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ATPase, GTPase, and RNA Binding Activities Associated with the 206-Kilodalton Protein of Turnip Yellow Mosaic Virus

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The 206-kDa protein of turnip yellow mosaic virus belongs to an expanding group of proteins containing a domain which includes the consensus nucleotide binding site GxxxxGKS/T. A portion of this protein (amino acids [aa] 916 to 1259) was expressed in *Escherichia coli* and purified by affinity chromatography to near homogeneity. In the absence of any other viral factors, it exhibited ATPase and GTPase activities in vitro. A mutant protein with a single amino acid substitution in the consensus nucleotide binding site (Lys-982 to Ser) exhibited only low levels of both activities, implying that Lys-982 is important for nucleoside triphosphatase activity. The protein also possessed nonspecific RNA binding capacity. Deletion mutants revealed that an N-terminal domain (aa 916 to 1061) and a C-terminal domain (aa 1182 to 1259) participate in RNA binding. The results presented here provide the first experimental evidence that turnip yellow mosaic virus encodes nucleoside triphosphatase and RNA binding activities.

Most animal and plant RNA viruses encode at least one protein harboring a well-conserved motif: the nucleoside triphosphate binding (NTPB) domain. This domain is often referred to as the RNA helicase domain on the basis of the assumption that this enzymatic activity might be a conserved requirement for the replication cycle of RNA viruses. However, experimental data confirming the helicase activity have been reported for only a few viruses (7, 13, 17, 20, 40). The function of the NTPB domain might not be the same in all cases, and various roles of certain NTPB domain-containing proteins in different viral processes, such as RNA replication (10, 26), vesicular trafficking of replication complexes (28), RNA encapsidation (23), and cell-to-cell movement of the virus (31), have been postulated.

On the basis of amino acid sequence comparisons, the NTPB domain-containing proteins of positive-strand RNA viruses have been classified into three major superfamilies (SF) (9, 10): SF1 includes proteins encoded by Sindbis-like viruses (e.g., nsP2 of Semliki Forest virus [27]), SF2 includes those encoded by poty-pesti-flavi-like viruses (e.g., the NS3-like proteins [13, 17, 40, and references therein]), and SF3 includes those encoded by picorna-like plant viruses (e.g., cowpea mosaic comovirus B polyprotein [26]) and animal viruses (e.g., poliovirus 2C protein [28]).

The three SFs share seven conserved segments designated I, Ia, II, III, IV, V, and VI (10). Segments I and II are the two most conserved and correspond to sites A and B, respectively of the NTPB motif characterized by Walker et al. (39). Segment VI, the third most conserved segment, might be a special type of nucleic acid binding domain, given its abundance in positively charged amino acids (9). The sequences present in segments Ia, III, IV, and V are less strictly conserved, and their roles are not clearly defined. The proteins containing at least three of the seven segments—of which segments A and B always form the NTPB motif—will be referred to here as NTPB-helicase domain-containing proteins.

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Turnip yellow mosaic tymovirus (TYMV) belongs to the Sindbis-like supergroup of viruses. Its positive-sense single-strand genomic RNA of 6.3 kb directs the synthesis of the viral nonstructural proteins expressed from two overlapping open reading frames, the 69- and 206-kDa proteins. The latter is a multifunctional protein whose computer-based sequence analysis suggests the presence of several domains that include a methyltransferase (33), a papain-like proteinase (3, 15, 32), a polymerase (16), and an NTPB-helicase domain (9). This latter domain spans the seven conserved segments and is referred to as TYMV-NTPB.

We have expressed the NTPB-helicase domain (amino acids [aa] 916 to 1259) of the 206-kDa protein in *Escherichia coli* and shown that the purified protein is endowed with ATPase, GTPase, and ATP binding activities and also possesses RNA binding capacity. Mutation of the invariant Lys-982 residue (numbering as in reference 24) in the site A of the NTPB motif revealed the importance of this residue for nucleoside triphosphatase (NTPase) activity. Deletion analyses mapped two distinct domains involved in RNA binding. This is the first experimental demonstration of NTPase activity in a plant virus of the Sindbis-like supergroup.

