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Interaction of the Cauliflower Mosaic Virus Coat Protein with the Pregenomic RNA Leader

ORLENE GUERRA-PERAZA,† MARC DE TAPIA,‡ THOMAS HOHN,* AND MAJA HEMMINGS-MIESZCZAK

Friedrich Miescher-Institut, CH-4002 Basel, Switzerland

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Using the yeast three-hybrid system, the interaction of the *Cauliflower mosaic virus* (CaMV) pregenomic 35S RNA (pgRNA) leader with the viral coat protein, its precursor, and a series of derivatives was studied. The purine-rich domain in the center of the pgRNA leader was found to specifically interact with the coat protein. The zinc finger motif of the coat protein and the preceding basic domain were essential for this interaction. Removal of the N-terminal portion of the basic domain led to loss of specificity but did not affect the strength of the interaction. Mutations of the zinc finger motif abolished not only the interaction with the RNA but also viral infectivity. In the presence of the very acidic C-terminal domain, which is part of the preprotein but is not present in the mature CP, the interaction with the RNA was undetectable.

Cauliflower mosaic virus (CaMV) (Fig. 1) is the type member of the caulimoviruses, which, together with the animal hepadnaviruses and the plant badnaviruses, are classified as pararetroviruses. Pararetroviruses, retroviruses, and retrotransposons form the group of retroelements (25). Foamy viruses are intermediate between pararetroviruses and retroviruses (32). Two important features distinguish pararetroviruses from retroviruses. First, mature pararetrovirus particles contain a double-stranded DNA genome while retroviruses contain two copies of an RNA genome. Second, in retroviruses the DNA is integrated into the host genome and in pararetroviruses it remains as multiple copies of a circular minichromosome. However, both forms of viral DNA direct the production of terminally redundant RNA, which plays a dual role as a replicative intermediate and as mRNA (for a review, see reference 40).

The mechanism used by CaMV to package its genome remains unclear. It is likely that, as in hepadnaviruses, the pregenomic 35S RNA (pgRNA) is first encapsidated and subsequently reverse transcribed within the virion (36). However, encapsidation of DNA following reverse transcription remains a possible alternative (24). The mechanism by which the terminally redundant RNA is encapsidated in hepadnaviruses and retroviruses has been widely studied. In retroviruses, the pgRNA is first specifically recognized by the Gag protein and then encapsidated (47), while in hepadnaviruses reverse transcription and encapsidation start after the recognition of the pgRNA by the N-terminal part of the reverse transcriptase (5). The specificity of encapsidation is determined by both the RNA and the viral protein involved.

In plant pararetroviruses, the 5′ end of the pgRNA contains a long leader sequence with the potential to form a large stem-loop structure (14, 38). In CaMV, the formation of such an elongated hairpin structure (Fig. 1B) has been confirmed in vitro (22). A purine-rich sequence at the top of this hairpin is conserved in some caulimoviruses including CaMV, figwort mosaic virus (FMV), and carnation etched ring virus, and in badnaviruses such as rice tungro bacilliform virus, sugar cane bacilliform virus, and dioscorea alata bacilliform virus (21, 38). In CaMV, deletion of this sequence drastically retarded the occurrence of symptoms in infected plants but did not influence the transient expression of a reporter gene downstream of the mutated leader in host plant protoplasts (14). The function of this sequence is not known, but it has been proposed to act as a packaging signal or to be involved in dimerization of pgRNA (14, 21). Experiments performed in vitro, however, argue against the involvement of the purine-rich sequence in the dimerization process of the CaMV pgRNA leader (22).

Retroviral Gag polyproteins are cleaved by the viral protease into the matrix, capsid, and nucleocapsid proteins, which rearrange and assemble during maturation to form infectious particles (35). The zinc finger domains in the nucleocapsid of retroviruses are critical for viral replication and participate directly in genome recognition and encapsidation (1, 6, 12, 20, 34, 45). One of the best-studied examples is human immunodeficiency virus 1 (HIV-1) (18), for which the three-dimensional structure of the nucleocapsid bound to RNA stem-loop 3 of the HIV-1 leader has been determined (11).

