P6 protein of *Cauliflower mosaic virus*, a translation reinitiator, interacts with ribosomal protein L13 from *Arabidopsis thaliana*

Marina Bureau, Véronique Leh, Muriel Haas, Angèle Geldreich, Lyubov Ryabova, Pierre Yot and Mario Keller

Institut de Biologie Moléculaire des Plantes, UPR CNRS 2357, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France

The P6 protein of *Cauliflower mosaic virus* (CaMV) transactivates translation of the CaMV 35S polycistronic pregenomic RNA and its spliced versions, and thus allows synthesis of a complete set of viral proteins. Previous studies have shown that P6 interacts with plant L18 and L24 ribosomal proteins and initiation factor eIF3, and it has been proposed that these interactions are involved in the reinitiation of translation of polycistronic viral RNAs. This study characterizes a novel cellular partner of P6, the ribosomal protein L13 from *Arabidopsis thaliana*. Far-Western assays performed with several P6 deletion mutants have shown that L13 interacts with the miniTAV of P6, which represents the minimal domain for transactivation, suggesting that the P6–L13 interaction might also be involved in this process. L13 and L18 were found to bind to the same region within the miniTAV. Competition assays between L18 and L13 for binding to miniTAV suggest that interactions between P6 and these ribosomal proteins involve separate P6 molecules, and/or occur at different stages of translation or in the context of another function also mediated by P6.

INTRODUCTION

*Cauliflower mosaic virus* (CaMV) is a plant pararetrovirus whose circular double-stranded DNA genome (8-0 kbp) contains seven major open reading frames (ORF I–VII). Except for ORF VII, all ORF products (P1–P6) have been found in infected plants and their function(s) have been studied (for review see Haas et al., 2002a). The CaMV genome is transcribed by cellular RNA polymerase II into two major transcripts that are capped and polyadenylated: the 35S pregenomic RNA, which serves both as a template for reverse transcription and a messenger for the expression of P1–P6, and the 19S monocistronic RNA, which is specific for P6. The 35S RNA undergoes alternative splicing, leading to four distinct polycistronic mRNAs (Kiss-Laszlo et al., 1995) encoding proteins P3–P5. The 35S RNA has an unusually long leader sequence (> 600 nt) that contains seven small ORFs (sORF). The latter are located in an extended and stable hairpin, except for the first, sORF A, which is located upstream and in close spatial proximity to this secondary structure (Pooggin et al., 1999). These structural features are a hallmark of the pregenomic RNAs of almost all viruses of the caulimoviridae (Pooggin et al., 1999). Two distinct mechanisms controlled by cis- and trans-acting elements are involved in translation of the CaMV 35S RNA in order to circumvent the eukaryotic translation rules (Fütterer & Hohn, 1996). After translation of sORF A, a shunt mechanism operates to transfer ribosomes downstream of the hairpin and thus overcomes the inhibitory effect of the leader sequence on scanning (Pooggin et al., 2000; Ryabova & Hohn, 2000). After bypass of the hairpin, the major ORFs are translated by a termination–reinitiation mechanism (Bonneville et al., 1989; Scholthof et al., 1992). The ribosomal shunt is controlled mainly by cis-acting elements, whereas termination–reinitiation of translation is activated in trans by the P6 protein of CaMV, which is also referred to as TAV. P6 also may have a stimulatory effect on the ribosomal shunt mechanism (for review see Ryabova et al., 2002).

P6 is a multifunctional 62 kDa protein that plays a pivotal role in the CaMV infectious cycle. In particular it is involved in host-range determination (Schoolz & Shepherd, 1988), symptomatology (Daubert & Routh, 1990; Wintermantel et al., 1993; Broglio, 1995), and probably viral morphogenesis (Himmelbach et al., 1996). Moreover, P6 is also the major component of the membrane-free cytoplasmic inclusion bodies called viroplasms (Xiong et al., 1982),

1Present address: Laboratoire de Dynamique, Evolution et Expression des Génomes de Microorganismes, FRE CNRS 2326, Université Louis Pasteur, 28 rue Goethe, 67084 Strasbourg Cedex, France.