Cloning, expression, and purification of fusion proteins. Previous studies of TYMV had delineated the proteinase domain in the 206-kDa polyprotein (3, 15, 32) and identified its cleavage site (15). The putative NTPB-helicase domain is located between the proteinase and the cleavage site. Primers were designed to match each of the boundaries of the domain of interest, and the region situated between nucleotides 2840 and 3871 of pTYFL7 (2) was amplified by PCR. The 5′ primer was 5′ GGGGATCCATCTCACACGCCAAGAACTTG 3′; it included a *Bam*HI site (underlined). The 3′ primer was 5′ GG AAGCTTGAATTCAGGCCCCGTTGAGTTTGGGGCCG CG 3′ with *Hin*dIII and *Eco*RI sites (both underlined).

The construction of plasmids encoding different fusion proteins was carried out by standard molecular cloning procedures (34). The PCR-amplified fragment was blunt ended and cloned into *Sma*I-digested pBluescript SK+ (Stratagene). To express full-length fusion proteins with glutathione-*S*-transferase (GST), the same DNA fragment digested by *Bam*HI-*Eco*RI was cloned into the similarly digested expression vector pGEX-2T

(Pharmacia), yielding pGEX-NTPB. The correctness of the construct was verified by restriction mapping.

The point mutant GST-NTPB-K/S was cloned by the same procedure from a parental plasmid (pTYFL7K/S) described previously (32).

E. coli BL21(DE3) cells (Novagen) transformed with the plasmids described above were grown overnight at 37°C in LB medium (34) containing 0.2% glucose and 100 μg of ampicillin per ml. The culture was then diluted 1:100 in the same medium and grown at 37°C to an A_{600} of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The culture was transferred to 24°C, and growth continued for an additional 3 h.

After IPTG induction, synthesis of a 63-kDa polypeptide was observed (data not shown), as expected from the combined size of GST (26 kDa) and TYMV-NTPB (37 kDa). This protein reacted with anti-GST antibodies (Pharmacia) (data not shown).

To purify the bacterially expressed proteins, cells were harvested and stored at −20°C. After being thawed in ice, they were resuspended in 10 ml of lysis buffer (10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) per g of cell paste, incubated in ice for 20 min with 1 mg of lysozyme per ml, and sonicated at 0°C for five cycles of 30 s each. The disrupted cells were centrifuged in an Eppendorf microcentrifuge at 15,000 rpm for 30 min, and the supernatant was immediately loaded onto a resin affinity column (Pharmacia). All purification steps were performed in accordance with the manufacturer's instructions. The eluted fractions were analyzed by 0.1% sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentration was estimated on the basis of known concentrations of bovine serum albumin analyzed on the same gel. The purified proteins were stored at −80°C. After column purification, GST-NTPB was virtually homogeneous (data not shown).

Several attempts were made to remove the GST moiety of the fusion protein by using thrombin. Since less than 5% of the GST-NTPB fusion was cleaved, even after prolonged incubation with thrombin, the intact fusion protein was used in most of the assays. Nevertheless, a small quantity of TYMV-NTPB protein devoid of GST could be obtained that served to confirm the results obtained with the intact fusion protein.

ATPase and GTPase activities. The NTPase activity of the TYMV-NTPB protein liberated from the GST extension and of the GST-NTPB protein was measured as the amount of free radioactive P_i liberated by nucleoside triphosphate (NTP) hydrolysis. The assays were done essentially as previously described (6), except that the purified proteins (approximately 0.2 μg; 2.5 pmol) were incubated at 37°C. At the times indicated, the reaction was stopped with 250 μl of 7.5% activated charcoal (Sigma). The samples were centrifuged at 13,000 rpm for 5 min, and the radioactivity contained in a 200-μl aliquot of the supernatant was determined by liquid scintillation counting. Kinetic measurements (data not shown) indicated that the progress curve of the hydrolysis reaction was linear up to 20 min. At 5 μM ATP, the ATPase activities of the TYMV-NTPB and GST-NTPB proteins were the same (data not shown).

The GTPase activity of the GST-NTPB protein, tested by using [γ-^{32}P]GTP (222 TBq/mmol; NEN) was half that of the ATPase activity (data not shown).