The CaMV coat precursor protein is processed by the viral aspartic proteinase (49). After processing, derivatives carrying the central domain (p44, p39, and p37) are found as coat proteins associated with the virion, while the acidic N- and C-terminal portions have not been detected (27, 33). Each of the major coat proteins (CP) contains a basic region flanking a zinc finger motif ($C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$) that is conserved among retroelements (10). A synthetic peptide covering this region binds zinc ions (26). In FMV, the zinc finger is essential for virus viability (44).

The characteristics shared by the coat protein of CaMV and the Gag protein of retroviruses suggest the possibility that these viruses use similar mechanisms to encapsidate their pregenomic and genomic RNAs, respectively. In the present study, using the yeast three-hybrid system (46), we have found a specific interaction between the coat protein of CaMV and the conserved purine-rich sequence in the central part of the CaMV pgRNA leader. As in retroviruses, the zinc finger domain of the CaMV CP is critical for both interaction with CaMV pgRNA and viral infectivity.

* Corresponding author. Mailing address: Friedrich Miescher-Institut, Maulbeerstrasse 66, 4058 Basel, Switzerland. Phone: (061) 6976672. Fax: (061) 6973976. E-mail: hohn@fmi.ch.

† Permanent address: Centro de Bioplanta, Ciego de Avila, Cuba.

‡ Present address: IPCB-UMR 7519, 67084 Strasbourg, France.

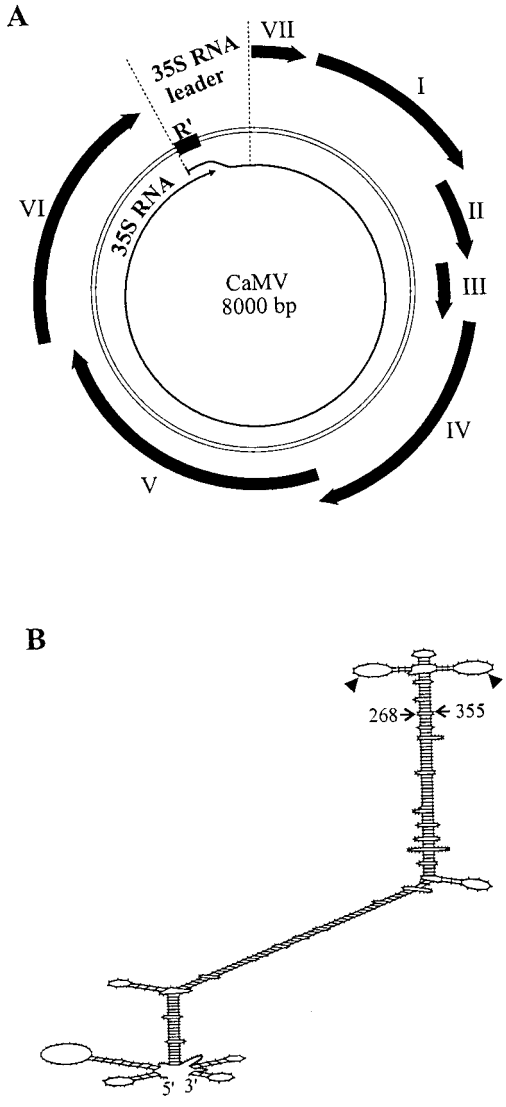

FIG. 1. (A) The genomic map of CaMV DNA is represented by a thin double line, with the box marked R′ indicating the region of the genome which is transcribed twice in the terminally redundant transcript. The major viral transcript, 35S RNA (pgRNA), is shown as a thin arrow inside the DNA. The thick arrows outside the DNA represent the major viral ORFs: ORF VII (unknown function), ORF I (movement protein), ORF II (aphid transmission factor), ORF III (DNA binding protein, perhaps playing a role in nucleic acid condensation during assembly), ORF IV (capsid protein), ORF V (protease and reverse transcriptase), and ORF VI (inclusion body matrix protein; translational transactivator). (Redrawn from reference 40.) (B) Secondary structure of the CaMV 35S RNA leader (Strasbourg strain). The plot represents the most stable secondary structure predicted by the program MFOLD at 25°C. The boundaries of the conserved purine-rich sequence are indicated by arrowheads. Numbers delimit the central part of the leader used in this study.