2Present address: UPR CNRS 9050, Département Récepteurs et Protéines Membranaires, ESBS, boulevard Sébastien Brant, 67412 Illkirch Cedex, France.

DOI 10.1099/vir.0.80242-0

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where the main steps of the replication cycle occur (Mazzolini et al., 1989).

Transactivation has been shown to depend on the interaction of P6 with polysomes and initiation factor eIF3 (Park et al., 2001), which is an essential component in the translation initiation process. P6 physically interacts with the g subunit of eIF3 (Park et al., 2001) and with the 60S ribosomal proteins L18 (Leh et al., 2000) and L24 (Park et al., 2001). L18 binds to the miniTAV domain (Leh et al., 2000), which corresponds to the minimal sequence required for translational transactivation (De Tapia et al., 1993), while L24 and eIF3 subunit g interact with an RNA-binding region located downstream of the miniTAV. The interaction between P6 and eIF3 on the 60S ribosomal subunit is apparently mediated by at least two ribosomal proteins, L18 and L24. The exact mechanism of P6-mediated reinitiation of translation is not yet fully understood, but it has been proposed that P6 could maintain eIF3 bound to the ribosome during the elongation phase by a shuttling process between the 40S and 60S ribosomal subunits (Park et al., 2001). Translocation of eIF3 back to the 40S ribosomal subunit shortly before the termination of translation would prevent the release of eIF3 from the ribosome and thus permit a ternary complex to reinitiate translation of the next ORF. L24 is one of the proteins of translation would prevent the release of eIF3 from the ribosome and thus permit a ternary complex to reinitiate translation of the next ORF. L24 is one of the proteins involved in bridging interactions between the 40S and 60S ribosomal subunits (Spahn et al., 2001). Whether the P6–L24 interaction is involved in ribosome formation during translation–reinitiation, and/or might affect the association of the two ribosomal subunits late in infection to favour reverse transcription of the CaMV genome, is still unclear (Park et al., 2001).

It has been shown by far-Western assays performed on proteins from a ribosome-enriched fraction of Arabidopsis thaliana that P6 binds to several polypeptides (Leh et al., 2000). This finding suggests that other components of the cellular translation machinery might be implicated in translation of the 35S RNA and its spliced versions. In this study, we have characterized a novel cellular partner of P6, the ribosomal protein L13 of the 60S ribosomal subunit. We also show that L13 and L18 bind to the same sequence of the miniTAV.

### METHODS

#### Construction of recombinant plasmids encoding CaMV P6 and its derivatives.

Recombinant plasmids containing ORF VI from the CaMV Cabb-JI strain (Delseny & Hull, 1983) and its derivatives have been described by Leh et al. (2000). Deletions of 60 nt were introduced along the miniTAV coding region of ORF VI using the site-directed mutagenesis strategy developed by Stratagene. Recombinant plasmid pET-VI, resulting from the insertion of ORF VI into a pET-3a modified pET-KaKS vector (Leh et al., 2000), was used as a template for PCR. PCR was performed using Pfu Turbo polymerase according to the manufacturer’s instructions, with designed internal oligonucleotides as primers (Table 1). After DNA amplification, the mixture was incubated with 2 U DpnI for 2 h at 37°C to destroy the template. The 5’ ends of the PCR products were phosphorylated by T4 polynucleotide kinase in the presence of 1 mM ATP for subsequent ligation. Error-free recombinant plasmids were identified by DNA sequencing.

The recombinant plasmid pGST-P6 was obtained by insertion of ORF VI into the BamHI restriction site of the linearized pGEX-2TK vector (Pharmacia Biotech).