The products of ATP or GTP hydrolysis were analyzed by polyethyleneimine-cellulose thin-layer chromatography. [α-^{32}P]ATP (167 TBq/mmol; Amersham) or [γ-^{32}P]ATP (167 TBq/mmol; ICN) was used to analyze ATP hydrolysis, and [γ-^{32}P]GTP was used for GTP hydrolysis. After 30 min, the reactions were stopped in ice with 0.25 M EDTA and aliquots (1 μl) were spotted onto a polyethyleneimine-cellulose plate (Schleicher & Schuell). Autoradiography of the plates served to identify the reaction products. The products of ATP hydrolysis were ADP (Fig. 1A) and P_i (data not shown). The product of GTP hydrolysis was P_i (Fig. 1B). Purified GST exhibited only background levels of either activity (Fig. 1A and B).

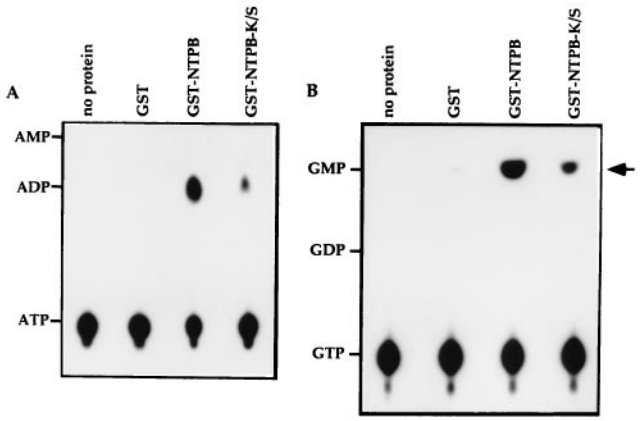

FIG. 1. Analysis of the NTPase activity of GST-NTPB and GST-NTPB-K/S by thin-layer chromatography with 0.5 M formic acid–0.5 M LiCl as the liquid phase for ATP and 0.5 M formic acid–1 M LiCl as the liquid phase for GTP. (A) ATPase activity measured with [α-^{32}P]ATP. After incubation, the reactions were stopped and the products were resolved by thin-layer chromatography and detected by autoradiography. Incubations were without protein or with 0.2 μg of GST, GST-NTPB, or GST-NTPB-K/S; the positions of ATP, ADP, and AMP are presented. (B) GTPase activity was measured with [γ-^{32}P]GTP. The conditions and the samples are as indicated in panel A. The positions of GTP, GDP, and GMP are shown. The arrow to the right indicates the position of P_i analyzed in a parallel lane.

ATPase activity was strictly dependent on the presence of $MgCl_2$, since no activity was detected in the absence of $MgCl_2$ or in the presence of 20 mM EDTA. Removal of the NaCl from the reaction buffer had no effect on the NTPase activity.

To determine the K_m of the GST-NTPB protein for ATP, the amount of ATP hydrolyzed after 10 min, i.e., in the linear phase of the reaction, was performed under standard conditions without NaCl at nonlabeled ATP concentrations varying from 0 to 10 μM. Free P_i was determined by liquid scintillation counting as outlined above. The K_m of ATP hydrolysis calculated from Lineweaver-Burke double-reciprocal plots was 12.5 μM, with an apparent V_{max} of 60 nmol min^{-1} mg of GST-NTPB^{-1} (data not shown). This K_m is in the same range as that of the yeast RAD3 DNA helicase (36), the RNA helicase A from HeLa cells (21), and the well-studied DNA and RNA helicase of simian virus 40 (5). In contrast, members of the poty-pesti-flavivirus RNA helicase family have K_m values for ATP ranging between 20 and 210 μM (37, 38, 40, 41).