MATERIALS AND METHODS

The components of the three-hybrid system, including the controls used in our experiment, were kindly provided by Marvin Wickens, University of Wisconsin (46).

Hybrid RNAs. Two annealed complementary oligonucleotides covering bases 268 to 355 of the CaMV 35S RNA leader (Strasbourg strain) (17) were cloned into the *Sma*I site of pIII/MS2-2 (46). Sense and antisense orientation clones are called LS and LAS, respectively. The iron response element (IRE) RNA was cloned in the same position by SenGupta et al. (46). RNA secondary-structure analysis was performed using the *MFOLD* program (Wisconsin package version 6.0; Genetics Computers Group, Madison, Wis.) at 25°C.

Hybrid proteins. Hybrid protein 1 (see Fig. 2A), containing the DNA binding domain (LexA) and the RNA binding domain of the MS2 coat protein, is integrated into the genome of the yeast strain L40-coat. To obtain hybrid protein 2, the full-length open reading frame IV (ORF IV) (pIV) and its variants (CPs, numbered with the amino acid positions at which they start and end) were cloned in frame with the GAL4 activation domain in plasmid pGAD 424 (Clontech) (4). The *Nco*I-*Sal*I pIV fragment from plasmid p(1–489) (31) was cloned directly into *Nco*I- and *Sal*I-restricted pGAD 424. CP77–454 and CP77–411 were described previously (24). CP77–489 was obtained by replacing the *Ban*II-*Bam*HI fragment of CP77–454 with the *Ban*II-*Bam*HI fragment of pIV. CP225–454, CP272–454, CP315–454, CP347–454, CP391–454, and CP402–454 were amplified by PCR with the 5′-end oligonucleotides 5′-gcgatgtacaccgaattcttaggac-3′, 5′-gaaaagaacg aattcaagacagaactgg-3′, 5′-cagtttagaattcgcggcaaagatagtc-3′, 5′-gtgttgtgaattcggaga agcttcaacag-3′, 5′-ccgatcaggaaaagaattcaagccc-3′, and 5′-gctcaaagcaagaattctgccca aaaggcaag-3′, respectively, and the 3′-end oligonucleotide 5′-gaatgaatagatcttgaa ctccttcatagg-3′. The PCR fragments were cut in *Eco*RI and *Bgl*II sites present at their 5′ and 3′ ends, respectively, and cloned into the corresponding sites in pGAD 424. All clones were derived from the Strasbourg strain of CaMV. The zinc finger mutations were introduced in the context of CP272–454 by using PCR-mediated site-directed mutagenesis.

CP315–489 was also amplified by PCR with the same 5′-end oligonucleotide used for CP315–454 and the 3′-end oligonucleotide 5′-acgttcggatcctgctcagtctga gtctgag-3′. The PCR fragment was cut with *Eco*RI and *Bam*HI and cloned into *Eco*RI and *Bgl*II sites in pGAD 424.

The cloning of ORF III into pGAD 424 is described in reference (30). ORF V was amplified by PCR with the 5′-end oligonucleotide 5′-gaacccggggatcctgat ggatcatctacttctgaagact-3′ and the 3′-end oligonucleotide 5′-tgaactgcagtagatctcgg atttcaattaggaattaacct-3′. The product was cloned *Bam*HI-blunt into plasmid pBluescript *Bam*HI-*Sma*I and then cut with *Xho*I and filled in with the DNA polymerase I large (Klenow) fragment. Thereafter, it was cut with *Bam*HI and cloned into pGAD 424. The vector pGAD 424 was cut with *Sal*I, filled in with the DNA polymerase I Klenow fragment, and then cut with *Bam*HI. ORF VII was amplified by PCR with the 5′-end oligonucleotide 5′-tggtgaattcgccatgaatcggttta ag-3′ and the 3′-end oligonucleotide 5′-gtactgtcgacaggatcctttattgttccagaa-3′ followed by digestion with *Eco*RI-*Bam*HI and cloned into pGAD 424 digested with the same enzymes. To obtain the ORF VI clone, the *Xma*I-*Pst*I fragment from pGBTTAV (24) was cloned directly into *Xma*I-*Pst*I-restricted pGAD 424.