#### Cloning of the A. thaliana L13 coding sequence into pET-KaKS and pGEX-2TK vectors.

The sequence encoding the

<table>
<thead>
<tr>
<th>Name*</th>
<th>Sequence 5’–3’</th>
<th>Recombinant vectors</th>
</tr>
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<tbody>
<tr>
<td>P6331</td>
<td>TCCCTGATGAGGAGCTAAAATAAAA</td>
<td>pET-KaKS-P6A112–131</td>
</tr>
<tr>
<td>P6366</td>
<td>GGTATTTCAACACCTACTATC</td>
<td>pET-KaKS-P6A132–151</td>
</tr>
<tr>
<td>P6391</td>
<td>GCATTCTGCTGGAGGAG</td>
<td>pET-KaKS-P6A152–171</td>
</tr>
<tr>
<td>P6454</td>
<td>TACGATGACTGGGGGTGGTAC</td>
<td>pET-KaKS-P6A172–191</td>
</tr>
<tr>
<td>P6451</td>
<td>AAGGGGGGAGGG</td>
<td>pET-KaKS-P6A192–211</td>
</tr>
<tr>
<td>P6453</td>
<td>AGCATAAGGCCCTACAAGAGGC</td>
<td>pET-KaKS-P6A212–231</td>
</tr>
<tr>
<td>P6452</td>
<td>CTTGTTGGCAACTCCGGAAC</td>
<td>pET-KaKS-P6A212–231</td>
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<tr>
<td>P6451</td>
<td>AGGCGCTTAACAGCCAC</td>
<td>pET-KaKS-P6A232–242</td>
</tr>
<tr>
<td>P6450</td>
<td>AGGCGCTTAACAGCCAC</td>
<td>pET-KaKS-P6A232–242</td>
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*Forward primers are indicated by +, reverse primers by −. Number in forward and reverse primer names used for mutagenesis of ORF VI refers to the first or last nucleotide hybridizing with P6 sequence.*
ribosomal protein L13 of *A. thaliana* (ecotype Columbia) was amplified by PCR from a cDNA library cloned into the HybridZAP vector (the library was provided by B. Lesure, INRA-UMR 215, Castanet-Tolosan, France) with 5‘- and 3'-specific oligonucleotides containing BamHI/KpnI and SalI/Cell restriction sites, respectively, at their 5’ termini (5’-GGACCTTGGAGGATCCCATGAAGCACAAGATGTTA- TC3’ and 5’-GGCACTTGGAGGATCCCATGCTCTGCACCTTTTCTCCTTTT- CTC-3’). After digestion with appropriate endonucleases, the DNA fragment was cloned into the pET-KaKS vector. The resulting pET-L13 recombinant plasmid generates a fusion protein containing, at its N terminus, a decapeptide, which can be phosphorylated in vitro by heart muscle bovine kinase.

The recombinant pGST-L13 plasmid was obtained by insertion of a BamHI/ EcoRI-digested PCR fragment (generated with the primers 5’-GCAGTTACCGGGATCCATGAAGCACAAGATGTTACGTTCCCTG- ATCCGAGGATCCCTCCTTTTCTCCTTTTCTCCTTTTCTCCTTT-3’) and 5’-GGTTGGGAATTCCTCACTTCTTCTCTTCTTTCTCAGC-3’) into BamHI- and EcoRI-cleaved pGEX-2TK.

**Expression and phosphorylation of recombinant proteins.** L13, GST–P6, P6 and its deleted versions were expressed in *E. coli* BL21/DE3 (Novagen) modified by transformation with the plasmid pUBS520 (Brinkmann et al., 1989) carrying the *e. coli* gene argU. L18 and GST were expressed in *E. coli* BL21/DE3 (pLys S) from pETKH-L18 (Leh et al., 2000) and pGEX-2TK vectors, respectively.

*E. coli* strains were transformed by electroporation with recombinant plasmids. Once the culture reached the exponential phase, expression of heterologous proteins was induced with 1 mM IPTG for 2 h. Bacteria were collected by centrifugation at 4000 g for 10 min, resuspended in HMK buffer (20 mM Tris/HCl pH 7–5, 100 mM NaCl, 12 mM MgCl₂), and lysed by sonication (two pulses for 20 s at 50 W). After centrifugation at 12 000 g for 10 min, the supernatant was discarded and the inclusion bodies containing the proteins of interest were resuspended in 500 μl HMK buffer.

Recombinant proteins were labelled in the presence of [γ-32P]ATP [3000 Ci mmol⁻¹; 10 mCi ml⁻¹ (370 MBq)] and 20 μg bovine heart muscle protein kinase for 1 h at room temperature, according to the manufacturer’s instructions (Sigma). Non-incorporated radioactive ATP was eliminated by filtration through a Sephadex G-50 column (Amersham Pharmacia Biotech). The eluate (300 μl) was treated for 30 min with a mixture of RNase A (40 μg) and DNase (100 U) at 37°C. Degradation of nucleic acids was verified after fractionation of an aliquot (20 μl) on a 1-5 % agarose gel and ethidium bromide staining.

