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The two-hybrid system was used to test for pairwise interactions between the tobacco vein mottling virus (TVMV)-encoded RNA-dependent RNA polymerase (or NIb protein) and two other TVMV-encoded proteins: the NIa protein, which consists of genome-linked protein (VPg) and proteinase domains, and the viral coat protein (CP). Using this approach, we find that the NIb protein interacts with both the NIa protein and the CP in yeast cells. Moreover, we find that a mutation in the conserved GDD domain of the NIb protein diminishes the NIb–CP interaction but not the NIb–NIa interaction. Likewise, mutations in the vicinity of the NIa protein to which the genomic RNA is covalently attached eliminate the NIb–NIa interaction. We conclude that the NIb protein interacts with the VPg domain of the NIa protein and that this interaction requires a functional RNA attachment site. This interaction may be important for the initiation of viral RNA synthesis in infected cells. We also conclude that the CP interacts with the NIb in a manner that is sensitive in changes in the highly conserved GDD motif. The role of this interaction in the functioning of the NIb protein or the CP is unclear, but may involve regulation of viral RNA synthesis in infected cells. © 1995 Academic Press, Inc.

INTRODUCTION

Potyviruses are positive-sense, single-stranded RNA plant viruses that are members of the Picorna superfamily of viruses. Potyvirus genomes consist of some 10^4 nt and encode a single polyprotein of more than 3000 amino acids (reviewed in Riechmann *et al.*, 1992). The polyprotein is processed by three virus-encoded proteinases to yield a number of different protein products. Most of these products can be assigned specific biochemical activities. For example, three proteins (the P1, HCpro, and NIa proteins) all possess proteinase activities that are involved in the various proteolytic cleavages needed to process the potyvirus-encoded polyprotein (Dougherty and Carrington, 1988; Carrington *et al.*, 1989; Verchot *et al.*, 1991). The P1 protein is also an RNA binding protein (Brantley and Hunt, 1993; Soumounou and Laliberté, 1994). The CI protein possesses a characteristic RNA:RNA helicase activity (Lain *et al.*, 1990; Eagles *et al.*, 1994). The NIb protein is presumed to be an RNA-dependent RNA polymerase, based upon sequence similarity with other polymerases (Domier *et al.*, 1987).

In addition to these activities, other roles for particular potyvirus-encoded proteins have been identified or suggested. The P1 protein has been proposed to be an accessory factor involved in replication of viral RNA (Verchot and Carrington, 1995). The HCpro protein is required for the acquisition and transmission of potyviruses by insect vectors (Pirone and Thornbury, 1984; Hellmann *et al.*, 1985) and may have other roles as well (Atreya and Pirone, 1993; Dolja *et al.*, 1993). The CI protein is the principal component of distinctive cylindrical inclusions that accumulate in the cytoplasm of infected cells (Dougherty and Hiebert, 1980). In addition to being a site-specific proteinase, the NIa protein (or its N-terminal half) is also the genome-linked protein (or VPg; Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990, 1991), that protein found covalently attached to the 5′-terminus of the viral RNA. In addition to its role in the assembly and structure of the virion, the coat protein (CP) is involved in the movement of virus in plants (Dolja *et al.*, 1994) and the aphid-mediated transmission of virus from plant to plant (Atreya *et al.*, 1990, 1991).

Although most potyvirus-encoded proteins have been characterized to a significant extent, many questions remain concerning the integration and regulation of activity of these different proteins during the infection process. To better understand the interrelationships between different potyvirus proteins, we have initiated a study of the pairwise interactions between different potyvirus-encoded proteins in yeast cells using the so-called two-hybrid system (Fields and Song, 1989). Here, we describe

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interactions between the NIb protein of the potyvirus to-bacco vein mottling virus (TVMV) and two other TVMV-encoded proteins, the CP and the NIa protein. Our results indicate that interactions between these proteins are diminished by specific mutations in conserved domains in the NIb or NIa proteins, suggestive of interactions important in the regulation of biochemical activities in infected cells.

METHODS

Recombinant DNA manipulations

Recombinant DNA manipulations and DNA sequencing were carried out using standard protocols (Sanger *et al.,* 1977; Sambrook *et al.,* 1989). Plasmids were grown and maintained in *Escherichia coli* strain TB1 (ara, Δlac-proAB, rpsL, hsdR [rk−, mk+], ϕ80:lacZΔM15).

The TVMV NIa, NIb, and CP genes, as well as the NIa and NIb mutants, were cloned into the plasmids pGAD2F (Chien *et al.,* 1991) and pMA424 (Ma and Ptashne, 1987a) after amplification by the polymerase chain reaction (PCR). For this, oligonucleotide primers were designed to incorporate *Bgl*II restriction enzyme sites at the 5′- and 3′-ends of the respective genes; in addition, the reading frame in the *Bgl*II site at the 5′-end of each gene was designed to match that of the *Bam*HI sites in pGAD2F and pMA424. Reactions contained 100 ng of template DNA (pXBS7; Domier *et al.,* 1989), 50 ng of each primer, 125 μM each of dATP, dCTP, dGTP, and dTTP, 1× reaction buffer (supplied by GIBCO BRL, Gaithersburg, MD) containing 1 mM MgCl$_2$, and 1 unit of *Taq* DNA polymerase. Reaction mixtures were incubated for 35 cycles, each of which consisted of 1 min at 92°, 1 min at 55°, and 2 min at 72°. After gel purification, PCR products were subcloned into *Eco*RV-digested pBluescript (KS$^+$) (Stratagene, LaJolla, CA). *Bgl*II fragments containing the appropriate genes were isolated from these recombinants and cloned into *Bam*HI-digested pGAD2F and pMA424.

