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Interaction between Cauliflower Mosaic Virus Inclusion Body Protein and

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The cauliflower mosaic virus (CaMV) inclusion body protein (pVI) is able to specifically interact with the viral capsid precursor protein (pIV). By using the yeast two-hybrid system and a blot assay, the pIV region required for the recognition of pVI was mapped to the lysine-rich domain. This region of only 48 amino acids when fused to dihydrofolate reductase (DHFR) mediated pVI and DNA binding *in vitro.* Competition experiments confirmed that pVI and DNA bind to the same region of pIV. Since pVI is absent from the mature virus, models are discussed in which pVI plays an accessory role in CaMV assembly, in addition to its function in transactivating translation. © 1996 Academic Press, Inc.

INTRODUCTION

Cauliflower mosaic virus (CaMV) has an icosahedral capsid of 50 nm diameter and is the type member of the caulimovirus family with a double-stranded, relaxed circular DNA genome of 8 kb. This plant virus infecting crucifereae has been classified as a pararetrovirus since it shares important features with other retroid elements. CaMV replicates its genomic DNA via an RNA intermediate by reverse transcription (Hull and Covey, 1983; Guilley *et al.,* 1983; Pfeiffer and Hohn, 1983; Mazzolini *et al.,* 1985). The genes encoding the capsid protein (ORF IV) and enzymatic functions (ORF V) are positioned adjacent to each other, providing the virus with the *gag–pol* core arrangement similar to that of all retroviruses (reviewed by Rothnie *et al.,* 1994).

The pIV polyprotein of CaMV is extensively processed by a viral aspartic protease encoded at the 5′ region of ORF V (Franck *et al.,* 1980; Torruella *et al.,* 1989). Two of the processed products, p37 and p44 are found as main components in the capsid in a ratio of approximately 6 to 1 (Al Ani *et al.,* 1979). The N-terminal acidic region (28% glutamic acid + aspartic acid) of the pIV precursor is absent from mature p37 and p44. While the N-terminus of p44 is known to be located at amino acid 76 of the full-length pIV, the precise position of the C-terminus remains uncertain (Martínez-Izquiedo and Hohn, 1987). Structural studies using cryoelectron microscopy and three-dimensional image reconstruction showed that the virus capsid contains 420 subunits positioned in a $T =$ 7 symmetry. The virus particles are composed of three concentric protein layers surrounding a solvent-filled central cavity (Cheng *et al.,* 1992). The outer layer I contains 60% of the viral proteins and includes the putative antiparallel β-barrel or "jellyroll" structure that is present in most isometric viruses. Layers II and III contain the adjacent lysine-rich region (Brunt *et al.,* 1975) and a Cys/His motif (Covey, 1986). The lysine-rich region of the pIV binds in a sequence nonspecific manner to nucleic acids (Franck *et al.,* 1980; Berg, 1986) that are located between layers II and III. The interaction between pIV and the genome most likely contributes to the great stability of the virus capsid. Among caulimoviruses (Covey, 1986) and many retroid viruses, the Cys/His motif ($C–X_2–C–X_4–H–X_4–C$) is conserved and probably involved in specific RNA binding during packaging of the pregenomic RNA into virions. Multiple mutations of the conserved cysteine and histidine residues in CaMV (De Tapia, unpublished observations) and figwort mosaic virus (FMV), another member of the caulimovirus family (Scholthof *et al.,* 1993), resulted in a noninfectious virus, indicating the crucial role of this motif. The inner layer III is exposed to the solvent-filled, central cavity (Cheng *et al.,* 1992).

Although the physical structure of CaMV virions has been resolved in some detail, only little is known about the mechanism of virus assembly. It has been suggested that the cytoplasmic, membrane-free inclusion bodies (viroplasm) are the site of virus assembly (Shepherd, 1976). The main component of the viral inclusion body matrix is the multifunctional viral pVI (Covey and Hull, 1981). This protein is involved in many other features of the virus life cycle, including determination of the host range (Daubert *et al.,* 1984), severity of symptoms (Schoelz *et al.,* 1986), and regulation of translation. In

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this latter function, the ORF VI product has been termed transactivator (TAV; Bonneville *et al.,* 1989; Zijlstra and Hohn, 1992; De Tapia *et al.,* 1993). Electron microscopic studies showed that the inclusion bodies are surrounded by ribosomes at an early stage of infection (Shepherd, 1976), indicating an important, active role for these structures in the virus life cycle. In fact, inclusion bodies have been demonstrated to be the site of accumulation of all viral gene products and of viral DNA synthesis (Pfeiffer and Hohn, 1983). The close proximity of pVI and pIV in the inclusion bodies led early to speculation about pVI being involved in virus assembly (Hull *et al.,* 1987).

In this article, experimental evidence is provided that pIV and pVI are able to interact. The region of the pIV required for interaction with pVI was characterized as a step towards a better understanding of the initial steps of CaMV assembly.