**Western blotting analysis.** Proteins from recombinant bacteria were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (Schleicher and Schuell). The membrane was blocked for 30 min in 5 % non-fat dried milk in PBS buffer (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7-3) containing 1% Tween 20 and then incubated overnight at 4°C with specific rabbit polyclonal antibodies raised against the protein of interest and resuspended in 20 μl of dissociation buffer as described above, and the proteins contained in the supernatant were separated on SDS-PAGE. The radiolabelled protein was detected by autoradiography for 1–3 days at −20°C using an intensifying screen, and the competitor was visualized by Coomassie blue staining of the polyacrylamide gel.

**Results**

**CaMV P6 protein specifically interacts with the ribosomal protein L13 of *A. thaliana***

A previously observed polypeptide of about 30 kDa from CaMV-infected *A. thaliana* plants that strongly interacted with P6 (Leh et al., 2000) was submitted to microsequencing of its N terminus. The resulting sequence of 16 aa (Fig. 1a) was compared with a non-redundant *A. thaliana* protein library using the BLASTP search program (Altschul et al., 1997). An identity of 93 % was obtained between the sequenced peptide and the N terminus of the 60S ribosomal protein L13 encoded by the AtRNABBC1 gene of *A. thaliana* (Bertauche et al., 1994). When L13 was compared with other recently sequenced genes from *A. thaliana* the identity ranged from 75 to 100 % [GenBank (protein bank) accession nos AAK55698.1 and CAB62014.1, respectively]. Sequencing did not reveal the initiator methionine, suggesting that this amino acid is removed after translation, as already described for its homologue from rat (Olvera & Wool, 1994). L13 is a small basic protein (206 aa) with a theoretical molecular mass of 24 kDa. The difference between the calculated mass and that observed by denaturing polyacrylamide gel electrophoresis is surprising, because the molecular mass of L13 of *Brassica napus* estimated by denaturing gel electrophoresis is identical to the theoretical one (Saez-Vasquez et al., 2000). The 13 protein of *A. thaliana* has a similar electrophoretic behaviour when expressed in *E. coli*, thus excluding the possibility that its slower migration is due to post-translational modification at the putative glycosylation site (Fig. 1a). Therefore, it is more likely that the amino acid composition and/or conformation did not allow complete denaturation of the protein in the presence of SDS.
To confirm the specificity of the interaction between P6 and L13, the sequence encoding L13 was amplified from a cDNA library of *A. thaliana* and cloned into a modified pET3a prokaryotic expression vector in fusion at its 5′ end with a sequence encoding a phosphorylation site. The resulting recombinant plasmid (pET-L13) was expressed in...
E. coli heterologous proteins in and thus improves the expression level of arginine-enriched (Brinkmann et al., 2000). After sonication, proteins from bacteria transformed with the recombinant plasmid (lane L13) or the empty vector (lane E. coli) were separated by SDS-PAGE (Fig. 1b, top left panel), transferred onto a nitrocellulose membrane and submitted to a far-Western assay using 32P-labelled P6 fused at its N terminus to glutathione-S-transferase (GST–P6) as overlay. The autoradiograph showed a major band at the position of L13 (Fig. 1b, bottom panel, lane L13), whereas no interaction was observed with proteins from control bacteria (Fig. 1b, bottom panel, lane E. coli). Additional far-Western assays performed with GST–P6 (89 kDa) blotted onto a membrane and using 32P-labelled L13 as overlay confirmed that P6 specifically interacts with L13, as a radioactive signal was detected at the level of GST–P6 (Fig. 1b, bottom right panel). Bands below this signal probably correspond to interactions between L13 and GST–P6 degradation products as these polypeptides could also be detected using a polyclonal anti-P6 serum (Fig. 1b, upper panel).