To create the GDD → ADD mutation in the NIb gene (this mutant is termed hereafter as mNIb), an *Eco*RV–*Spe*I fragment obtained from a full-length TVMV cDNA clone (this fragment spans nucleotides 5421 to 9256 of the TVMV genome) was subcloned into pBluescript (KS$^+$) that had been digested with *Eco*RV and *Spe*I. The resulting subclone was introduced into *E. coli* strain TG-1 and single-stranded DNA containing the TVMV sequences prepared using the helper phage M13KO7 as described by Kunkel *et al.* (1987). Mutagenesis was then performed using the "Sculptor" mutagenesis kit from Amersham (Arlington Heights, IL), using the oligonucleotide 5′-CAATTATAAGATCATC GGCATTGGCAAAG-3′ and the protocol recommended by the manufacturer. The authenticity of the mutation was confirmed by DNA sequencing.

The creation of the NIa-6652 mutant and the four muta-tions in the VPg domain of the NIa are described elsewhere (Klein *et al.,* 1994; J. F. Murphy, P. G. Klein, A. G. Hunt, and J. G. Shaw, manuscript in preparation).

To rule out artifacts caused by *Taq* polymerase-in-duced mutations, the NIb and mNIb genes were cloned two or three separate times, respectively, into pMA424; in all cases, the results with different independent plasmids were indistinguishable. Likewise, the NIa and CP genes were cloned two separate times into pGAD2F; as with the NIb clones, all of the independent NIa and CP clones yielded similar results.

Transformation of yeast

The yeast strain GGY1:171 (Gil and Ptashne, 1987) was transformed with plasmid DNA as described by Ito *et al.* (1983). Transformants were identified by plating on minimal media (Chien *et al.,* 1991; Sherman *et al.,* 1986) containing either sucrose (2%) or galactose (2%) + glycerol (2%) + ethanol (2%) as carbon sources. To examine the integrity of plasmids present in the transformed yeast strains, plasmid DNA was isolated from cultures grown under selection, as described by Hoffman and Winston (1987), and analyzed with diagnostic restriction enzymes. To confirm the production of the appropriate fusion protein, cell-free extracts from transformed yeast cells were analyzed by immunoblot analysis after separation by SDS–PAGE (data not shown); procedures for detecting TVMV-encoded proteins by immunoblot analysis have been described elsewhere (Maiti *et al.,* 1993).

β-galactosidase assays

Two techniques were used to evaluate expression of the *gal1-lacZ* gene in GGY1:171. Yeast cells that were able to grow on selective media were streaked onto the same media containing 40 μg/ml 5-bromo-4-chloro-3-in-doyl β-D-galactoside. Transformants that contained high levels of β-galactosidase appeared as blue colonies on these plates, whereas transformants with little or no β-galactosidase were white.

To quantitate β-galactosidase activity in transformed yeast cells, yeast cultures (grown in selective media at 28°) were harvested and β-galactosidase was determined as described by Yocum *et al.* (1984). In this study, the units of activity were calculated using the formula $U = 10^3 \cdot OD_{420}/t \cdot v \cdot OD_{600}$, where OD_{420} is the absorbance at 420 nm measured at the end of the reaction, OD_{600} is the cell density of the culture at the time of harvest, t is the duration of the β-galactosidase assay in min, and v is the volume (in ml) of culture used for the assay (usually 5 ml).

RESULTS

Analysis of interactions between the TVMV NIa, NIb, and coat proteins

We set out to identify possible interactions between the TVMV NIb protein and other TVMV-encoded proteins

using the two-hybrid system (Fields and Song, 1989). To this end, the TVMV genes highlighted in Fig. 1 (the NIa, NIb, and CP genes) were subcloned into pGAD2F and pMA424, respectively. pGAD2F is a yeast expression vector that carries the GAL4 transcription activation domain driven by the *adh1* promoter (Chien *et al.,* 1991). Similarly, pMA424 carries the GAL4 DNA binding domain driven by the same promoter (Ma and Ptashne, 1987a). Each of these plasmids is designed to accept DNA fragments at a *Bam*HI site at the C-termini of the activation and DNA binding domains of these plasmids. The NIb and CP genes were inserted in their entirety into these plasmids, whereas in the case of the NIa gene the C-terminal glutamine was not included. The various pairs of pGAD2F and pMA424 derivatives described in Table 1 were then introduced into the yeast strain GGY1:171 (Gill and Ptashne, 1987) and transformants carrying each plasmid were isolated on minimal media lacking histidine and leucine.

GGY1:171 contains a *gal1* promoter–*lacZ* fusion gene that can be controlled by the GAL4 transcriptional regulator, as well as by GAL4 activation and DNA binding domain fusion protein combinations that are capable of interacting. Thus, the β-galactosidase activity present in transformants can be used as an indicator of interactions between fusion protein partners (Fields and Song, 1989). Representative colonies from each transformation were grown on media containing 5-bromo-4-chloro-3-indoyl β-D-galactoside to obtain a qualitative estimate of β-galactosidase activity in the different transformants. When this was done, cells that carried the NIb coding region in pMA424 and either the CP or the NIa coding regions in pGAD2F were blue (Fig. 2). Cells transformed with one recombinant plasmid and an unaltered partner plasmid (either pGAD2F or pMA424) were invariably white (not shown). Therefore, this qualitative test suggested interactions between the NIb protein and the NIa and coat proteins.

