FEBS Letters 346 (1994) 217–220

FEBS 14091

The immobilized movement proteins of two tobamoviruses form stable ribonucleoprotein complexes with full-length viral genomic RNA

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Received 15 April 1994

Abstract

The movement proteins of two tobamoviruses (tobacco mosaic virus, TMV, common strain U1 and cruciferous TMV strain) containing amino-terminal hexahistidine affinity tags were overexpressed in *Escherichia coli* and purified by metal chelate affinity chromatography. Purified recombinant proteins were immobilized to a Ni^{2+}-chelate adsorbent and their ability to interact with full-length genomic TMV RNA was tested. Here we report that binding of viral RNA to hexahistidine fusion movement proteins results in the formation of stable ribonucleoprotein complexes.

Key words: Tobacco mosaic virus; Viral cell-to-cell movement; Movement protein; RNA binding protein; Immobilized metal affinity chromatography

1. Introduction

It is widely accepted that plant virus-encoded movement proteins (MPs) are required for active transport of viral RNA from infected cells to adjacent healthy ones (reviewed in [1–5]). However, the molecular mechanism(s) by which MPs operate are obscure.

It has been reported that virus-specific informosome-like ribonucleoprotein particles (vRNP) are produced in TMV-infected [6] and potato virus X-infected [7] plants. vRNP differed from mature virions in buoyant density and contained genomic and subgenomic RNAs. It has been suggested that vRNP are involved in the movement of infection throughout the plant, i.e. that they serve as a transport form of the viral genetic material [6,8]. More recent studies revealed that the TMV-U1 30 kDa movement protein (TMV MP30) overexpressed in *E. coli* binds cooperatively to short single-stranded nucleic acid fragments [9]. Binding of TMV MP30 to ssDNA and RNA was reported to be strong and sequence nonspecific. Virus-encoded movement proteins of cauliflower mosaic virus (CaMV), red clover necrotic mosaic virus (RCNMV) and alfalfa mosaic virus (AMV) were also observed to have single-stranded nucleic acid binding activity [10–12].

While preceding studies focused on gel retardation and UV light cross-linking experiments with relatively short radiolabelled DNA and RNA fragments, MP interactions with full-length viral RNA molecules have been addressed only in case of RCNMV. It has been demonstrated by UV cross-linking that the 36 kDa movement protein of RCNMV binds to the synthetic RCNMV RNA 2 (1448 nt) [11]. However, the UV light cross-linking assay can hardly be employed to analyse complexes with large RNA molecules such as the genomic TMV RNA (6395 nt). In the present study a new approach was taken toward the investigation of the movement protein–RNA interactions. Ability of viral genomic RNA molecules to bind to hexahistidine [$(His)_6$] fusion movement proteins was tested using immobilized metal affinity chromatography (IMAC) [13,14].

A complete nucleotide sequence of the TMV strain which systemically infects plants of the Cruciferaceae family (CrTMV) has recently been determined by our group [15]. The CrTMV genome was found to encode a 29.4 kDa movement protein (CrTMV MP29). Here we have studied the interactions of immobilized $(His)_6$CrTMV MP29 and $(His)_6$TMV MP30 with CrTMV RNA and TMV U1 RNA, respectively.

2. Materials and methods

2.1. Enzymes, bacterial strains and plasmids

Restriction endonucleases and other enzymes used for DNA manipulations were purchased from Biopol (Moscow, Russia), Bion (Novosibirsk, Russia), and Fermentas (Vilnius, Lithuania). *E. coli* strain M15[pREP-4], plasmid pQE-9, and Ni^{2+}-NTA agarose (QIAGEN, DIAGEN, Dusseldorf, Germany) were kindly provided by Prof. E. Maiss.

2.2. Expression of $(His)_6$MPs in E. coli

Recombinant constructs expressing fusion $(His)_6$ movement proteins

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Abbreviations: TMV, tobacco mosaic virus; MP, movement protein; CrTMV MP29, 29 kDa movement protein of the cruciferous TMV strain; TMV MP30, 30 kDa movement protein of the common TMV U1 strain; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; IMAC PNABA, immobilized metal affinity chromatography protein-nucleic acid binding assay; NTA, nitrilotriacetic acid.