The specificity of the L13–P6 interaction was also investigated in a GST-pulldown experiment. For this purpose the sequence encoding L13 was amplified by PCR from pET-L13 and inserted into pGEX-2TK. The sequence of the resulting clone, pGST-L13, revealed two differences from the original sequence of L13 cDNA. The initiator codon was changed into an ATT (isoleucine), and the second codon (normally encoding a lysine) was substituted by AAT specific for asparagine; the mutation of these two amino acids is not expected to have an effect on the GST-pulldown assay.

The proteins GST–L13, GST–P6, His–P6 (a histidine-tagged P6) and GST were expressed in E. coli and used in the GST-pulldown assays (Fig. 1c). GST–P6 protein was bound to Sepharose 4B beads, incubated with 32P-labelled L13 protein and then, after several washes, the retained complexes were analysed by SDS-PAGE. After transfer, the membrane was subjected to autoradiography (Fig. 1c, bottom left) and staining with Coomassie blue (Fig. 1c, upper panel). L13 specifically interacted with P6, as evidenced by the strong radioactive signal at the level of a polypeptide of approximately 30 kDa compared with the signals obtained when pulldown assays were performed with His–P6, GST and bacterial proteins as negative controls (Fig. 1c, bottom panel). Pulldown assays carried out with GST–L13 bound to the beads and 32P-labelled P6 as overlay confirmed this result, as labelled P6 was retained on the beads only in the presence of GST–L13 (Fig. 1c, bottom right), whereas almost no radioactivity was detected in any of the control experiments. The other radioactive signals in the GST–L13 lane presumably correspond to cleaved products of the P6 protein. The latter were already detected when proteins were analysed on polyacrylamide gel after labelling of the inclusion bodies prepared from E. coli expressing P6 (data not shown), in particular the polypeptide of 42 kDa, a P6-cleavage product which is also found in CaMV-infected plants (Xiong et al., 1982; Maule et al., 1989).

**L13 interacts with the miniTAV domain of P6**

To define the region of CaMV P6 involved in binding of L13, deleted versions of the P6-coding CaMV ORF VI were cloned into the pET-KaKS vector (Leh et al., 2000) except for mutant A, where the coding sequence was inserted into pGEX-2TK (Fig. 2a). Their capacity to interact in vitro with 32P-labelled L13 was tested by far-Western assays (Fig. 2b, bottom panel). Only N- and C-terminally truncated P6 and P6 mutants containing the miniTAV domain (residues 112–242) were able to bind the ribosomal protein L13 strongly, indicating that L13 interacts with the miniTAV of P6. Far-Western assays with the miniTAV itself could not be performed because this polypeptide is very poorly expressed in E. coli. Mutant H, corresponding to the C-terminal half of P6, interacted weakly with L13, whereas mutants G and ΔTAV encompassing this region did not, raising the question whether this interaction is specific. The possibility that interaction between L13 and P6 was mediated by nucleic acids (the miniTAV is known to have RNA-binding properties; Cerritelli et al., 1998) can be excluded because these results were obtained when far-Western experiments were performed in a buffer containing a cocktail of RNase and DNase.

**Ribosomal proteins L13 and L18 bind to the same domain within miniTAV**

Previously, it has been demonstrated that L18 (24 kDa) of A. thaliana also interacts with the miniTAV of P6 (Leh et al., 2000), raising the question whether L13 and L18 bind to distinct sites within this domain. We therefore carried out competition assays using the GST-pulldown technique. The fusion protein GST–P6 was bound to Sepharose 4B beads and thoroughly washed to remove excess protein. Equal volumes of the matrix were then incubated with 32P-labelled L13 in the presence of increasing quantities (0–8 µg) of total soluble protein from recombinant bacteria expressing A. thaliana L18 or the P14 protein of Beet necrotic yellow vein virus (BNYVV), used as a non-specific competitor (Fig. 3); these proteins were expressed at the same level in E. coli (data not shown). Free L13 molecules were eliminated by centrifugation, and protein complexes bound on the beads were dissociated and fractionated by SDS-PAGE. The autoradiograph revealed that L13 was dissociated from P6 by addition of L18. A decrease in the amount of 32P-labelled L13 bound to the beads (Fig. 3, bottom left) was associated with binding of increasing quantities of L18 on the matrix as evidenced by the staining of the polyacrylamide gel (Fig. 3, top left). As estimated from measuring the radioactivity in the 32P-labelled L13 bands, 95% of L13 was released from P6 in the presence of large amounts of L18. The radioactivity remaining on the matrix is probably due to an
interaction between L18 and P6-bound L13 and/or between L18 bound to P6 and L13 after its release from P6, as L18 and L13 interact with each other in vitro (see below). On the other hand, BNYVV P14 protein did not impair the interaction between L13 and GST–P6 as the radioactivity bound on the Sepharose 4B beads in the absence of P14 was almost totally recovered, even when large amounts of this protein were mixed with 32P-labelled L13 prior to its binding to the matrix (Fig. 3, bottom left). In parallel, no P14 could be detected after staining of the gel (Fig. 3, top)