These studies were extended using a more quantitative (and sensitive) assay for β-galactosidase. Several individual colonies from each transformation were grown in liquid media until stationary phase, harvested, and β-galactosidase activity was determined. The results obtained (Table 1) were similar to those seen with the qualitative plate assay (Fig. 2). With one exception (the combination of pMA424-CP and pGAD2F-NIb), doubly transformed cells that carried the NIb coding region in one plasmid and either the CP or NIa coding regions in the appropriate partner contained substantial β-galactosidase activity (between 10 and 20 units of β-galactosidase activity). Doubly transformed yeast cells that contained a single fusion protein along with the corresponding unaltered domain (either DNA binding or activation) contained low levels of β-galactosidase activity. These results indicate that the TVMV NIb protein interacts, in yeast cells, with both the CP and NIa proteins.

In addition to these observations, we found that yeast cells that carried the same gene in both plasmids also had significant β-galactosidase activity (between 8.8 and 14.4 units; Table 1). Thus, the TVMV NIa protein interacts with itself in yeast, and the NIb and coat proteins interact with themselves as well.

In the experiment described in Table 1, the yield of β-galactosidase was much greater in cells that carried the NIb gene fused to the DNA binding domain than in cells with NIb activation domain fusions. A similar disparity is often seen in studies such as this (see, for example, Brown *et al.,* 1994, and Preker *et al.,* 1995) and probably reflects improved folding, localization, or binding when foreign proteins are fused to one GAL4 domain and not the other. In light of this, further experiments were done only with combinations in which the NIb gene was fused to the DNA binding domain.

Effects of a mutation in the NIb polymerase domain

To further characterize the interactions inferred from the results in Fig. 2 and Table 1, a number of mutants were incorporated into this study. One of these was an NIb mutant in which G_{2575} (amino acid position 2575 in the TVMV polyprotein) was changed to A (see Fig. 3A). G_{2575} is part of the GDD motif that is a hallmark of RNA-dependent RNA polymerases (Poch *et al.,* 1989), and this particular mutation abolishes viral infectivity (K. Levay, unpublished results), presumably by eliminating polymerase activity. This mutation dramatically diminished the interactions of the TVMV NIb protein with the CP, as yeast cells carrying the appropriate sets of plasmids contained 18% of the β-galactosidase activity seen with the combination that included the wild-type NIb (Fig. 4, Table 2). However, it did not affect the interaction of NIb with NIa or with itself, as cells with plasmid combinations carrying the mutant NIb and either NIa or wild-type NIb genes contained β-galactosidase activities similar to cells that carried the same combinations, but with the wild-type NIb gene (Fig. 4, Table 2). These results thus indicate that the $G_{2575} \rightarrow A$ alteration did not affect the accumulation or localization of the NIb-containing fusion proteins in yeast or otherwise introduce gross structural alterations in the NIb protein.

### Effects of mutations in the NIa proteinase and VPg domains

To examine the possible roles of the VPg and proteinase domains of the NIa protein in the interactions inferred in Fig. 2 and Table 1, several other mutants were studied (see Fig. 3B for a summary of these). In one mutant (designated NIa-6652), a four-amino-acid insertion at position 2148 was created. This insertion lies in a region of the TVMV NIa proteinase equivalent to the proposed substrate binding domain of the TEV NIa pro-

VPg ... P1 | HC-Pro | P3 | CI | NIa | NIb | CP ... A_n
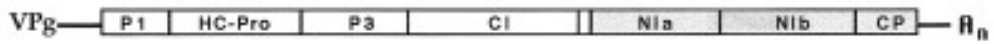
```
      1801
  I  F  G  K  S  R  R  R  L          M  D  D  L  V  R  T  K  D
atctttGGCAAGAGTAGACGCCGACTT   NIa   ATGGACGATTTGGTCCGCACTaaagat
                                                    2223
```

```
      2225
  I  F  G  E  K  R  K  W  M          R  E  T  V  R  F  Q  K  D
atctttGGGGAGAAGCGAAAATGGATG   NIb   CGTGAAACTGTGAGATTTCAAaaagat
                                                    2740
```

```
      2741
  I  F  S  D  T  V  D  A  G          L  L  G  V  K  G  V  K  D
atctttAGTGATACAGTAGATGCTGGG    CP   CTTCTGGGTGTTAAGGGGGTGaaagat
                                                    3005
```