SSDI 0014-5793(94)00477-D

of two TMV strains (TMV-U1 and CrTMV) were generated by cloning the PCR-amplified fragments into the pQE-9 plasmid vector which belongs to the pDS family of plasmids [16]. The amplified sequences of TMV MP30 and CrTMV MP29 were digested with *Bam*HI/*Pst*I and *Bam*HI/*Hin*dIII, respectively. Restriction fragments were ligated into the corresponding sites of the expression vector pQE-9. The *E. coli* strain M15 containing multiple copies of the lac repressor-producing plasmid pREP-4 was transformed with the resulting plasmids pQE-9/MP30 and pQE-9/MP29. Selected transformants with correctly ligated coding inserts were further used for $(His)_6$MP production.

Glycerol stock cultures of the recombinant bacteria were used to inoculate 2 × YT medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The culture was incubated at 37°C with vigorous shaking until A_{600} has reached 0,7. For the induction of MP expression, IPTG was added to a final concentration of 1 mM. The cultures were grown further for 3 h at 37°C. Induced cells were harvested by centrifugation at 4000 × *g*, stored overnight at −70°C and lysed in buffer A (6 M guanidine hydrochloride, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0).

2.3. Purification of $(His)_6$MPs

The obtained lysates were loaded onto a Ni^{2+}-NTA column (0.8 × 9 cm, 3 ml resin) at a flow rate of 20 ml/h. The column was pre-equilibrated with 100 ml of buffer A. After sample application, the column was developed with step reductions in pH. Nonspecifically bound proteins were washed out with buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8,0) and buffer C (same composition, pH 6.0). Recombinant $(His)_6$MPs were eluted with buffer D (same composition, pH 5.0). Collected fractions were immediately neutralized with NaOH and analysed by SDS-PAGE [17]. The purification procedure yields about 5 mg of $(His)_6$MP from 200 ml of induced culture.

2.4. RNA binding experiments

Radiolabelled 800 nt RNA fragments were prepared by in vitro transcription of the linearized plasmids with T7 polymerase in the presence of [α^{32}P]UTP. Purified $(His)_6$MPs were refolded by step-wise dilution of 8 M urea in a dialysis procedure against 0,01 M Tris-HCl (pH 7.5). Protein–synthetic RNA cross-linking by UV light was carried out as described in [9].

Six steps were involved in the protein–viral RNA interaction studies: (i) the purified movement proteins (4 mg) were immobilized on the column with Ni^{2+}-NTA agarose in buffer B (pH 8.0); (ii) the adsorbent-bound proteins were refolded using a Gilson MixoGrad gradient mixer (Flow rate: 10 ml/h; Linear Gradient: 8 M–0.5 M urea in 0.01 M Tris-HCl, pH 7.5; Time: 2.5 h); (iii) the full-length viral genomic RNA (20 μg) was applied to the column in 0.01 M Tris-HCl, pH 7.5; (iv) the column was washed with 0.01 M Tris-HCl, pH 7.5 at a flow rate of 1 ml/min; (v) the salt gradient elution was performed on the resulting protein–RNA complexes (Flow rate: 15 ml/h; Linear Gradient: 0.05 M–0.8 M NaCl in 0.01 M Tris-HCl, pH 7.5; Time: 1.5 h); (vi) $(His)_6$MPs were eluted from the column with buffer D. The absorbance at 260–280 nm was measured, and collected fractions were analysed by PAGE for the presence of $(His)_6$MPs and viral RNA. Protein–viral RNA-binding experiments were carried out at 4°C.