Fig. 2. Mapping of the domain on P6 that interacts with L13. Interaction assays were performed with full-length P6 (520 aa) and various P6 deletion mutants shown in (a). Empty boxes and thin broken lines correspond to the P6 sequence and to in-frame deletions in ORF VI, respectively. N- and C-terminal deleted mutants are represented by the first and last amino acids, which are not deleted; internal deletions are represented by a delta followed by the deleted amino acids. The black box corresponds to the miniTAV domain, boxes with black and white squares to RNA-binding domains, the striped box to a zinc finger motif, and the white box with black dots to the double-stranded RNA-binding domain. (b) Protein extracts from bacteria expressing P6 or its truncated versions or empty vector (lane E. coli) were separated by SDS-PAGE (15 %) and transferred onto a nitrocellulose membrane, and the membrane was treated with antibodies raised against P6 (top) or submitted to a far-Western assay using 32P-labelled L13 as overlay (bottom). Interactions were deduced from the pattern of the autoradiograph.
left). The control experiment performed using GST instead of the fusion protein GST–P6 as ligand showed that 32P-labelled L13 was not unspecifically bound to the matrix (Fig. 3, right). Together, these results demonstrate that L13 and L18 specifically compete with each other for binding to P6 and thus that they recognize the same or overlapping sites within miniTAV.

To provide further insight into this question, a series of P6 mutants, in which 20 aa long deletions were systematically introduced in the miniTAV, were tested for their ability to bind in vitro to L13 and L18. Nearly equal loading of the P6 mutants was evidenced by Western blotting performed with anti-P6 antibodies (Fig. 4, upper panels). Deletions of the N- (Δ112–131) and C-termini of the miniTAV (Δ192–211, Δ212–231 and Δ232–242) did not impair binding of either L13 or L18 to P6, whereas removal of the central part of this domain (Δ132–151, Δ152–171 and Δ172–191) totally abolished the interaction. Taken together, these results demonstrate that L13 and L18 bind to the same domain in P6 and that the latter is located in the central part of the miniTAV (residues 132–191).

**Fig. 3.** Competition between L13 and L18 for binding to P6 miniTAV domain. Competition experiments were performed by GST-pulldown assays using GST–P6 fusion protein (left panel) or GST (right panel) alone bound to a Sepharose-4B column and 32P-labelled L13 protein mixed with increasing amounts of soluble proteins from recombinant bacteria containing either L18 ribosomal protein of *A. thaliana* or P14 protein from BNYVV (0–8 μg). The labelled L13 protein retained on the matrix after incubation was dissociated, fractionated by SDS-PAGE and detected by autoradiography. Non-radioactive competitors were revealed by Coomassie staining of the gel.

**Fig. 4.** Mapping of the domain on miniTAV of P6 that interacts with ribosomal proteins. Interaction assays were performed with mutant in the miniTAV domain corresponding to stepwise deletions of 20 aa segments along the miniTAV. After separation by SDS-PAGE (15% polyacrylamide) and transfer onto a nitrocellulose membrane, P6 mutants were detected with anti-P6 antibodies (upper panels) or tested for the capacity to interact with 32P-labelled L13 (a) or L18 (b) (bottom panels).
Both ribosomal proteins can interact with each other in far-Western experiments