1

2

4
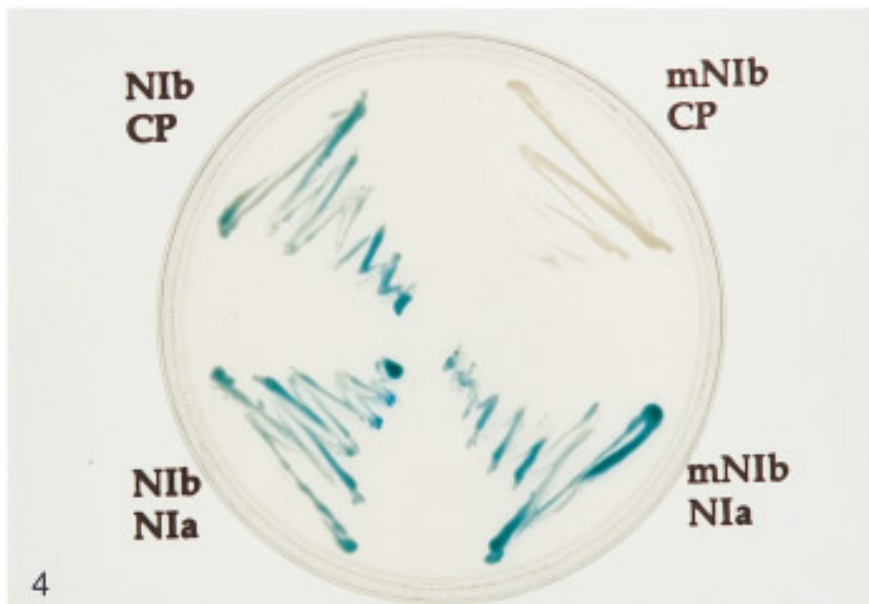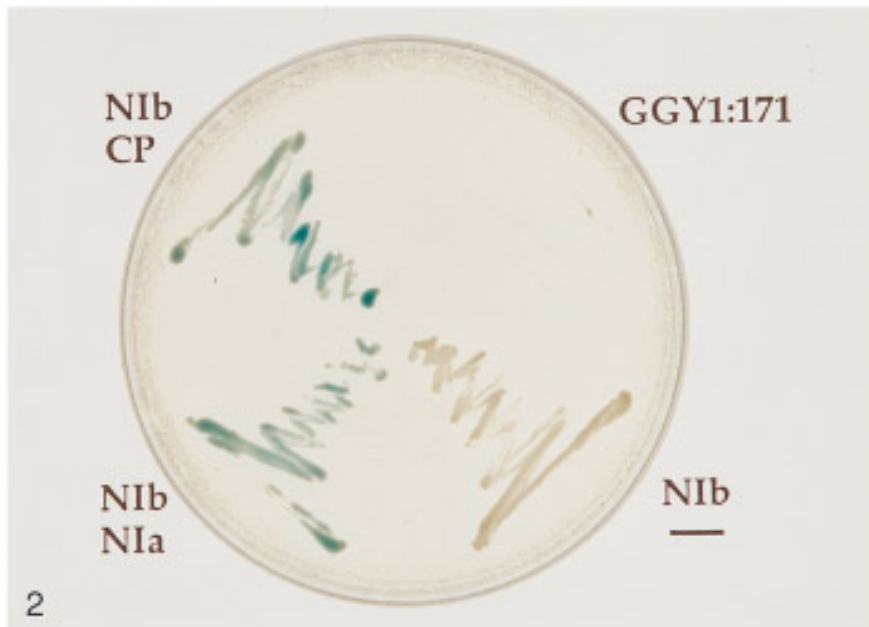
TABLE 1

Interactions between the TVMV NIb Protein
and the NIa and Coat Proteins

TVMV gene in			
DNA binding domain[a]	Activation domain[b]	β-gal activity (units)[c]	Interaction[d]
NIb	None[e]	0.41	−
NIb	NIb	10.2	+++
NIb	NIa	20.9	++++
NIb	CP	11.2	+++
None	NIb	0.40	−
NIa	NIb	6.3	++
CP	NIb	1.50	±
NIa	NIa	8.8	++
CP	CP	14.4	+++
CP	None	0.43	−
None	CP	0.52	−
NIa	None	0.48	−
None	NIa	1.53	±

[a] TVMV gene present in pMA424.

[b] TVMV gene present in pGAD2F.

[c] Units of β-galactosidase activity, determined as described under Methods. These values are the averages of measurements done with at least six independent cultures; variation was less than 10% in these measurements.

[d] Qualitative estimate of the interaction. ++++, >20 units of β-galactosidase activity; +++, 10–19.9 units; ++, 5.0–9.9 units; +, 2.1–5.0 units; ±, 1.51–2.0 units; −, <1.51 units.

[e] No TVMV gene was inserted in cases indicated as "none."

teinase (Dougherty et al., 1990). This mutation abolishes virus infectivity and proteinase activity (Klein et al., 1994). In four other NIa mutants, specific amino acid residues in the vicinity of the tyrosine residue to which the genomic RNA is attached were changed (see Fig. 3C).

The NIa-6652 mutation had little effect on the interaction of NIa with NIb (Table 3), as cells containing plasmid combinations that included this mutation had levels of β-galactosidase activity similar to cells with combinations that included the wild-type NIa gene (Fig. 4, Table 3). In contrast, the four VPg mutants all displayed markedly reduced interactions with the NIb protein (Table 3). In particular, yeast cells that carried three of these mutants along with the wild-type NIb protein contained little or no β-galactosidase activity, whereas cells that carried the fourth (the Y1ΔF) mutant had

about 23% of the activity seen with the NIa–NIb combination. In contrast, cells that carried these mutants along with the wild-type NIa protein contained levels of β-galactosidase activity similar to those seen in cells with the wild-type NIa in both plasmids (Table 3). Thus, these four mutants were not impaired in their interactions with a wild-type NIa protein, indicating that these individual mutations had no effect on the accumulation of the NIa-containing fusion proteins in yeast or on the subcellular localization of such fusion proteins.