Yeast culture. L40-coat strain (46) was grown in synthetic dropout (SD) minimal medium without tryptophan. Following transformation using the lithium acetate method (19), positive transformants were selected on SD medium lacking tryptophan and leucine and/or uracil. The transformants were allowed to grow at 30°C for 3 to 4 days.

β-Galactosidase filter assay. A total of 20 colonies were restreaked on fresh plates and were allowed to grow for a further 2 days. They were then transferred to a nitrocellulose filter, frozen in liquid nitrogen, thawed, and assayed for the appearance of blue color by using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as the substrate of β-galactosidase, as described previously (43), and incubated at 30°C.

β-Galactosidase liquid assay. Five different transformants were grown in minimal medium for 18 h to obtain an aliquot that was diluted in fresh medium to give an optical density of approximately 0.25. The aliquots were allowed to grow for 3 h to obtain an optical density of between 0.5 and 0.8, frozen in liquid nitrogen, and thawed four times for lysis. The yeast lysates were assayed for β-galactosidase enzymatic activity (43) using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (Fluka). The enzymatic activity was determined in five independent transformants and in at least three independent experiments. One unit of β-galactosidase was defined as the amount which hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell (37).

Mutations of the zinc finger in the virus. Following cloning of the *Hpa*I-*Sma*I fragment of pCa540 (which contains the genome of the CM4-181 CaMV strain) (13, 39) into plasmid pTZ 19R, mutations were introduced using site-directed mutagenesis. The mutants obtained were recloned into the pCa540 context by exchanging *Mlu*I-*Xma*I fragments and were analyzed by DNA sequencing. The infection was carried out using a procedure described previously (13, 39).

RESULTS

CaMV CP interacts with the central part of the pgRNA leader. The yeast three-hybrid system (46) is a genetic method to detect and analyze RNA-protein interactions, whereby the binding of a bifunctional RNA to each of two hybrid proteins activates the transcription of a reporter gene in vivo (Fig. 2A). We used this system to test whether there is a specific interaction between any of the CaMV proteins and the conserved purine-rich sequence from the central region of the CaMV pgRNA (35S RNA) leader (nucleotides 268 to 355 [Fig. 1B and 2B]). The schematic representation of the fused transcript expressed from vector pIII/MS2-2 is represented in Fig. 2C. This hybrid RNA was called leader sense (LS). The same