Since L13 and L18 recognize and bind to the same region of miniTAV, it is possible that they are in proximity in the 60S subunit and could bind to each other. To test for such binding, we performed far-Western experiments with L13 and L18 proteins expressed in E. coli, using as controls for non-specific interactions protein P19 of Tomato bushy stunt virus (TBSV) and GST, both also overexpressed in bacteria. Note that P19 migrates as a polypeptide of about 25 kDa. Similar amounts of these proteins were separated by SDS-PAGE as evidenced by Coomassie blue staining of the gels (Fig. 5, top) and blotted onto nitrocellulose membrane. After a step of renaturation in PBS/Tween buffer with 5 % milk, the membranes were incubated with either $^{32}$P-labelled L13 (Fig. 5, left) or $^{32}$P-labelled L18 (Fig. 5, right). In each case, results show a strong signal of molecular mass corresponding to that of L18 and L13, whereas no signal was detected with either P19 or GST. The other bands on the autoradiograph correspond to non-specific interactions between the overlay and bacterial proteins, as they are detected with all extracts and, in particular, with proteins from E. coli transformed with the empty vector (lane E. coli). This result demonstrates that L13 and L18 interact specifically, suggesting that they could be near one another on the surface of the 60S subunit.

**DISCUSSION**

Previous studies have shown that the capacity of the CaMV P6 protein to mediate the reinitiation of translation of polycistronic mRNAs relies on its interactions with several polypeptides of the cellular translation machinery. Three of the cellular partners have already been characterized: the ribosomal proteins L18 (Leh *et al.*, 2000) and L24 (Park *et al.*, 2001) and subunit g of initiation factor eIF3 of A. thaliana (Park *et al.*, 2001). In this study we have identified a novel cellular partner of P6, protein L13 of the A. thaliana 60S ribosomal subunit. Far-Western and GST-pulldown experiments, using L13 or P6 alternatively as bait and prey, demonstrate that L13 binds to P6. Investigation of the interaction domain on P6 revealed that L13 interacts with the P6 miniTAV domain, which also represents the functional core for translational transactivation (De Tapia *et al.*, 1993). It appears that L13 also associates, albeit weakly, with mutant H (residues 305–520) but not with either mutant G, which extends from the miniTAV to the C terminus (residues 242–520) or mutant miniTAV, which corresponds to the complete P6 protein without the miniTAV. This interaction might be non-specific, but we cannot totally exclude the possibility that the C-terminal region of P6 encompasses sequence(s) that might be part of the interaction domain. In the latter case the failure of mutants G and miniTAV to bind L13 might be due to the fact that the overall conformation of the P6 sequence in the mutant did not properly expose the L13 binding domain. Leh *et al.* (2000) and Park *et al.* (2001) have demonstrated that ribosomal protein L18 also interacts with the miniTAV, but they observed no binding with regions located downstream.

A detailed mutational analysis revealed that the L13 and L18 proteins bind to the same 60 aa long sequence within miniTAV. This interaction domain encompasses the consensus sequence of the RNase H nucleic acid-binding site that can bind in vitro to RNA–DNA heteroduplexes and double-stranded RNA (Cerritelli *et al.*, 1998). However, treatment of both the membrane and the overlay with nucleases did not impair the interaction of either L13 or L18 with P6, suggesting that binding of these ribosomal proteins to P6 is not mediated by nucleic acids. The involvement of a protein sequence in an interaction with several partners is often observed for viral proteins, and has already been described for CaMV P6 (Park *et al.*, 2001).
These authors demonstrated that both L24 and eIF3 interact with RNA-binding domain A located just downstream of miniTAV, and that these factors compete with one another for binding to this region. As expected, L18 also competes with L13 for binding to miniTAV as both proteins occupy exactly the same sequence and presumably cannot bind simultaneously to the same P6 molecule.

Currently, the only information available concerning the topography of L13 in eukaryotic ribosomes comes from studies performed on the rat 60S subunit using immobilized enzymes and cross-linking experiments (Marion & Marion, 1987). L13 from rat presents a high degree of identity with other eukaryotic L13 and, in particular, with its plant counterpart (75 %). The current model for the spatial rearrangement of proteins within the 60S rat liver ribosomal subunit has L13 linked to L18 by the intermediary rearrangement of proteins within the 60S rat liver ribosomal subunit near the neck region (Ban et al., 2000). It is tempting to speculate that L13 and L18 might bind to P6 dimers, as it was recently shown that P6 self-interacts in vitro (Haas et al., 2002b) and in vivo (Li & Leisner, 2002), and it has been proposed that the dimers represent the biologically active form of P6 (Haas et al., 2002b; Li & Leisner, 2002). Nevertheless, it should be noted that dimerization of P6 is not a prerequisite for L13 and L18 binding as the N-terminal domain, which is absolutely required for P6–P6 interaction, is dispensable for L13 binding.