DISCUSSION

We have used the two-hybrid system to test the possibility of interactions between the TVMV NIb protein and the CP and NIa proteins, respectively. Using this approach, we have been able to detect such interactions, as well as CP–CP, NIa–NIa, and NIb–NIb interactions (Table 1). Importantly, mutations in conserved domains of the NIa or NIb proteins abolished some of these interactions. In particular, a mutation in the conserved polymerase-related GDD motif of the NIb protein largely abolished the NIb–CP interaction (Table 2), and mutations in the VPg domain of the NIa protein reduced or eliminated the NIa–NIb interaction (Table 3). We conclude from these studies that the TVMV CP interacts with the NIb protein and that a functional polymerase is needed for this interaction. We also conclude that the NIa and NIb proteins interact in a manner that is disrupted by changes in the VPg domain in the NIa protein.

The β-galactosidase levels that we observe in this study are lower than values reported for experiments studying the activity of intact GAL4 derivatives [a deletion mutant that contains both of the domains carried in the plasmids used here yielded β-galactosidase activities of about 450 (Ma and Ptashne, 1987b)]. However, our values fall well within the range noted for interacting partners (generally, between 4 and 220; see, for example, Legrain et al., 1993, and Kämper et al., 1995). The relatively low values we measure may reflect interactions that are not particularly strong. However, this is not a likely explanation, as we see modest β-galactosidase activities in cells that test the CP–CP interaction, an interaction that should be strong, based on the propensity of potyviral CPs to form extended helical aggregates

FIG. 1. Structures of the TVMV genes in pGAD2F and pMA424. The genetic map of TVMV is shown at the top of the illustration, and the NIa, NIb, and CP genes studied here are shaded. The amino acid and nucleotide sequences at the N- and C-termini of each gene, as cloned into the two-hybrid vectors, are shown beneath the genetic map of TVMV. Non-TVMV nucleotides derived from the oligonucleotides used for cloning are shown in lowercase, and the TVMV amino acid coordinates of the N- and C-termini of each gene are shown above the appropriate amino acid.

FIG. 2. The TVMV NIb protein interacts with the NIa and coat proteins in yeast cells. Yeast cells that carry pMA424:NIb + pGAD2F (NIb/−), pMA424:NIb + pGAD2F:NIa (NIb/NIa), or pMA424:NIb + pGAD2F:CP (NIb/CP) were streaked onto selective media containing 5-bromo-4-chloro-3-indoyl β-D-galactoside and grown for 36 hr at 30°. Untransformed GGY1:171 cells were tested as well.

FIG. 4. The GDD → ADD mutation in the NIb abolishes the NIb–CP interaction. Yeast cells that carry pMA424:NIb + pGAD2F:CP (NIb/CP), pMA424:NIb + pGAD2F:NIa (NIb/NIa), pMA424:mNIb + pGAD2F:CP (mNIb/CP), and pMA424:mNIb + pGAD2F:NIa (mNIb/NIa) were streaked onto selective media containing 5-bromo-4-chloro-3-indoyl β-D-galactoside and grown for 36 hr at 30°.

A

```
                 2573        2579
    NIb      A N G D D L I

    mNIb     A N a D D L I
```

B

```
            2132                                    2151
    NIa       G Q C G S P L V S I I D G N I L G I H S

    NIa-6652  G Q C G S P L V S I I D G N I L G i p r g I H S
```

C

```
                      1857                    1870
    NIa           V N M Y G V S P D E Y S Y V
    NIa Y1860 -> F V N M f G V S P D E Y S Y V
    NIa Y1860 -> S V N M s G V S P D E Y S Y V
    NIa YG -> GY   V N M g y V S P D E Y S Y V
    NIa Y1867 -> F V N M Y G V S P D E f S Y V
```
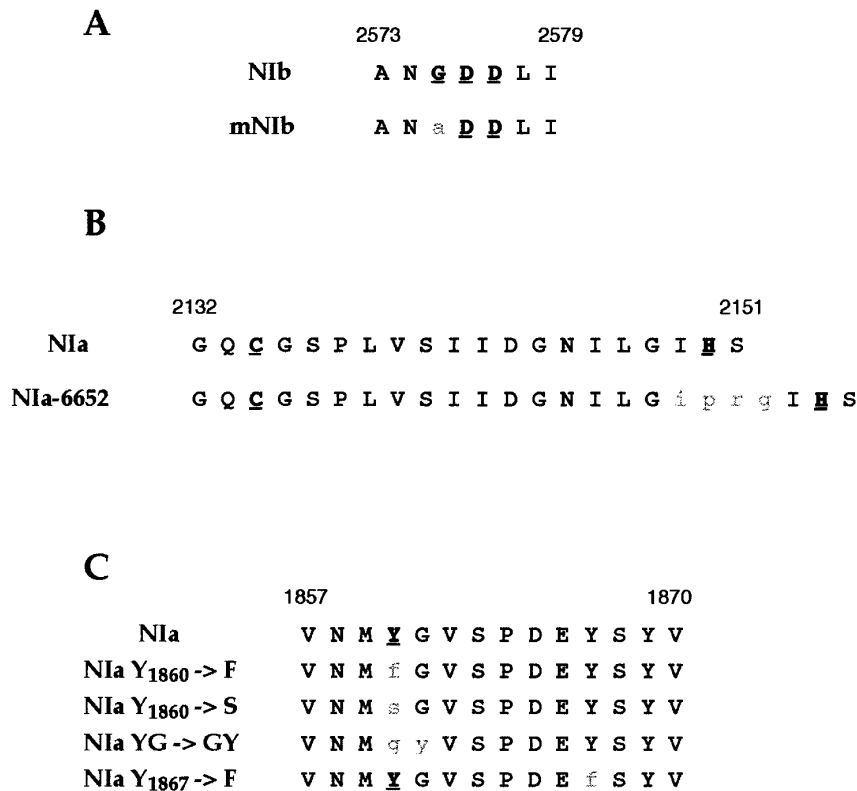
FIG. 3. Structures of NIb and NIa mutants. (A) Amino acids 2573–2579 (using the TVMV polyprotein coordinates defined by Domier *et al.,* 1986) of the wild-type (NIb) and mutant (mNIb) NIbs. Constituent amino acids of highly conserved GDD motif are underlined and in boldface type. The changed amino acid residue in the mNIb mutant is in lowercase gray type. (B) Amino acids 2132–2151 of the wild-type NIa and the NIa-6652 mutant. The cysteine residue (C_{2134}) that is part of the putative catalytic triad and the conserved histidine (H_{2150}) that is presumably part of the substrate binding site (Dougherty *et al.,* 1990) are underlined and in boldface type, and the four inserted amino acids in the NIa-6652 mutant are represented as lowercase letters in gray type. (C) Amino acids 1857–1870 of the wild-type NIa and four VPg domain mutants. The designations of each mutant are shown to the left. The tyrosine to which the TVMV genomic RNA is covalently attached (Y_{1860}; Murphy *et al.,* 1991) is underlined and in boldface type. The changed amino acid residues in the various mutants are in lowercase gray type.