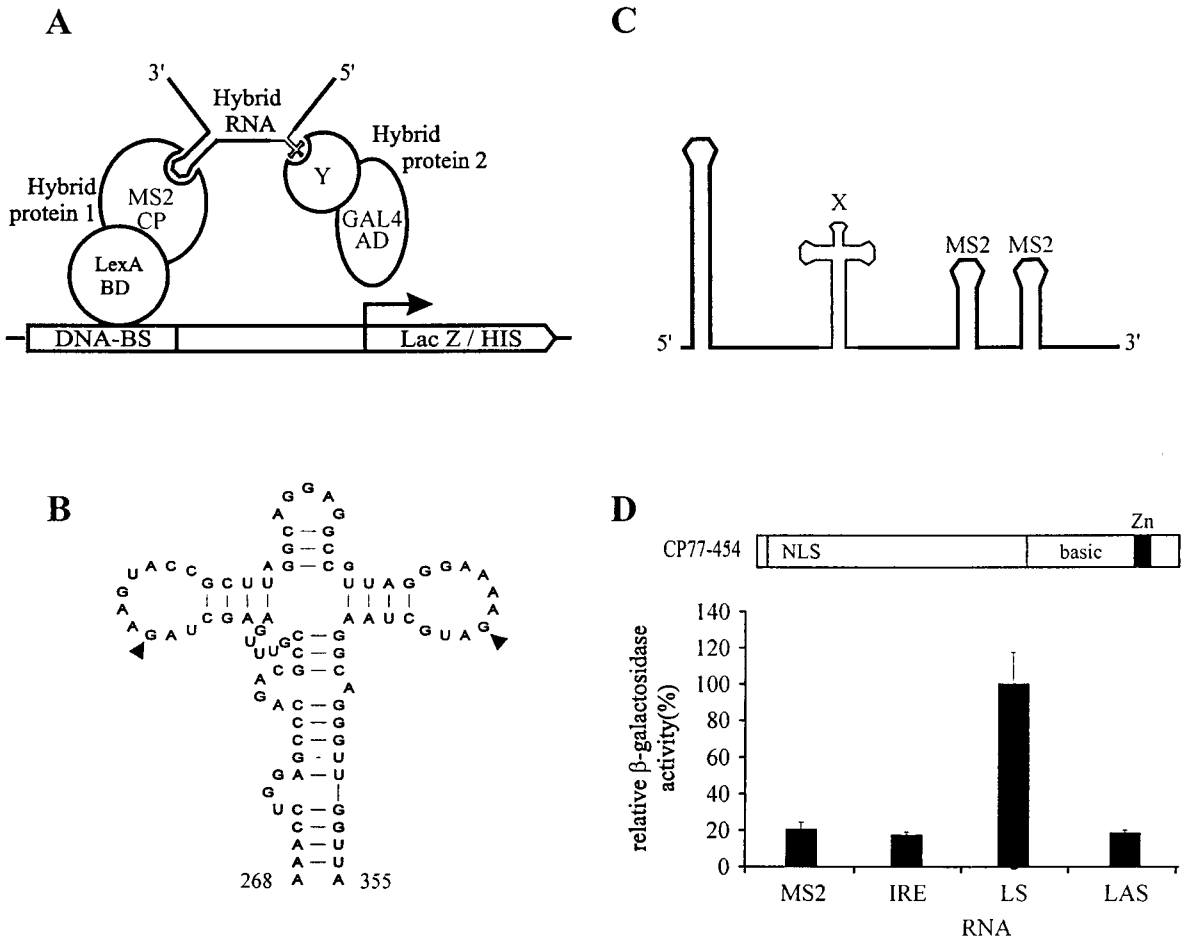

FIG. 2. (A) Scheme of the yeast three-hybrid system. MS2 CP, coat protein of the MS2 phage; LexA BD, LexA DNA binding domain; GAL4 AD, GAL4 transcriptional activation domain; Y, protein under investigation; LacZ/HIS, β-galactosidase and histidine reporter genes. (B) Sequence and predicted secondary structure of the central part of the leader (268 to 355) used in the LS hybrid RNAs. The conserved purine-rich sequence is delimited by arrowheads. (C) Hybrid RNA. The 5′ and 3′ regions flanking X RNA include RNase P leader and the duplicated MS2 RNA sequence, respectively. (D) Interaction between CP77–454 and the hybrid RNAs. Bars represent relative β-galactosidase activity measured in extracts from yeast cells expressing CP77–454 and one of the hybrid RNAs shown. The mean and standard deviation from three independent experiments are shown. NLS, nuclear localization signal; basic, stretch of basic amino acids; Zn, zinc finger.

sequence in antisense orientation (LAS), the MS2 RNA alone, and an RNA containing the IRE (29) were also tested.

The coding regions of the essential CaMV ORFs I, III, IV, V, and VI and also of ORF VII (Fig. 1A) were fused in frame with the GAL4 activation domain of vector pGAD 424. For ORF IV, which encodes CP, construct CP77-454 was used (Fig. 2D). The CP77–454 clone had previously been used successfully in the yeast two-hybrid system to detect CP-CP and CP-pVI interactions (24). Cotransfection of the resulting plasmids with the vector expressing the LS RNA led to expression of the β-galactosidase gene as seen by filter assay only for the viral coat protein construct. In this positive case, the colonies turned blue after 1 h.