MATERIALS AND METHODS

Plasmids

For expression of ORF VI in *Escherichia coli,* the *Hin*dIII–*Pst*I fragment from pHELP7 (Bonneville *et al.,* 1989) was cloned into the *Bam*HI, *Pst*I sites of plasmid pQE11 [pDS56 derivative (Bujard *et al.,* 1987), QIAGEN], the *Hin*dIII and *Bam*HI sites being filled in with Klenow polymerase before ligation. Thereby six adjacent His residues (6 × His) were fused to the N-terminus of pVI, which served as an affinity tag for purification (Stüber *et al.,* 1990). Capsid protein (ORF IV) deletion mutants for expression in *E. coli* were derived by PCR amplification of cloned CaMV sequences. The sense primers contained an *Nco*I restriction site including an ATG initiation codon. An ATG initiation codon was introduced at codon 76 for the N-terminal deletions starting at codon 77. The antisense primers had built-in TGA stop codons and *Bam*HI restriction sites. PCR products were cut with these restriction enzymes for cloning in pET3d vector (Novagen). All clones were derived from CaMV Strasbourg strain, with the exception of pIV1–454 (strain 4184) and sequenced. DHFR was expressed from plasmid pQE13 (QIAexpress). For affinity purification of the lysine-rich region, ORF IV was amplified by PCR between codons 363–411 and 363–404 and cloned into the *Bgl*II and *Hin*dIII sites of pQE13, yielding a DHFR fusion protein with a N-terminal 6 × His tag (DHFR–pIV363–411). Nucleotides of ORF IV at positions 1124–1129 and 1160–1165 were changed in mutant Del1 to unique *Mlu*I and *Pst*I sites, respectively, by using PCR. The mutants Del2, -3, and -5 were derived from Del1 by standard cloning techniques. For Del4, the *Mlu*I–*Pst*I fragment of Del1 was exchanged for an oligonucleotide covering the ORF IV codons 377–380. All constructs derived from PCR and cloning of oligonucleotides were verified by sequencing. For assays in the yeast two-hybrid system the *Hin*dIII–*Pst*I ORF VI fragment from pHELP7 was cloned in-frame into the *Bam*HI and *Pst*I sites of the yeast shuttle vector pGBT9 (Bartel *et al.,* 1993) using the same strategy described above for cloning into pQE11. Capsid protein sequences from pET plasmids were opened at the *Nco*I site, and recessed 3′ ends were filled with Klenow fragment, cut with *Bam*HI, and cloned in the yeast two-hybrid vector pGAD424 at the *Sma*I and *Bam*HI restriction sites. As control plasmids for the yeast two-hybrid system we used pEE5 [GAL4(1–147)–SNF1] and pNI12 [SNF4–GAL4(768–881)] described by Fields and Song (1989).

Expression of CaMV pIV and its derivatives in *E. coli*

Capsid protein (pIV) deletion mutants cloned in pET3d vector (Novagen) were transformed into BL21 (DE3) cells. Liquid cultures were started with several bacterial colonies in LB medium supplemented with 0.4% glucose and containing 50 μg/ml ampicillin. The cultures were grown to A_{260} = 0.6–1.0 and induced with 10 μg/ml isopropyl-β-D-thiogalactopyranosid (IPTG) for 2 hr at 37°C (Studier and Moffatt, 1986). The bacterial pellets were washed and resuspended in lysis buffer (20 m*M* Tris–Cl, pH 7.5, 10 m*M* EDTA, 1 m*M* DTT, and 1 m*M* phenylmethanesulfonyl fluoride). Bacterial cells were treated with lysozyme (2 μg/ml of culture) for 30 min and frozen on dry ice. After thawing, lysates were sonicated, mixed with 0.1 vol of 5 *M* NaCl, stored on ice for 1 hr, and spun at 12,000*g* for 10 min. Pellets contained pIV inclusion bodies which were washed, dissolved in lysis buffer containing 8 *M* urea, and dialyzed overnight against the lysis buffer. After dialysis, samples were cleared by centrifugation at 12,000*g* for 10 min and soluble proteins were used for the blot assay.

Expression and purification of pVI under nondenaturing conditions

Briefly, the protocol was essentially as described by the manufacturer (QIAGEN) and Hochuli *et al.* (1988). Soluble, cytoplasmic pVI was expressed from the pQE11 construct in M15 cells at a reduced growth temperature of 27°C. After a 4-hr induction time with 1 m*M* IPTG, $ZnSO_4$ was added to a final concentration of 20 μ*M*. Cells were harvested and resuspended in sonication buffer (50 m*M* NaH_2PO_4 pH 8.0, 300 m*M* NaCl, 0.1% Tween-20, 10 m*M* β-mercaptoethanol, 10% glycerol). After 5 min incubation with 1 mg/ml lysozyme on ice, the mixture was frozen in dry ice and then thawed in cold water. Cells were sonicated and the 10,000*g* supernatant was loaded onto a Ni^{2+}–NTA column. The tag consisting of six N-terminal His residues allowed high affinity binding of pVI to the column resin. After extensive washing with sonication buffer, pH 8.0, containing 30 m*M* imidazole and 20% glycerol, the pVI was eluted from the column by increasing the imidazole concentration to 250 m*M*.

The eluate was dialyzed overnight against 50 m*M* NaH_2PO_4 pH 8.0, 200 m*M* NaCl, 0.1% Tween-20, 10 m*M* β-mercaptoethanol, 20 μM $ZnSO_4$, and 30% glycerol and stored in aliquots at −80°C. Protein concentrations were determined using the Bradford method.

Purification of DHFR and DHFR-fusion proteins expressed in *E. coli*

6 × His affinity-based purification of DHFR and DHFR-fusion proteins under denaturing conditions was performed as described previously (De Tapia *et al.,* 1993). For total cell extracts, cell pellets were boiled for 7 min in SDS–PAGE sample buffer (Sambrook *et al.,* 1989).

SDS–PAGE and staining of proteins

SDS–PAGE of protein samples was performed as described by Laemmli (1970). Proteins were stained with Coomassie brilliant blue dye or silver salts according to Sambrook *et al.* (1989).

Western blot analysis

Proteins were transferred from SDS–PAGE onto a nitrocellulose membrane (Schleicher & Schüll) via semidry electroblotting (1 hr, 1 mA/cm^2 gel) with an apparatus from JKA Biotech Denmark. The membrane was saturated with 5% nonfat dry milk in TBS (20 m*M* Tris–Cl, pH 7.5, 0.5 *M* NaCl) for 1 hr at room temperature and incubated for 1 hr with rabbit anti-pIV antiserum (raised against CaMV p37; Martínez-Izquierdo and Hohn, 1987) diluted 1:3000 in 5% nonfat dry milk in TBS. The membrane was washed twice for 15 min with TTBS (TBS containing 0.05% Tween-20), decorated for 45 min with goat anti-rabbit (IgG, H + L) horseradish peroxidase-linked (HRP) secondary antibody diluted 1:5000 in nonfat dry milk in TBS, and washed three times for 10 min with TTBS. The antigen was detected by chemiluminescent immunodetection as described by the manufacturer (ECL system, Amersham).