Competition for ATP hydrolysis by other NTPs was performed under standard conditions with a reaction mixture containing [γ-^{32}P]ATP, 5 μM ATP, and 5 μM each competitor separately. The amount of ATP hydrolyzed after 15 min was compared to that of a reaction mixture without a competitor (Table 1). The most efficient competitors were ADP, consistent with end product inhibition, and adenosine 5′-γ-thiotriphosphate (Sigma). In the presence of equimolar concentrations of ATP and either ADP or adenosine 5′-γ-thiotriphosphate, the ATPase activity dropped to 50% of the value of the reaction mixture containing ATP only. GTP had no effect on the ATPase activity, even when added at a molar ratio of 10:1 or 20:1 over ATP, presumably because the affinity of the enzyme for ATP is much higher than that for GTP.

TABLE 1. Effects of different competitors on GST-NTPB ATPase activity[a]

Competitor	ATPase activity (%)
None	100[b]
dATP	83
ADP	50
AMP	93
ATPγS[c]	42
GTP	100

[a] The complete reaction mixture contained [γ-^{32}P]ATP and unlabeled ATP at 5 μM, and an unlabeled competitor at 5 μM was added where indicated. Reaction time was 15 min.

[b] ATPase activity of 100% corresponds to the amount of ATP hydrolyzed in the absence of a competitor.

[c] ATPγS, adenosine 5′-γ-thiotriphosphate.

The NTPase activity of NTPB-helicase proteins is typically stimulated by nucleic acids (12, 19, 37, 38). To determine whether this is also true of the TYMV protein, three different RNAs were tested for the capacity to enhance the ATPase activity of GST-NTPB. Addition of poly(A) or poly(U) or of a TYMV RNA fragment at 0.2 mg/ml stimulated the ATPase activity about twofold during the first 10 min of the reaction. This effect disappeared in the presence of 200 mM NaCl (data not shown), suggesting that nucleic acid-protein interaction was responsible for the stimulation observed. Similar results have been reported for other proteins belonging to the NTPB-helicase family (7, 19). The level of RNA stimulation of the ATPase activity varies among the members of the three NTPB-helicase SFs. The highest stimulatory effect (3- to 15-fold) has been described for the enzymes belonging to SF2, whereas ~2-fold stimulation was observed for the nsP2 protein of Semliki Forest virus (27) and the NS polyprotein of rubella virus (11), members of SF1, as is TYMV, and poliovirus 2C protein (a member of SF3 [28]). Despite this variation, all of these viral proteins show single-strand RNA-stimulated ATPase activity typical of RNA helicases.

Site-directed mutagenesis of the conserved lysine residue. The NTP-binding motif (39) consists of two consensus sequences, the A and B sites found in many ATP- and GTP-utilizing proteins (9, 35). The A site (GxxxxGKS/T) forms a phosphate-binding loop, as demonstrated by X-ray crystallography (35), and the B site (consensus sequence DE) is thought to chelate the Mg^{2+} of the Mg-NTP complex (25). The conserved A and B sites are also found in the TYMV 206-kDa protein between aa 976 and 983 and between aa 1042 and 1043, respectively (9, 24). Involvement of the A site in the NTPase activity of TYMV-NTPB was tested by replacing invariant Lys-982 with Ser. Previous results (15a, 42) have shown that an infectious transcript derived from a full-length clone of TYMV carrying this point mutation was unable to replicate in rapeseed protoplasts.

The mutant gene was cloned into pGEX-2T, and GST-NTPB-K/S was expressed and purified as described for GST-NTPB. The ATPase and GTPase activities of this mutant were severely impaired (Fig. 1); as quantified by liquid scintillation counting (data not shown), only 15 to 20% of the wild-type activity remained. Therefore, Lys-982, which is invariant in all NTP-binding proteins, is important for NTPase activity. This is compatible with data obtained with other NTP-utilizing proteins showing that mutation of the conserved Lys abolished or strongly debilitated the NTPase activity in vitro; the degree of influence on ATPase activity of the mutation in this position also depends on the amino acid introduced (6, 14, 23, 27).