To confirm that this interaction was due to the bridge formed by the hybrid RNA between MS2 CP and CaMV CP, several control plasmids were used, i.e., plasmids that code for the hybrid RNA alone, the GAL4 activation domain alone, or the fusion between the GAL4 activation domain and the coat. No signal was obtained in any of these cases (results not shown). Although colonies expressing the GAL4-CP77–454 fusion and either MS2, IRE, or LAS RNA turned blue in the β-galactosidase filter assay after prolonged incubation (5 or 6 h), the rapid appearance of the blue color (1 to 2 h) with the GAL4-CP77–454 protein and the LS RNA indicated a specific interaction. Relative β-galactosidase activities resulting from the interaction between CP and the different RNAs are shown in Fig. 2D. The interaction between CP and the LS RNA is about five times stronger than that obtained with either the MS2 RNA alone or the MS2 RNA fused to the IRE or LAS, indicating that CP interacts preferentially with the central part of the leader sequence.

Both the zinc finger and the basic domain of CP are important for the specific interaction with the leader. To map the region of the CaMV CP responsible for the specific interaction with the LS RNA, a series of N- and C-terminal deletion mutants (Fig. 3A) were introduced in frame with the GAL4 activation domain (hybrid protein 2 [Fig. 2A]) into the pGAD 424 vector (see Materials and Methods). All the constructs were transfected alone or together with the plasmid expressing the LS RNA in the yeast strain L40-coat. Colonies containing either the plasmids expressing CaMV CP or the plasmid expressing the LS RNA alone did not turn blue after more than 6 h when analyzed using the β-galactosidase filter assay (results not shown). When both plasmids were present, colonies turned blue in 1 to 2 h (Fig. 3A, right side). The interaction was lost when the zinc finger motif was removed (CP77–411). CP391–

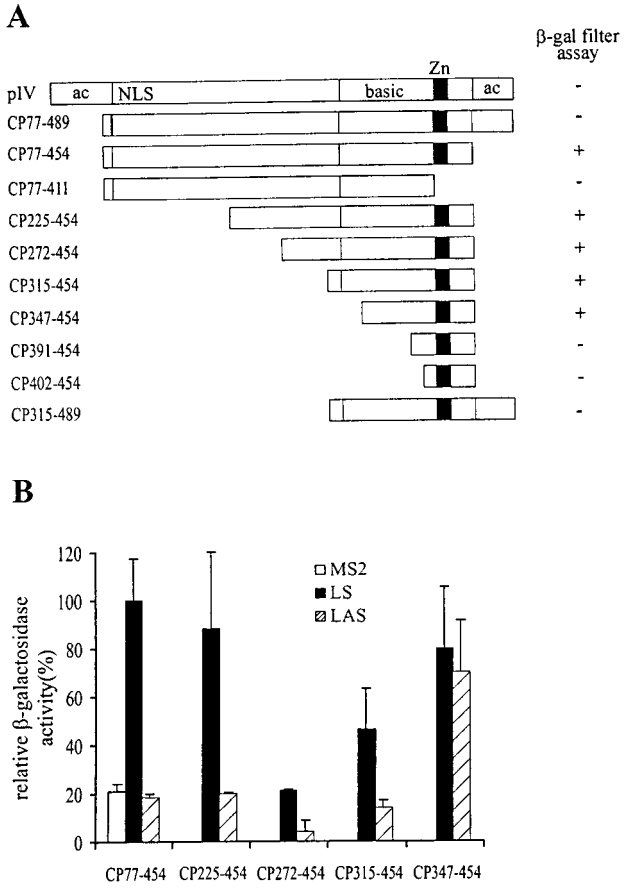

FIG. 3. (A) Schematic representation of the CaMV ORF IV-encoded protein (pIV; coat protein) and the N- and C-terminal deletion mutants under study. Two acidic regions (ac), the basic region (basic), the nuclear localization signal (NLS), and the zinc finger (Zn) are indicated. Truncated ORF IV derivatives are represented by CP and numbers corresponding to the first and last amino acids of the pIV protein that they contain. The proteins represented here are fused in frame to the C-terminal part of the GAL4 AD (results not shown). The interaction of each fused protein with the LS RNA, detected using the β-galactosidase (β-gal) filter assay, is shown + or − on the right. (B) Relative β-galactosidase activities resulting from the interactions between different CP mutants and LS or LAS hybrid RNAs. The interaction between the coat protein and the MS2 RNA is also shown for CP77–454. Standard deviation is indicated.