Far-Western analysis

In a modified overlay method (Bregman *et al.,* 1989) pVI was used as a probe followed by immunodetection of pVI binding. Protein pIV and its derivatives (1 μg) were separated by SDS–PAGE, electrotransferred to a nitrocellulose filter and renatured overnight at 4°C in renaturing buffer composed of 5% nonfat dry milk in HMK-buffer (10 m*M* Hepes, pH 7.5, 2.5 m*M* $MgCl_2$, 75 m*M* KCl [Hatton *et al.,* 1992]). All the subsequent incubations were carried out at 4°C. Filters were incubated for 2 hr in renaturation buffer containing pVI (20 ng/ml). Protein pVI was omitted from the negative control on a duplicate filter. Filters were washed with renaturing buffer and incubated with anti-pVI antiserum (raised against MiniTAV protein; De Tapia *et al.,* 1993; Himmelbach, 1995) diluted 1:3000 in 5% nonfat dry milk in TBS (20 m*M* Tris–Cl, pH 7.5, 500 m*M* NaCl). After washing with 5% nonfat dry milk in TTBS (TBS containing 0.05% Tween-20), the membrane was decorated with goat anti-rabbit horseradish peroxidase-linked (HRP) secondary antibody. The filter was washed in TTBS and bound pVI was visualized by chemiluminescent immunodetection as described by the manufacturer (ECL system, Amersham). Densitometric measurement was performed with the Bio-Rad Model GS-670 imaging densitometer and the program molecular analyst.

Northwestern assay

RNA binding was determined essentially as described previously (De Tapia *et al.,* 1993). The NaCl concentration in the standard binding buffer was 0.3 *M* during incubation with the ^{32}P-labeled RNA probe and washing steps. The RNA sequence of the probe was GGGUACCCUCGAGCGC$(N)_{20}$GCGGCCGCAGGAUCCAG (N = G, A, U, C).

Southwestern assay

Recombinant pIV purified from bacterial inclusion bodies was separated on SDS–PAGE and transferred onto nitrocellulose membranes and renatured. The immobilized proteins were incubated for 1 hr at room temperature with ^{32}P-labeled plasmid DNA (25,000 cpm/ml) in HMK-buffer containing 5% nonfat dry milk. The probe used was obtained by random priming (Sambrook *et al.,* 1989) on plasmid DNA containing the entire viral genome (pCa100). The filters were washed three times for 10 min at room temperature in HMK-buffer and subjected to autoradiography.

Competition assay

Protein pIV (2 μg) was applied to a nitrocellulose membrane in a dot-blot chamber by vacuum. The membrane was essentially treated as described for the blot assay, except that competitor (pUC12 plasmid DNA and single- and double-stranded oligonucleotides 36 nucleotides in length [GATCCTCAGTCTGAGTCTGAGTCTTCAGAAGTAGAT]) was added as indicated to 5% nonfat dry milk in HMK-buffer and the pVI concentration was 20 ng/ml.

Yeast strain and methods

Yeast host strain SFY526 is deleted for *GAL4* and *GAL80* and contains a *GAL1-lacZ* reporter gene integrated at *URA3* (Bartel *et al.,* 1993). Yeast cells were grown in YPD complete medium or minimal supplemented synthetic dextrose medium (SD; Sherman, 1991). Yeast transformation was performed by the high-efficiency lithium acetate method using single-stranded DNA as a carrier (Schiestl and Gietz, 1989). Yeast transformants were grown on selective SD medium, trans-

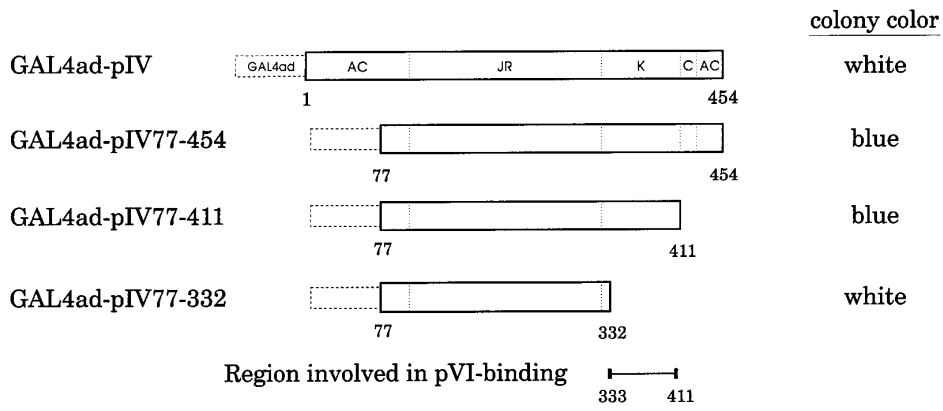

FIG. 1. Interaction of pVI with the pIV deletion mutants in the yeast two-hybrid system. The pIV derivatives are represented as a fusion to the GAL4 activation domain (GAL4ad, not to scale). Regions are defined arbitrarily and show the N- and C-terminal acidic regions (AC), the proposed β-barrel or "jellyroll" region (JR), lysine-rich region (K), and the retroviral-like Cys/His motif (C). The yeast reporter strain was analyzed for interaction between the GAL4 DNA-binding fusion to pVI and the different GAL4ad–pIV fusions. Results of the β-galactosidase filter assay are given in the last column. The region involved in binding to pVI is shown as a bar. The amino acid positions of pIV are indicated.

ferred onto nitrocellulose or Whatman No. 1 filters, and assayed for β-galactosidase (Breeden and Nasmyth, 1985).