ATP binding assay. To further substantiate these findings, we investigated whether a nonhydrolyzable analog of ATP could bind to the protein. GST-NTPB (0.3 μg) was mixed with the standard buffer for ATPase assay in 10 μl containing 10 μM 5′-adenylylimidodiphosphate ([α-^{32}P]AMP-PNP; 1.85 TBq/mmol; ICN) instead of ATP. After incubation for 15 min at room temperature, the reaction was stopped by addition of EDTA at 7 mM. UV cross-linking was performed by irradiating the reaction mixture for 30 min at 254 nm (UV Stratalinker 2400) in an open Eppendorf tube placed on ice at 4 cm from the UV lamps. The AMP-PNP protein complexes were then analyzed by SDS-PAGE and autoradiography. A single radiolabeled band appeared whose migration position corresponded to that of the GST-NTPB protein (data not shown). At 100 μM or higher, ADP inhibited binding of AMP-PNP to the protein (data not shown). Hence, as expected from its ATPase activity, GST-NTPB also possesses ATP binding activity.

RNA binding activity of TYMV-NTPB and its derivatives. Since TYMV-NTPB has homology with the NTPB-helicase family of proteins and since the ATPase activity was stimulated by single-strand RNAs, it seemed conceivable that the protein would have RNA binding capacity. This was tested by Northwestern binding assays (8, 18, 29) using a ^{32}P-labeled RNA probe derived from the TYMV genome as previously described (8), except that final washing of the blot was performed with a buffer containing 100 mM NaCl.

Wild-type TYMV-NTPB, either as genuine NTPB devoid of the GST moiety or as GST-NTPB, bound RNA, whereas GST did not (Fig. 2B). No difference was observed when using other RNA probes deriving either from TYMV or from potato virus Y, suggesting that TYMV-NTPB has sequence-independent affinity for RNA.

To map the region(s) of the protein responsible for RNA binding, four deletion mutants were constructed (Fig. 2A). It has been suggested that motif VI of NTPB-helicases, which contains basic amino acids, might provide a nucleic acid-binding region (9). On the basis of this assumption, two C-terminal deletion mutants of GST-NTPB were constructed as follows: pGEX-NTPB was digested with either *Dra*III (nucleotide 3637) (2) or *Xho*I (nucleotide 3278) (2) and *Eco*RI, blunt ended with T4 DNA polymerase, gel purified, and self-ligated. The resulting plasmids, pGEX-NTPBΔ1 and pGEX-NTPBΔ2, encode fusion proteins that, respectively, lack 77 and 198 aa from the C terminus of the GST-NTPB protein. The RNA binding capacities of these deletion mutants were determined, and the positions of the proteins were visualized by Ponceau S staining. GST-NTPBΔ1, which lacks 23% of the protein, including motif VI, exhibited weak binding activity, whereas GST-NTPBΔ2, which lacks 58% of the protein, including motifs III to VI, possessed strong RNA binding activity (Fig. 2C).

These results suggested that intact GST-NTPB possesses at least one N-terminal region involved in RNA binding, a suggestion also supported by the weak binding capacity of GST-NTPB-K/S (Fig. 2C), but they did not exclude the existence of a second C-terminal domain encompassing motif VI. To test this hypothesis, two complementary N-terminal deletions were produced by using the PCR procedure. The 5′ primers used were 5′ GGGGATCCCTCGAGCTCGTCATAATTCTCGG C 3′ and 5′ GGGGATCCAAGTGGCTCTCCTCGGCTAAC GGC 3′; both contained a *Bam*HI site (underlined). The 3′ primer used was the same as that used for amplification of the full-length protein. The resulting plasmids, pGEX-NTPBΔ3 and pGEX-NTPBΔ4, encode fusion proteins that lack 145 and 266 aa, respectively, from the N terminus of the GST-NTPB protein (Fig. 2A). Mutant protein GST-NTPBΔ3 lacks 42% of the wild-type protein (including motifs I, Ia, and II), where-