3. Results

3.1. Cloning, expression and purification of hexahistidine fusion movement proteins $(His)_6$MPs

The coding sequences of CrTMV MP29 and TMV MP30 were enzymatically amplified *in vitro* and ligated into the pQE-9 His-Tag expression vector. *E. coli* M15[pREP-4] cells transformed with pQE-9/MP29 and pQE-9/MP30 plasmids encoding corresponding $(His)_6$MPs were found to produce proteins with an approximate molecular weight of 30 kDa at levels corresponding to 10% of total cell protein (Fig. 1, lanes +).

Numerous attempts to solubilize overproduced proteins in aqueous buffers for further native processing by IMAC were unsuccessful. Recombinant $(His)_6$MPs were therefore solubilized in 6 M guanidine hydrochloride and purified on the Ni^{2+}-NTA adsorbent under denaturing conditions. The contaminating *E. coli* proteins were subsequently washed from the column and $(His)_6$MPs were eluted by step reduction of pH. Collected fractions were applied to 12.5% SDS-PAGE. The yielded proteins were observed to be more than 90% pure (Fig. 1, lanes PUR).

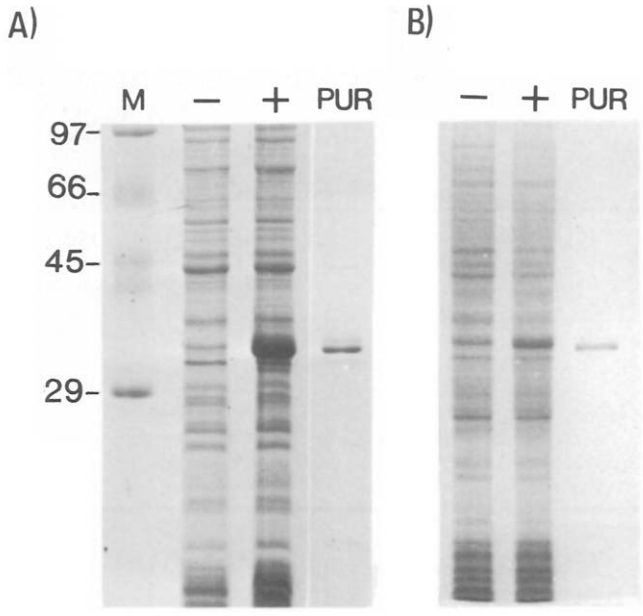

Fig. 1. Expression and purification of $(His)_6$CrTMV MP29 (A) and $(His)_6$TMV MP30 (B). Lanes +, protein from induced *E. coli* M15 cells harboring expression plasmids; lanes −, negative controls without IPTG induction; lanes PUR, purified $(His)_6$movement proteins after processing by IMAC; lane M, molecular weight marker proteins (in kDa). Proteins were separated on 12.5% SDS-polyacrylamide gel and bands were visualized by Coomassie brilliant blue.

3.2. Recombinant $(His)_6$MPs interact with viral genomic RNA

In a series of preliminary experiments, the UV cross-linking of $(His)_6$MPs with ^{32}P-labelled 800 nt transcripts was carried out. Both $(His)_6$CrTMV MP29 and $(His)_6$TMV MP30 were found to bind to synthetic RNA fragments (data not shown).

The IMAC Protein–Nucleic Acid Binding Assay (IMAC PNABA) was developed to study the formation of MP-viral RNA complexes. Purified protein preparations were subjected to slow linear gradient refolding on the Ni^{2+}-NTA column. Refolded Ni^{2+}-bound movement proteins were tested for their ability to interact with full-length viral genomic RNA.

The elution profile of the $(His)_6$CrTMV MP29-CrTMV RNA complex is shown in Fig. 2A. After the absorbance of the flow-through RNA has returned to its base-line level, the salt gradient was applied to the col-

umn. When the NaCl concentration has reached the value of 0.4 M, a sharp peak of released RNA indicated the dissociation of the MP–RNA complex. The immobilized $(His)_6$CrTMV MP29 was further eluted by step decrease in pH and collected fractions were applied to PAGE. SDS-PAGE analysis revealed that fractions corresponding to the flow-through RNA peak and the salt elution peak contained no protein material. Furthermore, the full-length genomic CrTMV RNA was detected by 3% PAGE in these fractions (data not presented). Similar results were obtained with $(His)_6$TMV MP30 and TMV RNA of the common U1 strain (Fig. 2B).