RESULTS

Interaction of CaMV pIV and pVI revealed by the yeast two-hybrid system

A genetic test for *in vivo* protein–protein interaction in the yeast two-hybrid system (Bartel *et al.,* 1993) was used to study the interaction between pIV and pVI. The ORF VI sequence was linked to the C-terminus of the GAL4 DNA-binding sequence in the yeast vector pGBT9, thereby yielding a GAL4bd-pVI fusion protein. The vector pGAD424 was used to generate GAL4 activation domain fusions with pIV (GAL4ad–pIV) and derivatives of it (GAL4ad–pIV77–454, 77–411, and 77–332; Fig. 1). Both types of expression plasmids were cotransformed into the yeast reporter strain and assayed for reporter gene activity by staining colony lifts with X-Gal. Colonies showing β-galactosidase reporter gene expression indicated reconstitution of the GAL4 transcriptional activator function by interaction between pVI and pIV sequences. Reporter gene activity was observed in cells coexpressing the fusions to pVI and pIV sequences 77–454 or 77–411 (Fig. 1). No activity was observed if the GAL4bd–pVI plasmid or the GAL4ad–pIV plasmids were used alone and if they were used together with the empty partner plasmids or together with partner plasmids coding for extraneous hybrid proteins (pEE5 and pNI12; Fields and Song, 1989).

The combination of pVI-fusion and the complete pIV-fusion was inactive, probably due to toxicity, improper folding, occlusion of the interaction site, or masking of the nuclear targeting signal (Fig. 1). However, the results obtained with the N-terminally truncated pIV derivatives and the Far-Western assay described below, indicate that the failure to detect binding of pVI with the complete pIV (1–454) in the two-hybrid system is a false negative result. Quantification of β-galactosidase activity from yeast cells grown in selective liquid medium gave no conclusive data. This could be explained by the toxicity of constitutively expressed pIV or pVI. Expression of pIV was shown to be toxic for bacteria (e.g., Fütterer *et al.,* 1988), and toxicity in *E. coli* was more apparent in liquid media than on plates and this is likely also to be true in yeast. In addition pVI was recently shown to be active in transactivation in yeast (Sha *et al.,* 1995).

The presumptive pVI binding region located on pIV was mapped by comparing the effect of different C-terminal pIV truncations. Removal of residues up to position 411 had no effect, while removal of residues up to position 332 abolished the interaction. This indicates that the sequence between amino acid 333 and 411, comprising the lysine-rich domain, is required for the interaction with pVI, while the N- and C-terminal acidic regions and the Cys/His motif are dispensible (Fig. 1).

Mapping of the interaction domain using a Far-Western blot assay

The results from the yeast two-hybrid system were confirmed by the observation that immobilized pIV retained its ability to bind to soluble pVI and DNA. Deletion derivatives of pIV were therefore expressed in *E. coli* and partially purified as bacterial inclusion bodies. Coomassie blue staining (Fig. 2A) and Western blot analysis (Fig. 2B) showed that the different pIV derivatives were present in comparable amounts. The pIV derivatives were separated by SDS–PAGE, blotted on nitrocellulose filters, refolded, and incubated with purified pVI. Bound pVI was visualized by immunodetection using rabbit anti-pVI