(McDonald *et al.,* 1976; Jagadish *et al.,* 1991; B. Amsden and J. G. Shaw, unpublished observations). There are several other possible explanations for the modest values of β-galactosidase activity we observe, such as decreased stability or inefficient localization of the fusion proteins into the nucleus. In any case, the fact that the β-galactosidase levels we observe fall within a range taken as indicative of an interaction in other studies, along with the observation that a test of the CP–CP interaction yields a value comparable to those measured with tests of other interactions, indicates that the β-galactosidase activities we observe are indicative of interactions in yeast cells. Moreover, the various controls and the effects of the different mutations that we have produced indicate that the observed interactions are not artifactual; they rule out a proclivity of the NIa, NIb, and coat proteins to bind nonspecifically to the GAL4 domains encoded by the two-hybrid plasmids.

Several of the interactions that we observe reflect known properties of potyvirus coat, NIa, and NIb proteins. As mentioned in the preceding paragraph, potyvirus CP subunits

very likely interact with other CP subunits during virus assembly, and the CP–CP interaction in yeast cells is probably indicative of this property. NIa and NIb proteins accumulate in nuclear inclusions in cells infected with some potyviruses (Dougherty and Carrington, 1988), and the NIa–NIa and NIb–NIb interactions we observe may reflect this. However, nuclear inclusions have not been detected in TVMV-infected cells, and it is possible that the observed interactions are indicative of a role for homodimers or higher order structures in the functioning of these proteins.

The NIa–NIb interaction we observed may also be indicative of a tendency of these proteins to accumulate in nuclear inclusions. However, the observation that mutations near the RNA attachment site in the NIa diminish the NIa–NIb interaction suggests an alternative explanation. As is the case with picornaviruses (Takeda *et al.,* 1986; Tobin *et al.,* 1989; Lama *et al.,* 1994), potyvirus VPgs are probably involved in the early stages of (−) and (+) strand RNA synthesis and thus should interact with the polymerase at some stage during RNA replication. We see such an interaction in yeast cells, one that is

disrupted by mutations in the VPg domain of the NIa protein. Our results thus suggest that the TVMV-encoded RNA-dependent RNA polymerase directly contacts the VPg in the vicinity of the RNA attachment site.

We have not identified NIb mutants that are likewise impaired in the NIa–NIb interaction. However, the observation that the GDD → ADD mutation in NIb did not affect this interaction suggests (but does not prove) that the domain that includes this conserved motif is not in direct contact with the NIa protein. More extensive mutational analyses of the NIb protein will be needed to identify the domain in this protein that interacts with the NIa protein.

The interaction between the TVMV CP and NIb proteins is intriguing, as the known or putative biochemical and biological properties of these proteins do not suggest the likelihood of such an interaction. Equally interesting is the effect of a mutation in the conserved GDD motif on this interaction. The diminishing effect of this mutation suggests that the CP interacts with the active site of the NIb protein or with other regions of the NIb that may be affected by changes in the GDD motif. This result raises several interesting possibilities regarding possible functions of the CP beyond its involvement in virus assembly (Jagadish *et al.,* 1991), movement of virus within the plant (Dolja *et al.,* 1994), and aphid transmission (Atreya *et al.,* 1990, 1991). The TVMV CP may regulate the relative levels of (+)- and (−)-strand TVMV RNAs in infected cells, as has been proposed for the alfalfa mosaic virus CP (Van der Kuyl *et al.,* 1991). Alternatively, the TVMV CP may be a part of the replication apparatus, perhaps serving to facilitate the movement of RNAs to or from the active site. These and other possibilities await

TABLE 2

Effect of the $G_{2575} \to$ A Mutation on the Interactions of the TVMV NIb Protein with the NIa and Coat Proteins

TVMV gene in			
DNA binding domain[a]	Activation domain[b]	β-gal activity (units)[c]	Interaction[d]
NIb	None[e]	0.4	−
NIb	NIb	10	+++
mNIb[f]	NIb	9.5	+++
NIb	NIa	21	++++
mNIb	NIa	19	++++
NIb	CP	11	+++
mNIb	CP	2.0	±

[a] TVMV gene present in pMA424.
[b] TVMV gene present in pGAD2F.
[c] Units of β-galactosidase activity. See the legend to Table 1 for details.
[d] Qualitative estimate of the interaction. See the legend to Table 1 for details.
[e] No TVMV gene was inserted.
[f] The $G_{2575} \to$ A mutant is designated mNIb.