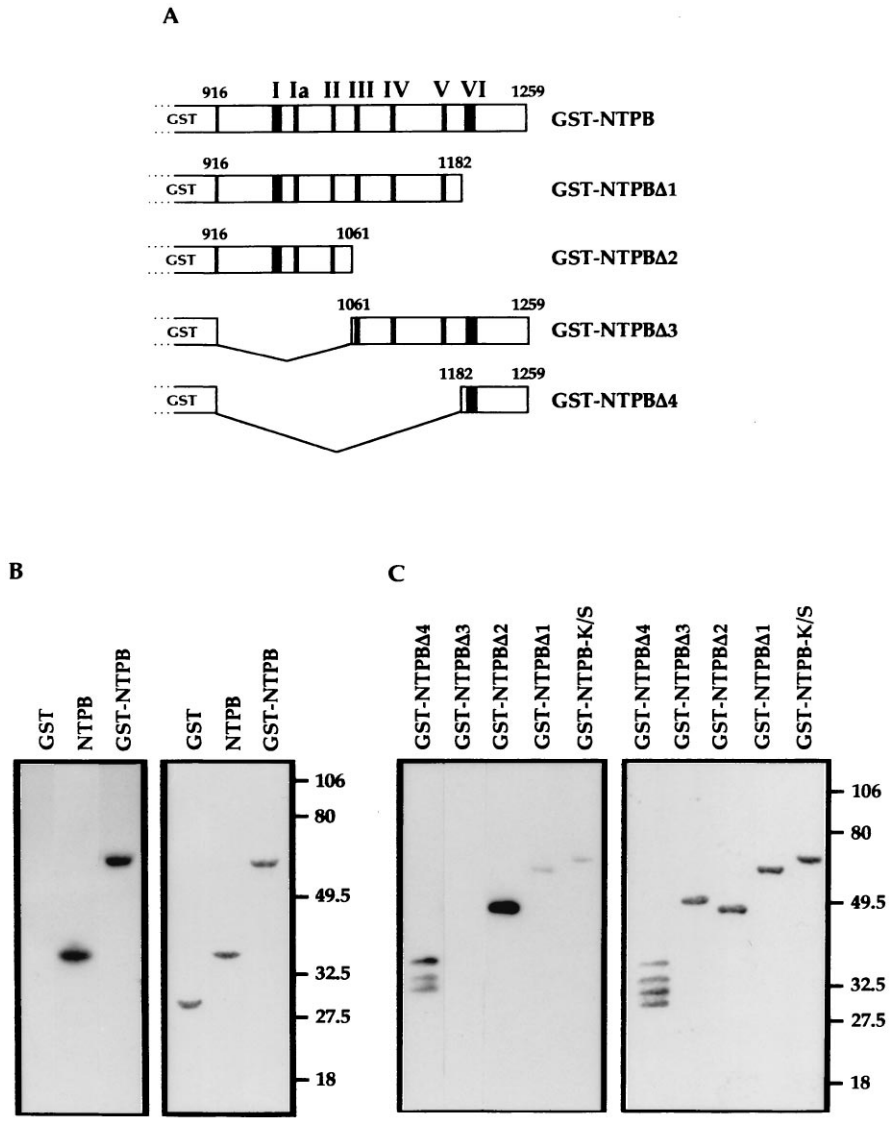

FIG. 2. RNA-binding activities of TYMV-NTPB and its derivatives. (A) Schematic representation of the GST-NTPB family of proteins encoded by different recombinant plasmids. The positions of the first and last amino acids of each NTPB region (numbering according to reference 24) are indicated. Motifs conserved among RNA helicases (designated according to references 9 and 10) are shown as black areas. Lys-982 is contained in segment I. (B) RNA-binding activities of NTPB and GST-NTPB analyzed by Northwestern blotting. The various purified proteins (2 μg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). After several incubations in the appropriate renaturation buffer (8), the blot was hybridized with a ^{32}P-labeled RNA probe (specific activity, 2×10^6 to 3.3×10^6 cpm/μg), washed, dried, and exposed to an X-ray film. The probe (a 200-nucleotide fragment from the 5′ end of TYMV RNA) was obtained by in vitro transcription with [α-^{32}P]CTP (15 TBq/mmol; Amersham) and T7 RNA polymerase (Bethesda Research Laboratories). Left panel: autoradiography of the transferred GST, NTPB, and GST-NTPB proteins after hybridization with the RNA probe. Right panel: Ponceau S staining of the same blot. (C) RNA binding activities of the mutant proteins. From left to right, the lanes contained GST-NTPBΔ4, GST-NTPBΔ3, GST-NTPBΔ2, GST-NTPBΔ1, and GST-NTPB-K/S. Left panel: autoradiography of the membrane after hybridization with the RNA probe. Right panel: same blot stained with Ponceau S. All lanes are from the same gel. The positions and sizes (in kilodaltons) of the prestained protein markers are shown at the right.