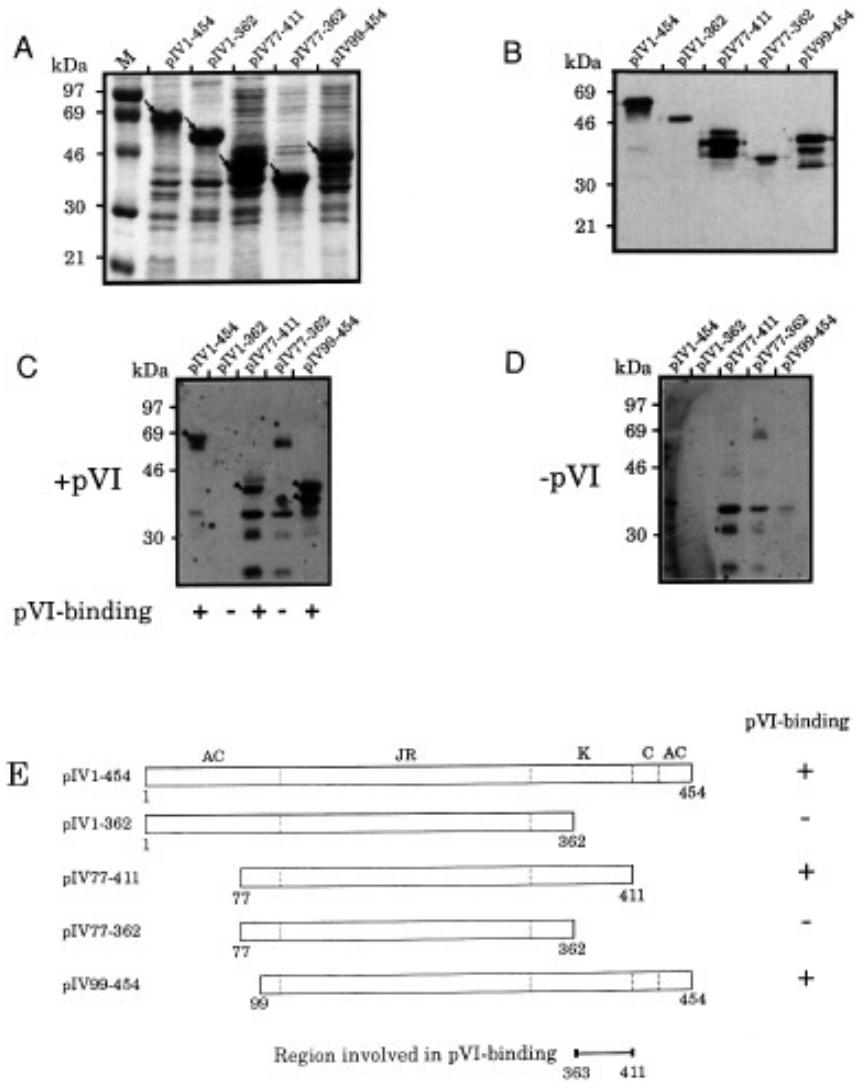

FIG. 2. Mutational analysis of pIV/pVI binding by using a Far-Western blot assay. A series of truncated pIV derivatives (arrows) purified from bacterial inclusion bodies was separated on SDS–PAGE and stained with Coomassie brilliant blue dye (A). Arrows indicate the position of pIV-derived proteins. A duplicate gel was blotted on nitrocellulose (B). The different pIV derivatives were revealed by immunodetection using rabbit anti-pIV antiserum. For the blot assay, 1 μg of the pIV derivatives was separated on duplicate SDS–PAGE, blotted on nitrocellulose filters and refolded. Filters were incubated with pVI (C) or without pVI (D). Binding of pVI was visualized by immunodetection based on rabbit anti-pVI antiserum and anti-rabbit HRP-antibody. Bands specific for pVI are indicated in (C) with arrowheads. (E) Schematic representation of the pIV derivatives and of the region involved in pVI-binding deduced from this experiment. Abbreviations for the pIV regions as in Fig. 1.

antiserum (Fig. 2C). Bands derived from pIV–pVI interaction were absent in a duplicate filter assayed without pVI (Fig. 2D). Background bands seen in both blots are probably due to interactions of the polyclonal anti-pIV antiserum with bacterial proteins copurified with pIV in some of the inclusion body preparations. The lower band of the doublet observed for polypeptide 99–454 in the Western and Far-Western blots (Fig. 2B and 2C) is probably a degradation product.

The assays revealed that pIV comprising residues 1–454, as well as N- and C-terminal deletions up to codons 99 and 411, respectively, interact with pVI. A C-terminal truncation to codon 362, on the other hand, did not bind, indicating that the interaction domain was either deleted or masked. These results are consistent with those obtained with the yeast two-hybrid system. However, in the Far-Western blot assay the N-terminus of pIV (residues 1–76) was not inhibitory for the interaction with pVI, ruling out the possibility that this region per se prevents binding in the two-hybrid system.

The pVI-binding domain of pIV is active in a different protein context and overlaps with a DNA-binding domain

The lack of binding capacity of the pIV mutants C-terminally deleted up to codon 362 could reflect removal

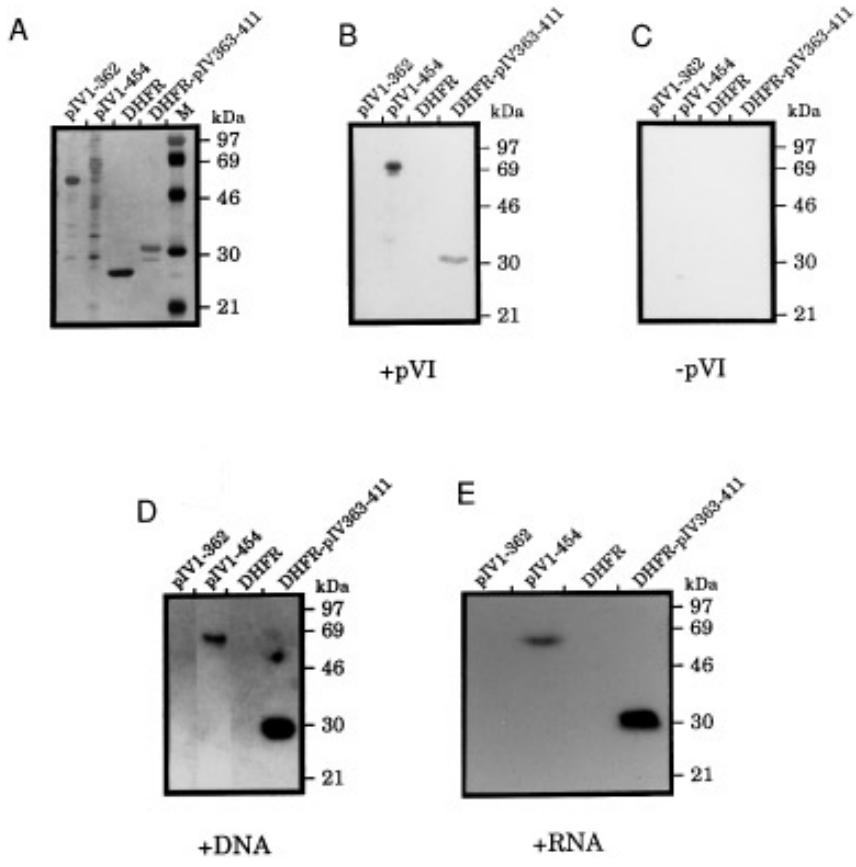

FIG. 3. Nucleic acid and pVI binding to the lysine-rich region. (A) Silver-stained SDS–PAGE of pIV1–362, pIV1–454 derived from bacterial inclusion bodies, affinity purified DHFR, and a DHFR fusion to pIV region 363–411 (DHFR–pIV363–411). Proteins were electrophoresed, transferred onto a nitrocellulose filter, renatured, and incubated with pVI (B) or without pVI (C). Binding of pVI was visualized by immunodetection as described in Fig. 2. The filters were decorated with labeled DNA (D) and RNA (E).

of the binding domain or masking of this domain by improper folding. To distinguish between these alternatives, the sequence of pIV between positions 363 and 411 was fused to the C-terminus of the DHFR sequence, tagged with six histidine residues, and affinity purified (Fig. 3A). The fusion protein reacted with pVI in the Far-Western experiment, while the unfused (6 × His) DHFR did not (Figs. 3B, 3C). Again, pIV (residues 1–454) used as a positive control bound pVI while the derivative truncated to residue 362 was not recognized. These experiments show that indeed the lysine-rich domain of pIV is directly involved in protein interactions with pVI.