454 and CP402–454 do contain the zinc finger motif, but the basic domain is drastically shortened. This might indicate that the basic region is also important for the interaction. The constructs pIV and CP77–489, as well as the very short constructs CP391–454 and CP402–454, did not show the blue color. In a previous analysis, full-size pIV and CP77–489 also failed to show any interaction with the ORF VI-encoded protein (pVI) in the two-hybrid system, although the truncated construct CP77–454 did so. The reason in both cases might be either that the C-terminal acidic domain destabilizes the protein (10) or that it masks the interaction domain. An additional construct, CP315–489 (Fig. 3A), which differs from the positively interacting CP315–454 only by inclusion of the C-terminal acidic domain, was confirmed by Western blot analysis to be stably expressed in yeast (results not shown). Since CP315–489 did not interact with the LS-RNA, masking of the interacting domain appears likely.

The clones that turned blue were analyzed further for their specificity in the interaction with LS RNA by using the β-galactosidase liquid assay. The activity of the β-galactosidase is shown relative to the interaction between the CP77–454 protein and the LS RNA (Fig. 3B). As shown above, the interaction of CP77–454 with the LS RNA is specific compared to that the MS2 and the LAS RNAs. The same was true for CP225–454, CP272–454, and CP315–454 when the interaction with the LS RNA was compared to that obtained with the LAS RNA. The strength of the interaction of CP272–454 and CP315–454 with the LS RNA was drastically reduced although still specific compared with LAS RNA. The specificity of the interaction was lost when the basic domain was partially deleted, as shown by the interaction between CP347–454 and the LS and LAS RNAs, respectively.

		Infectivity	β-gal filter assay
CaMV	**C**W**IC**N**IEGH**Y**A**N**E**C**	+	+
CaM1	A W I A N I E G A Y A N E A	*na*	-
Ca583	G W I C N I E G Y Y A N E G	-	-
Ca584	C G I C N I E G H Y A N E C	-	-
Ca586	C W I C N I E G H D A N E C	-	-
Ca587	C W I C N I E G C Y A N E C	-	-
Ca588	C W I C N I E G H Y A N E H	-	*na*

FIG. 4. Sequence of the CaMV zinc finger domain and mutants used to determine the infectivity in plants and to study the interaction with the LS RNA in the three-hybrid system. +, infectivity or appearance of blue color in the β-galactosidase filter assay; bold, amino acids that form the zinc finger; underlining, mutated amino acids; *na*, not analyzed.

Our results indicate that the zinc finger domain of the CP and part of the basic domain are required for the interaction with the LS RNA. The shortest protein capable of a specific interaction was CP315–454, which contains, in addition to the complete basic domain, part of a putative α-helix (amino acids 330 to 346) that might be involved in protein-protein interaction (9).

Mutations in the zinc finger of the CaMV capsid protein abolish both virus infectivity and interaction with the leader. To see whether the zinc finger motif of CaMV CP is important for the infectivity of this virus, we mutated some of the most important conserved amino acids, i.e., cysteine and histidine residues forming the zinc ion complexes, and additionally, aromatic amino acids considered important for the specificity of zinc finger interaction (10) (Fig. 4). Turnip (*Brassica rapa*) plants were inoculated mechanically with wild-type and mutated viruses (Ca583, Ca584, Ca586, Ca587, and Ca588). Symptoms appeared after 3 weeks on plants inoculated with wild-type virus. In contrast, even after 2 months, no symptoms appeared on plants inoculated with any of the mutant viruses.

The mutated motifs leading to noninfectious viruses (Fig. 4) were cloned in the context of CP315–454 (Fig. 3) in plasmid pGAD 424 and then cotransformed in yeast with the plasmid encoding the LS RNA. Transformants were tested using the β-galactosidase filter assay. No blue color appeared in any colony corresponding to the zinc finger mutants (Fig. 4).

