majority of virions that associate with the protoplasts during inoculation remain initially stuck to the plasma membrane in a passive state but are little by little endocytosed, so that more and more coat protein becomes available in the cytoplasm and the viral RNA polymerase becomes programmed. This would explain why Nassuth and Bol [1] found that the ratio of minus- to plus-strand RNA synthesized in incompletely infected protoplasts in the first 24 h after inoculation is exceptionally high, whereas we find that the crude enzyme isolated from such cells after that period produces almost exclusively plus-strand RNA. It is also conceivable that coat protein from the parental virions which stick to the plasma membrane reaches the enzyme and/or the templates after the cells are homogenized and in that way programmes the in vitro system to plus-strand synthesis.

Whatever the explanation for programming of viral RNA polymerase preparations from incompletely infected protoplasts may be, only the parental coat protein could be responsible for it, not the 32 kDa protein, which is certainly absent in these cells.

Because of our failure to eliminate completely the coat protein from the in vitro system for viral RNA synthesis we were still not able to vindicate or reject the hypothesis that coat protein is necessary for the initiation of minus-strand synthesis. Our next approach will be to prepare the enzyme system from protoplasts inoculated with the RNA species 1 and 2 to which only a small amount of coat protein or the subgenomic coat protein messenger, RNA 4, is added. In that way the concentration of endogenous coat protein could possibly be reduced to an insignificant level. It is even conceivable that protoplasts efficiently inoculated with a large amount of RNAs 1 and 2 without any further addition would yield sufficient primary translation products to make the enzyme activity detectable in an in vitro system. This would be the ideal system to test the early function of the coat protein.

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