Parallel DNA-binding experiments with pIV (residues 1–454) and the derivative truncated to residue 362 (Fig. 3D) suggested that region 362–454 is also involved in DNA binding. To confirm this, duplicates of the aforementioned transfer blot were probed with radiolabeled CaMV DNA. Both pIV (residues 1–454) and the DHFR fusion containing the lysine-rich region (residues 363–411) interacted with DNA, while DHFR itself and the pIV derivative lacking the lysine-rich domain did not bind (Fig. 3D). The proteins tested for DNA binding showed the same activity also for RNA binding (Fig. 3E). This shows, as expected, that the lysine-rich domain of pIV is not involved only in pVI binding, but also in DNA and RNA binding.

To test whether pVI and nucleic acids in fact compete for the same binding region on pIV, a dot-blot competition assay was performed. Preparations of pIV (residues 1–454) and a deletion mutant lacking the lysine-rich regions (residues 1–362) were immobilized on a nitrocellulose membrane by using a dot-blot chamber and incubated with pVI in the presence of increasing amounts of either plasmid DNA (pUC12), double-stranded (ds), or single-stranded (ss) oligonucleotides. The results in Fig. 4 confirm that pVI binds to pIV (residues 1–454) containing the lysine-rich region and not to the pIV mutant lacking residues 362–454. The addition of either plasmid DNA or ds oligonucleotides all reduced binding of pVI to background level. The ds oligonucleotides abolished binding of pVI at a lower absolute concentration than plasmid DNA. Single-stranded DNA oligonucleotides abolished pVI binding at a similar concentration as ds oligonucleotides. However, if compared on a molar basis plasmid

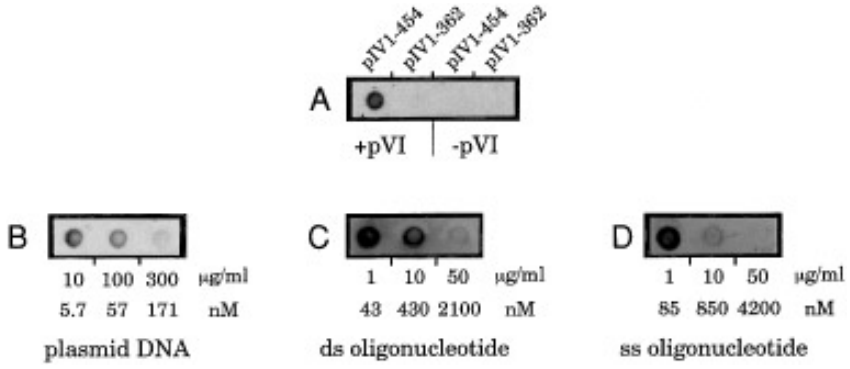

FIG. 4. Competition assay. A Far-Western dot–blot from pVI binding to immobilized pIV1–454 and pIV1–362 is shown in (A). For the competition, 2 μg of pIV1–454 protein was renatured and incubated in a dot-blot chamber with pVI in the presence of increasing amounts of competitor plasmid DNA (B), double-stranded (ds, C), and single-stranded (ss, D) oligonucleotides. For detection of pVI binding, see Fig. 2.

DNA competed the pVI binding most efficiently. Due to the presence of RNase in the experimental system, competition by RNA could not be tested.

These experiments show either that pVI and DNA compete for the same binding site of pIV or that the two binding sites are located so close together that DNA prevents the interaction of pVI with pIV.

Mutations in the binding region

Between positions 363 and 411, 50% of the amino acids are lysines (Fig. 5). Comparison of sequences of caulimoviruses [CaMV, carnation etch ring virus (CERV), FMV, and soybean chlorotic mottle virus (SoyCMV)] revealed that five of the lysine residues, as well as the cysteine[405], proline[406], and glycine[408] located in the C-terminal seven amino acids are conserved in all cases (marked bold in Fig. 5E). These amino acids are upstream of the Cys/His motif and would be part of a retrovirus-like nucleocapsid domain. A DHFR fusion with the lysine-rich region (363–404) was constructed and provided for convenience with unique *Mlu*I and *Pst*I sites resulting in three amino acid changes (Del1). With or without these modifications (DHFR–pIV363–404, Del1), a similar reduction of pVI binding was observed in comparison with the DHFR fusion to region 363–411. Del1 was therefore used to generate further deletions in the lysine-rich region (Del2–5; Fig. 5E). The Coomassie blue-stained gel indicates that similar levels of protein were obtained for all mutants (Fig. 5A). Total *E. coli* cell extracts were used for the blot assay, and pVI binding was quantified densitometrically and compared to the binding efficiency of the DHFR fusion with the lysine-rich region 363–411 (Figs. 5B, 5C, and 5E). The deletions Del1, -2, and -4 reduced binding of pVI to about 50% of the level of the whole lysine-rich region. The most drastic effects were found in Del3 (18% binding) and Del5, which completely lost the pVI binding activity. Del5 abolished also DNA-binding. By the deletions Del1–4 (Figs. 5D and 5E) DNA-binding was less affected than pVI binding. The high accumulation of charge seems to be sufficient for the binding of DNA. It is also possible that the region between positions 377 and 411 contains multiple DNA-binding sites.