To ensure that the observed binding was not due to high affiniy of RNA for chelated Ni^{2+} ions, a control experiment with no immobilized $(His)_6$protein was carried out. Fig. 2C shows the elution diagram of the TMV RNA from the Ni^{2+}-NTA column. More than 90% of the loaded RNA were immediately washed from the adsorbent in the flow-through material.

Another control experiment was carried out with Ni^{2+}-NTA-bound $(His)_6$proinsulin which was expected to lack RNA-binding function. Fig. 2D shows that the $(His)_6$proinsulin–TMV RNA complex was rather unstable and could be dissociated by as low salt concentration as 0.15 M.

4. Discussion

The CrTMV and TMV-U1 movement proteins containing engineered N-terminal hexahistidine affinity tags were expressed in *E. coli*. The obtained proteins were found to be practically insoluble in aqueous solutions even in the presence of detergents such as Triton X-100 and *n*-octyl β-D-glucopyranoside. Observed strong hydrophobic nature of recombinant MPs correlates with recently reported results indicating that the TMV MP has the characteristics of a hydrophobic, integral membrane protein [18].

Both $(His)_6$CrTMV MP29 and $(His)_6$TMV MP30 overexpressed in *E. coli* and purified by IMAC show RNA-binding activity while immobilized to the Ni^{2+}-NTA adsorbent. This activity was successfully recovered by linear gradient refolding of anchored $(His)_6$MPs after their denaturing purification. The RNA-binding func-