These results indicated that the zinc finger motif-mutated amino acids, including aromatics and the ones responsible for the interaction with zinc ions (cysteines and histidine), are crucial for virus infectivity and are also important for the interaction with the LS RNA in the three-hybrid system.

DISCUSSION

Several reports have described the interaction between CaMV CP and nucleic acids (7, 9, 15, 24, 48) but none have described the specific interaction between CP and RNA that is

believed to be responsible for the encapsidation of the pgRNA. To study this, we selected the purine-rich sequence in the middle part of the leader because of its conservation in most caulimoviruses and badnaviruses. Our approach was to use an in vivo method, the yeast three-hybrid system (46), that permits the analysis of protein-RNA interactions. We confirm that CP (CP77–454) interacts with RNA in general. However, the interaction is much stronger with the central part of the CaMV pgRNA leader (LS) than with other RNAs such as LAS, MS2 RNA, or IRE RNA. Similar results had been reported for the HIV-1 Gag protein, which interacts more strongly with the HIV-1 RNA viral encapsidation signal than with the IRE RNA or the encapsidation signal of either Moloney or Harvey murine sarcoma virus (2).

The zinc finger motif of the nucleocapsid protein is very important for infectivity in retroviruses. There are several studies showing that deletion or point mutation of the very highly conserved amino acids in this domain result in noninfectious viruses (see reference 47 for a review). Also, it has been previously shown for another pararetrovirus, FMV, that mutation of all the cysteines and the histidine of the zinc finger abolishes the infectivity of the virus (44). Here, we confirm this observation for CaMV CP. In addition, some of the zinc finger mutants were tested in the three-hybrid system, and none of them interacted with the central part of the leader. However, the zinc finger of CaMV CP alone is not sufficient for the specific interaction. The minimal protein CP315–454, which still interacts specifically with the LS RNA, contains the zinc finger, the basic domain, and a preceding region forming a putative helix containing two cysteines conserved in plant pararetroviruses. This region has been suggested to be important for the CP-CP interaction (9).

Our findings indicate that the interaction between the CP and the central part of the leader might play a role in the encapsidation of the CaMV pgRNA, similar to that in retroviruses. On the other hand, the encapsidation mechanism of CaMV might share some characteristics with the encapsidation mechanism of hepadnaviruses, since, as in hepadnaviruses but not in retroviruses, the reverse transcriptase of CaMV is expressed separately from CP (8), implying a mechanism to target it to the capsid (5, 41).

The central part of the CaMV RNA leader, which we found to interact specifically with the CaMV capsid protein, is not present in the 19S subgenomic RNA. However, it is still present in spliced forms of CaMV RNA (28). How could the spliced RNA be excluded from the packaging process? One possibility is that the leader structure of the spliced RNA is melted and occupied by scanning ribosomes while that of the unspliced RNA is not. Preservation of the central part of the unspliced leader structure is possible due to the shunt mechanism of translation initiation (16, 42), which excludes this region from the scanning process and hence from melting (23). Another possibility is that for successful packaging, not only the central part of the CaMV RNA leader but also other parts, e.g., the primer binding site, which is absent from spliced RNA, are required. Such a requirement might guarantee that the reverse transcriptase is included in the packaging and assembly process. Furthermore, RNA dimerization might be a prerequisite for efficient packaging (3) and the corresponding dimerization signal might be located within the intron.

Both terminal regions of the precoat protein (pIV) are very acidic and are removed during maturation. The N-terminal acidic region was implicated in inactivating the nuclear targeting signal, such that only CPs lacking this region are transported to the nucleus (31). Interestingly, the full-length pIV capsid preprotein and also other versions of this protein, CP77–489 and CP315–489, which are N-terminally truncated but contain the complete C-terminal sequence, did not interact with LS RNA in the three-hybrid system (Fig. 3A). CP315–489 does not contain the destabilizing N-terminal domain (A. Karsies, D. Leclerc, and T. Hohn, unpublished data), suggesting that the interaction of the coat protein with RNA is masked by the acidic C-terminal domain. Thus, the C terminus of pIV might have regulatory functions, e.g., preventing the binding of RNA and thereby guaranteeing its availability as mRNA until enough viral proteins (including the protease) are produced.

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