DISCUSSION

The two most abundant CaMV proteins, the inclusion body matrix protein (pVI, also known as the translational transactivator [TAV]) and the capsid protein (pIV), coaccumulate in the typical viral inclusion bodies (Shepherd *et al.,* 1979), which are large enough to be seen in the light microscope. This could be a consequence of the translation strategy, e.g., because the CaMV proteins are translated from polyribosomes located on the surface of the inclusion bodies. In fact, CaMV proteins pI, pII, pIII, pV and human interferon αD, if expressed from a transgene incorporated into the CaMV genome (De Zoeten *et al.,* 1989) are found in inclusion bodies. An alternative or additional reason could be that pIV and pVI have direct affinities for each other. The results presented in this study show that such an affinity exists. The interaction between pIV and pVI was demonstrated by using the *in vivo* genetic assay for protein/protein interaction in the yeast two-hybrid system and a Far-Western blot assay utilizing immobilized, refolded pIV protein and soluble, affinity-purified pVI protein. By these two independent approaches, the pIV protein region mediating binding to pVI consistently mapped to the lysine-rich region, which is also involved in DNA-binding and thought to be located at the inside of the mature virus shell. The binding sites for DNA and pVI are situated so close to each other that binding of DNA can exclude the binding of pVI.

We consider the interaction of pIV with pVI specific, since: (I) the pVI/pIV interaction was demonstrated *in vivo* in the yeast two-hybrid system in the presence of cellular proteins and nucleic acids; (II) the incubation of immobilized pIV with pVI in the blot assay was performed

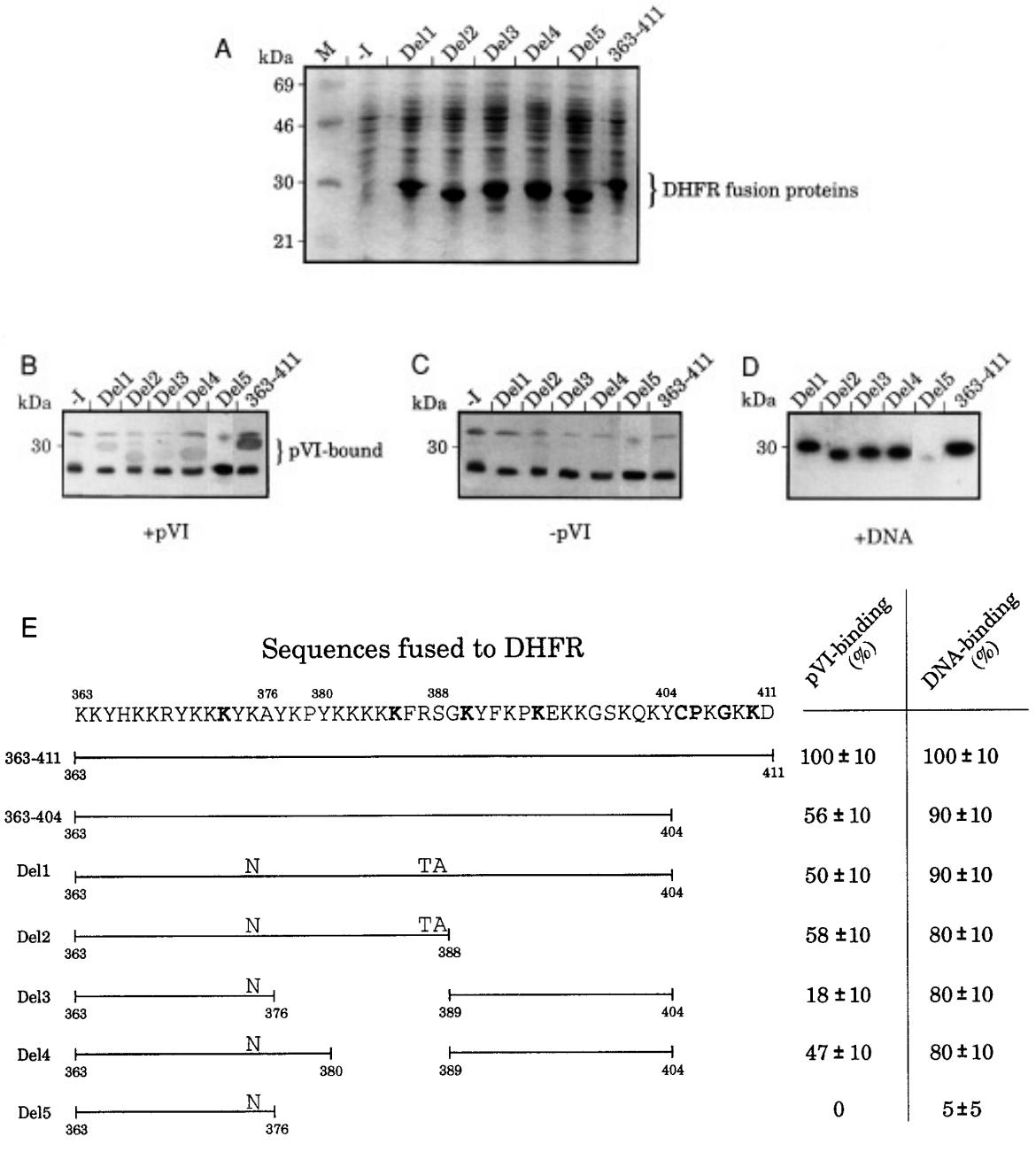

FIG. 5. Deletion analysis of the lysine-rich region of pIV fused to DHFR. (A) Whole cell extracts from *E. coli* before (−I) and after induction of expression of DHFR fusion proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue dye. Proteins were transferred onto nitrocellulose filters, renatured, and analyzed for pVI and DNA binding. Molecular weight markers (M) are indicated. Filters incubated with pVI (B) or without pVI (C) were analyzed for pVI binding by using the Far-Western assay. Bound pVI was visualized by immunodetection. (D) The filter was incubated with labeled DNA. (E) Schematic representation of the DHFR fusions. The sequence shows the CaMV pVI binding region. The amino acids conserved in caulimoviruses (CaMV, CERV, FMV, and SoyCMV) are in bold. Changes at positions 375 (K to N), 387 (R to T) and 388 (S to A) resulting from the introduction of *Mlu*I and *Pst*I restriction sites are indicated. Binding efficiency (%) given on the right side was evaluated by measuring the optical density/cm^2 of specific bands in at least two independent experiments.

in the presence of about 10^5-fold excess of carrier proteins; (III) the binding does not correlate strictly with charge (comparison of fusions with 363–411 and 363–404 shows that the deletion of the conserved C-terminal amino acids, without much affecting the charge of the protein, resulted in a 50% decrease pVI binding); (IV) further deletion of seven lysine positions in the fusion with 363–388 (Del2) did not display a gradual loss of pVI binding; (V) the internal deletion mutants Del3 and Del4 show a 5- and 2-fold weaker binding to TAV compared with DHFR-fusion to 363–411, although both proteins differ only in one positively charged amino acid of 23.