as GST-NTPBΔ4 lacks 78% of the protein but nevertheless conserves motif VI. Northwestern blot analyses showed (Fig. 2C) that the former protein was devoid of RNA binding capacity while the latter exhibited strong binding capacity. Furthermore, smaller versions of GST-NTPBΔ4 produced by uncontrolled degradation in bacteria still bound the probe. Presumably, these smaller versions correspond to C terminally truncated GST-NTPBΔ4 still retaining motif VI. It thus appears that motif VI is involved in RNA binding.

The results obtained with four proteins with deletions may be explained in the context of other identified RNA binding proteins, in which small deletions abrogate RNA binding even though the primary sequences responsible for binding are still contained within such deletion mutants, whereas larger deletions may restore this ability (1, 4, 29, 30). For example, deletion of 174 residues from the N terminus of the 54-kDa protein of the signal recognition particle (from dog pancreas) generates a 330-aa protein devoid of RNA binding activity, but an additional deletion of 122 aa from the N terminus renders the resulting protein capable of interacting with RNA (30). It is conceivable that removal of the last 77 aa from GST-NTPB (Fig. 2A) alters the structure of the resulting protein, GST-NTPBΔ1, rendering the new conformation almost inactive, whereas further deletion of 119 residues (GST-NTPBΔ2) may

allow the mutant protein to retain an active conformation. The same situation might prevail for the two N-terminal deletion mutants: GST-NTPBΔ3 (lacking 145 residues but retaining motifs III to VI) exhibits no detectable RNA-binding activity, whereas further deletion of 121 residues in GST-NTPBΔ4 (conserving motif VI) restores RNA binding activity. The results obtained with the deletion mutants suggest that two regions in TYMV-NTPB participate in RNA binding, one located near the N terminus and the other located near the C terminus. Deletion of either region destroys the activity; thus, both domains seem to act in concert to bind RNA, in agreement with other findings on RNA-binding proteins. For example, two distinct domains are responsible for RNA binding of hepatitis delta antigen, as is also the case for the 2C protein of poliovirus, and in each case deletion of either domain results in total loss of this activity (22, 29).

The 206-kDa protein of TYMV contains several postulated and/or demonstrated functional domains. Additional molecular dissection of these different domains should be possible by overexpression of such domains in heterologous systems, such as *E. coli*. In this approach, conserved sequences among proteins with known enzymatic activities can serve as signatures to predict functions of uncharacterized proteins. The only indication that was available concerning the putative existence of an NTPB domain when this study was undertaken was based on sequence comparisons and suggested that the 206-kDa protein belongs to a group of proteins able to bind and hydrolyze NTPs (9, 10).

Indeed, the portion of this protein carrying the NTPB-helicase motif, lying between the proteinase and its autocleavage site, has ATPase, GTPase, and ATP binding activities in vitro. The closely located proteolytic and NTPase activities of the 206-kDa protein function independently, since (i) the proteinase is active in the absence of the NTPB-helicase domain in vitro (32) and when expressed in *E. coli* (15) and vice versa (this report) and (ii) mutation of Lys-982 to Ser causes a dramatic decrease of the NTPase activity but has no influence whatsoever on the proteolytic activity (3, 15, 32).

The NTPase activity of TYMV-NTPB probably couples nucleotide hydrolysis to a function necessary for viral replication. The two biochemical roles of this protein, the RNA-stimulated NTPase and RNA binding activities, suggest that TYMV-NTPB is a strong candidate for helicase function. Only relatively large positive-strand RNA viruses (genomic size over 6 kb) encode putative RNA helicases (9). It is not known if tymoviruses possessing genomes of 6 to 7 kb need a helicase to replicate their RNA. Helicase activity could be responsible for the displacement of a previously synthesized RNA molecule, allowing concomitant synthesis of a new molecule. It could also be required to unwind the secondary structures at the 3′ end of the viral RNA prior to replication. Further experimental evidence and further mutation analyses are required to precisely decipher the role of each motif in a possible helicase function.

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