Concerning the role of the L13–P6 interaction, this can only be a matter of speculation in the absence of any information on the function of L13 during protein synthesis or in other processes. Several possibilities can be proposed if the hypothesis that L13 physically interacts with L18 on the ribosomal surface is tentatively accepted. Recently, Park et al. (2001) suggested that P6 mediates the interaction between L18 and eIF3 on the large ribosomal subunit and proposed that this interaction permits stalling of the initiation factor during the elongation process to favour the reinitiation of translation of downstream ORFs. The possibility that simultaneous binding of P6 to L13 and L18 reinforces this interaction cannot be excluded. On the other hand, studies in mammals have shown that L18 interacts with a double-stranded RNA-activated protein kinase (PKR) and that this interaction inhibits PKR activity. A putative PKR-like protein was identified in plant extracts using antibodies raised against human PKR (Langland et al., 1995, 1996) but no protein containing double-stranded RNA binding and kinase domains has been identified so far (although the two domains could be present on separate proteins in plants). The possible existence of a PKR-like activity in plants was reinforced by the discovery of a plant PKR inhibitor and the fact that its inactivation favours viral pathogenesis (Bilgin et al., 2003). PKR-like kinases are also involved in the expression of mRNAs bearing short ORFs in their leader (Kumar et al., 2003). PKR-like kinases are also involved in the expression of mRNAs bearing short ORFs in their leader (Kumar et al., 2003). PKR-like kinases are also involved in the expression of mRNAs bearing short ORFs in their leader (Kumar et al., 2003).

Saez-Vasquez et al. (2000) have demonstrated that a significant fraction of L13 from B. napus is located in the nucleus. Transient expression in tobacco BY-2 cells of A. thaliana L13 protein fused to EGFP showed that it also localizes in the nucleus (data not shown). It has been suggested that the tobacco L13 homologue might play a regulatory role in transcription, as a region of L13 can replace the activation domain of the transcription factor GAL4 in yeast double-hybrid experiments (Esturuch et al., 1994). Recently we have discovered that P6, which has previously been considered an exclusively cytoplasmic protein, also localizes in the nucleus of CaMV-infected cells and thus may shuttle between the nucleocytoplasmic compartments using the import–export cellular machinery (Haas et al., 2002b). The fact that several cellular genes are down- or upregulated in transgenic A. thaliana plants expressing CaMV P6 protein (Geri et al., 1999) might be indicative of a role for both P6 and L13 in the regulation of cellular gene transcription.

Ribosomal proteins L13, L18 and L24 and initiation factor eIF3 probably represent only some of the components of the translation machinery that are usurped by CaMV P6 protein to fulfill its role in translation reinitiation of polycistronic mRNAs. Results of far-Western experiments indicated that P6 interacts with about a dozen proteins from a ribosomal fraction. It will be interesting to see if other P6 partners are ribosomal proteins of the 40S subunit and/or other initiation factors potentially involved in reinitiation of translation. Involvement of numerous cellular partners suggests that ribosomes bear different P6-binding sites and, consequently, are capable of binding simultaneously to several P6 molecules. P6 might also interact with ribosomes in a coordinated manner during the translation process. Answering these questions will provide new insights concerning the reinitiation mechanism mediated by the CaMV P6 protein.
ACKNOWLEDGEMENTS

We are grateful to Kenneth Richards for critical reading of the manuscript, Philippe Hammann and Malek Alioua for automatic sequencing of plasmid constructions, Daniele Scheidecker for providing us with BNYVV P14 and TBSV P19 proteins and Monique Le Ret for protein microsequencing. We thank also Richard Wagner and colleagues for growing *A. thaliana* plants. This work was supported by the Centre National de la Recherche Scientifique and by the Université Louis Pasteur of Strasbourg.

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