These results suggest that correct folding of this region is important for the pVI/pIV interaction.

It is likely that the pVI/pIV interaction plays a role in the control of the assembly process. By considering details of the assembly process of other viruses, several possibilities for such a role arise:

(1) pVI could act as a type of chaperonin. In this case, its interaction would lead to a refolding of the capsid protein. Chaperonins contribute to proper folding of other virus capsid proteins, such as bacteriophage λ where the heat shock proteins GroEL and GroES play a crucial role in capsid assembly. In their absence, mainly aberrant capsid-like structures are formed (Georgopoulos *et al.,* 1973; Georgopoulos and Ang, 1990). Assembly experiments in the presence of pVI will help to clarify this question.

(2) pVI, in its aggregated form of the inclusion body, might act as a nucleation site for assembly. Specific assembly sites have been characterized for other retro- and pararetroviruses. C-type retroviruses, such as HIV-I, are assembled at the plasma membrane (Göttlinger *et al.,* 1989; Gelderblom *et al.,* 1989). Although this is a host cell structure, it is further specified by viral envelope proteins, incorporated as patches into the plasma membrane (Hunter and Swanstrom, 1990). B- and D-type retrovirus capsids, A-particles, and the HBV capsid are assembled in the cytoplasm (Hunter, 1994). In the case of intracisternal A-particles and HBV assembly was shown to occur at the surface of the endoplasmic reticulum (Kuff and Lueders, 1988; Bartenschlager and Schaller, 1993).

A similar role for pVI as a nucleation surface for CaMV capsid assembly has in fact been proposed by Hull *et al.* (1987). However, this model assumed that N-terminal domains of the capsid protein, which will finally be located on the outside of the capsid, interact with pVI. In contrast, we found the interaction site near the C-terminus of pIV, which is thought to be located at the inner surface of the capsid. The pVI binding site on pIV closely overlaps with the DNA-binding site. This renders it unlikely that pVI acts on CaMV assembly from the outside, although it is possible that major transitions of the capsid protein occur, which might even at some stages turn regions of the capsid inside out. Such dramatic conformational changes have been described, e.g., for T4 assembly in which epitopes on the inside surface of the shell are transiently exposed to the outside, and vice versa (Steven *et al.,* 1990, reviewed by Hendrix and Garcea, 1994).

(3) pVI could play the role of a scaffold protein in assembly, as was observed with most of the ds DNA bacteriophages (Hohn *et al.,* 1975, 1976; Fujisawa and Hearing, 1994; Hendrix and Garcea, 1994) and with some of the ds DNA animal viruses, as exemplified by adenovirus (D'Halluin *et al.,* 1980). In these cases, the scaffold protein, usually together with other accessory proteins, helps to assemble a "prehead" (Hendrix and Garcea, 1994). The scaffold protein remains transiently within the capsid and is then either digested, as in bacteriophage λ (Hohn *et al.,* 1975), or removed through holes in the prehead and reused as in bacteriophage P22 (King and Casjens, 1974). Then, DNA can be packaged into the prehead through a gate structure (Hohn and Hohn, 1974; Casjens and Huang, 1982). In many cases, interaction of the capsid protein with the scaffold protein prevents the formation of premature capsid protein–DNA complexes which would inhibit both the ordered capsid assembly and the properly timed DNA-packaging process. The competition of DNA and pVI for the CaMV capsid protein binding might hint that a similar mechanism exists for CaMV assembly.

If the scaffold mode of assembly occurs for CaMV, it implies that pVI is located at some stage inside of a prehead structure and leaves the prehead through holes during virus maturation. In fact holes in the virus shell are observed in three-dimensional reconstructions of CaMV. They have been interpreted as vestiges of pVI scaffold leaving the assembly complex (Cheng *et al.,* 1992). Such a model also suggests that a DNA-packaging enzyme could exist for CaMV. This role could be fulfilled by the minor capsid protein pIII, although attempts to find an associated ATPase activity have been unsuccessful so far (Mougeot, personal communication). Another consequence of the model would be that reverse transcription occurs outside the CaMV virions and the DNA is packaged into preformed virions. Interestingly, this model would explain why a large portion of replication activity was found with 20S complexes (Pfeiffer and Hohn, 1983) rather than with the virus at 80S.

In accordance with the assembly pathways of retroviruses, another mechanism was anticipated for the assembly of CaMV, namely the building of the capsid around the pregenomic RNA, possibly concomitant with replication (Marsh and Guilfoyle, 1987). This mechanism is also used for RNA viruses of all kingdoms, and some of the animal ds DNA viruses that replicate in the nucleus, such as polyoma and SV40 virus assemble around the minichromosome (Bina and Blasquez, 1983). In HBV and retroviruses the reverse transcriptase is copackaged (Bartenschlager and Schaller, 1992; Hunter, 1994) and reverse transcription occurs within the virions, thereby secluding this process from the cytoplasm.

The multitude of properties and functions of pVI is remarkable. It binds to pIV, to RNA, and to other copies of pVI (De Tapia *et al.,* 1993), forms inclusion bodies in which most or all of the virus proteins accumulate, transactivates translation, and apparently has a role in assembly. It will be interesting to learn whether all these properties and functions correspond to different domains

of a multifunctional protein or whether they are commonly derived from an as yet unknown master function.

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