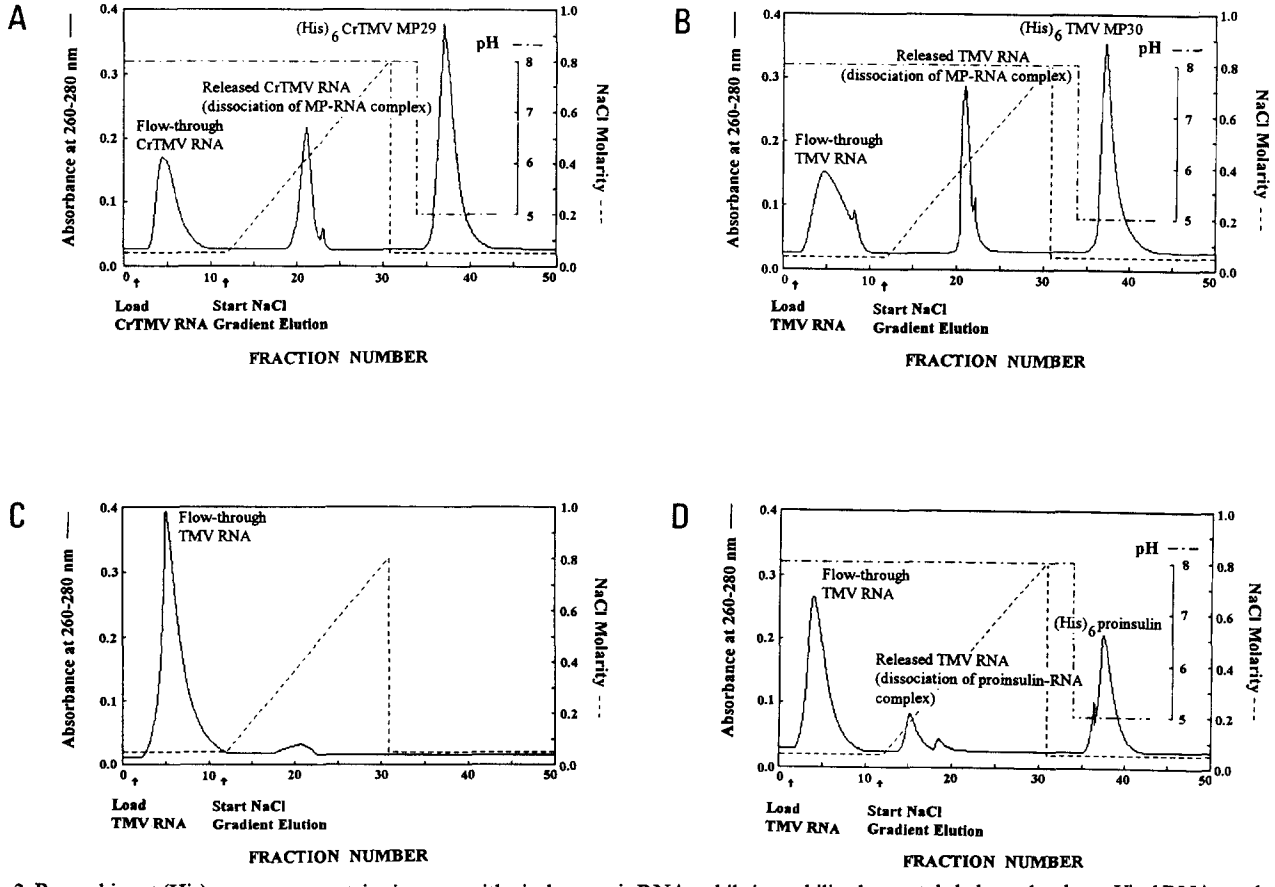

Fig. 2. Recombinant $(His)_6$movement proteins interact with viral genomic RNAs while immobilized to metal chelate adsorbent. Viral RNA was loaded onto a Ni^{2+}-NTA column containing immobilized $(His)_6$proteins. Unbound RNA was washed out and the protein–RNA complexes were dissociated by salt gradient elution. $(His)_6$proteins were further eluted from the column by decrease in pH. (A) Elution profile of the $(His)_6$CrTMV MP29–CrTMV RNA complex. (B) Elution profile of the $(His)_6$TMV MP30–TMV RNA complex; (C) no significant binding was observed when TMV RNA was loaded onto a Ni^{2+}-NTA column containing no protein material; (D) elution profile of the $(His)_6$proinsulin–TMV RNA complex.

tion of MPs was not affected by the presence of hexahistidine tags at the N-termini of recombinant proteins. Assuming that the first several N-terminal amino acids of MPs were not accessible for RNA due to interactions of the adjacent histidines with Ni^{2+}-NTA adsorbent, one can suggest that they are not important for RNA-binding activity. This is in agreement with preceding observations [9] that nucleic acid binding domain is not localized within the first 64 amino-terminal amino acids of TMV MP30.

A possible mechanism was proposed recently for cell-to-cell movement of viral genetic material [9,19]. It was suggested that the protein–RNA complex serves as a transport intermediate which shapes viral RNA for its translocation through the plasmodesmata. This model is supported by structural compatibility between the unusually thin in vitro TMV MP30-single stranded nucleic acid complexes [19] and the movement protein- induced increase in plasmodesmatal permeability [20]. The results of this study indicate that immobilized CrTMV and TMV-U1 MPs interact with full-length viral genomic RNAs which are likely to be natural substrates for MP binding.

The results presented here show that the IMAC PNABA can be applied to study the formation of complexes between immobilized $(His)_6$proteins and nucleic acids. At the same time, this technique may provide an effective and straightforward alternative for identification and isolation of the nucleic acids that might specifically bind to a cloned protein. Moreover, immobilized RNPs can be used in their turn as a tool to fish for protein factors which can interact with matrix-bound protein–nucleic acid complexes.

Acknowledgements: The authors thank S.Yu. Morozov for experimental advice during the course of this project, A.F. Fradkov for $(His)_6$proinsulin, V.A. Mavrodieva and I.I. Senin for much help during the work. Financial support from the International Science Foundation and the Russian Fund for Basic Research is gratefully acknowledged.

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