TABLE 3

Effects of Proteinase and VPg Domain Mutations on the Interaction of the TVMV NIb Protein with the NIa Protein

TVMV gene in			
DNA binding domain[a]	Activation domain[b]	β-gal activity (units)[c]	Interaction[d]
NIb	NIa	20	++++
NIb	NIa-6652	18	++++
NIb	$Y_{1860} \to$ F	4.5	+
NIb	YG → GY	1.1	−
NIb	$Y_{1867} \to$ F	1.5	±
NIb	$Y_{1860} \to$ S	1.3	−
NIa	NIa	8.8	++
NIa-6652	NIa-6652	8.0	++
NIa	$Y_{1860} \to$ F	4.0	+
NIa	YG → GY	5.5	++
NIa	$Y_{1867} \to$ F	8.4	++
NIa	$Y_{1860} \to$ S	6.4	++

[a] TVMV gene present in pMA424.
[b] TVMV gene present in pGAD2F; see the text for designation of NIa mutants.
[c] Units of β-galactosidase activity. See the legend to Table 1 for details.
[d] Qualitative estimate of the interaction. See the legend to Table 1 for details.

further experimental study, including further analysis of this interaction *in vivo* and *in vitro.*

ACKNOWLEDGMENTS

REFERENCES

Atreya, C. D., Raccah, B., and Pirone, T. P. (1990). A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virology* **178**, 161–165.

Atreya, C. D., and Pirone, T. P. (1993). Mutational analysis of the helper component–proteinase gene of a potyvirus: Effects of amino acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility. *Proc. Natl. Acad. Sci. USA* **90**, 11919–11923.

Atreya, P. L., Atreya, C. D., and Pirone, T. P. (1991). Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proc. Natl. Acad. Sci. USA* **88**, 7887–7891.

Brantley, J. D., and Hunt, A. G. (1993). The N-terminal protein of the polyprotein encoded by the potyvirus tobacco vein mottling virus is an RNA binding protein. *J. Gen. Virol.* **74**, 1157–1162.

Brown, N. G., Costanzo, M. C., and Fox, T. D. (1994). Interactions among three proteins that specifically activate translation of the mitochondrial COX3 mRNA in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **14**, 1045–1053.

Carrington, J. C., Cary, S. M., Parks, T. D., and Dougherty, W. G. (1989). A second proteinase encoded by a plant potyvirus genome. *EMBO J.* **8**, 365–370.

Chien, C.-T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The two-

hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* **88**, 9578–9582.

Dolja, V. V., Haldeman, R., Robertson, N. L., Dougherty, W. G., and Carrington, J. C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus. *EMBO J.* **13**, 1482–1491.

Dolja, V. V., Herndon, K. L., Pirone, T. P., and Carrington, J. C. (1993). Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *J. Virol.* **67**, 5968–5975.

Domier, L. L., Franklin, K. M., Hunt, A. G., Rhoads, R. E., and Shaw, J. G. (1989). Infectious in vitro transcripts from cloned cDNA of a potyvirus, tobacco vein mottling virus. *Proc. Natl. Acad. Sci. USA* **86**, 3509–3513.

Domier, L. L., Franklin, K. M., Shahabuddin, S., Hellmann, G. M., Overmeyer, J. H., Hiremath, S. T., Siaw, M. F. E., Lomonossoff, G. P., Shaw, J. G., and Rhoads, R. E. (1986). The nucleotide sequence of tobacco vein mottling virus RNA. *Nucleic Acids Res.* **14**, 5417–5430.

Domier, L. L., Shaw, J. G., and Rhoads, R. E. (1987). Potyviral proteins share amino acid sequence homology with picorna-, como-, and caulimoviral proteins. *Virology* **158**, 20–27.

Dougherty, W. G., and Carrington, J. C. (1988). Expression and function of potyviral gene products. *Annu. Rev. Phytopathol.* **26**, 123–143.

Dougherty, W. G., and Hiebert, E. (1980). Translation of potyvirus RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation, and cylindrical inclusion protein as a product of the potyvirus genome. *Virology* **103**, 174–182.

Dougherty, W. G., Parks, T. D., Smith, H. A., and Lindbo, J. A. (1990). Expression of the potyvirus genome: The role of proteolytic processing. *In* "Viral Genes and Plant Pathogenesis" (T. P. Pirone and J. G. Shaw, Eds.), pp. 124–139. Springer-Verlag, New York/Berlin.

Eagles, R. M., Balmori-Melian, E., Beck, D., Gardner, R. C., and Forster, R. L. S. (1994). Characterization of NTPase, RNA-binding and RNA-helicase activities of the cytoplasmic inclusion protein of tamarillo mosaic potyvirus. *Eur. J. Biochem.* **224**, 677–684.

Fields, S., and Song, O.-k. (1989). A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245–246.

Gil, G., and Ptashne, M. (1987). Mutants of GAL4 protein altered in an activation function. *Cell* **51**, 121–126.

Hellmann, G. M., Shaw, J. G., and Rhoads, R. E. (1985). On the origin of the helper component of tobacco vein mottling virus: Translational initiation near the 5′ terminus of the viral RNA and termination by UAG codons. *Virology* **143**, 23–34.

Hoffman, C. S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli. Gene* **57**, 267–272.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163–168.

Jagadish, M. N., Ward, C. W., Gough, K. H., Tulloch, P. A., Whittaker, L. A., and Shukla, D. D. (1991). Expression of potyvirus coat protein in E. coli and yeast and its assembly into virus-like particles. *J. Gen. Virol.* **72**, 1543–1550.

Kamper, J., Reichmann, M., Romeis, T., Bolker, M., and Kahmann, R. (1995). Multiallelic recognition: Nonself-dependent dimerization of the bE and bW homeodomain proteins in Ustilago maydis. *Cell* **81**, 73–83.

Klein, P. G., Klein, R. R., Cerezo-Rodriguez, E., Hunt, A. G., and Shaw, J. G. (1994). Mutational analysis of the tobacco vein mottling potyvirus genome. *Virology* **204**, 759–769.

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.

Lain, S., Riechmann, J. L., and Garcia, J. A. (1990). RNA helicase: A novel activity associated with a protein encoded by a positive strand RNA virus. *Nucleic Acids Res.* **18**, 7003–7006.

Lama, J., Paul, A. V., Harris, K. S., and Wimmer, E. (1994). Properties of purified recombinant protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase 3D^{pol}. *J. Biol. Chem.* **269**, 66–70.

Legrain, P., Chapon, C., and Galisson, F. (1993). Interactions between PRP9 and SPP91 splicing factors identify a protein complex required in prespliceosome assembly. *Genes Dev.* **7**, 1390–1399.

Ma, J., and Ptashne, M. (1987a). A new class of yeast transcriptional activators. *Cell* **51**, 113–119.

Ma, J., and Ptashne, M. (1987b). The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. *Cell* **51**, 137–142.

Maiti, I. B., Murphy, J. F., Shaw, J. G., and Hunt, A. G. (1993). Plants that express a potyvirus proteinase gene are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* **90**, 6110–6114.

McDonald, J. G., Beveridge, T. J., and Bancroft, J. B. (1976). Self-assembly of protein from a flexuous virus. *Virology* **69**, 327–331.

Murphy, J. F., Rhoads, R. E., Hunt, A. G., and Shaw, J. G. (1990). The VPg of tobacco etch virus RNA is the 49-kDa proteinase or the N-terminal 24-kDa part of the proteinase. *Virology* **178**, 285–288.

Murphy, J. F., Rychlik, W., Rhoads, R. E., Hunt, A. G., and Shaw, J. G. (1991). A tyrosine residue located within the small nuclear inclusion protein links TVMV VPg to its RNA. *J. Virol.* **65**, 511–513.

Pirone, T. P., and Thornbury, D. W. (1984). The involvement of a helper component in non-persistent transmission of plant viruses by aphids. *Microbiol. Sci.* **1**, 191–193.

Poch, O., Sauvaget, I., Delarue, M., and Trodo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* **8**, 3867–3874.

Preker, P. J., Lingner, J., Minvielle-Sebastia, L., and Keller, W. (1995). The FIP1 gene encodes a component of a yeast pre-mRNA polyadenylation factor that directly interacts with poly(A) polymerase. *Cell* **81**, 379–389.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1992). Highlights and prospects of potyvirus molecular biology. *J. Gen. Virol.* **73**, 1–16.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Shahabuddin, M., Shaw, J. G., and Rhoads, R. E. (1988). Mapping of the tobacco vein mottling virus VPg cistron. *Virology* **163**, 635–637.

Sherman, F., Fink, G. R., and Hicks, J. B. (1986). "Methods in Yeast Genetics." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Soumounou, Y., and Laliberté, J.-F. (1994). Nucleic acid-binding properties of the P1 protein of turnip mosaic potyvirus produced in Escherichia coli. *J. Gen. Virol.* **75**, 2567–2573.

Takeda, N., Kuhn, R. J., Yang, C.-F., Takegami, T., and Wimmer, E. (1986). Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected Hela cells. *J. Virol.* **60**, 43–53.

Tobin, G. J., Young, D. C., and Flanegan, J. B. (1989). Self-catalyzed linkage of poliovirus terminal protein VPg to poliovirus RNA. *Cell* **59**, 511–519.

Van der Kuyl, A. C., Neeleman, L., and Bol, J. F. (1991). Role of alfalfa mosaic virus coat protein in regulation of the balance between viral plus and minus strand RNA synthesis. *Virology* **185**, 496–499.

Verchot, J., and Carrington, J. C. (1995). Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. *J. Virol.* **69**, 3668–3674.

Verchot, J., Koonin, E. V., and Carrington, J. C. (1991). The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase. *Virology* **185**, 527–535.

Yocum, R., Hanley, S., West, R., Jr., and Ptashne, M. (1984). Use of lacZ fusions to delimit regulatory elements of inducible divergent gal1-gal10 promoter in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **4**, 1985–1998.