

Strain-Specific Interaction of the Tobacco Etch Virus NIa Protein with the Translation Initiation Factor eIF4E in the Yeast Two-Hybrid System

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Received September 28, 1999; returned to author for revision October 27, 1999; accepted April 28, 2000

The NIa protein of potyviruses provides VPg and proteolytic functions during virus replication. It has also been shown to confer host genotype-specific movement functions in plants. Specifically, NIa from tobacco etch virus (TEV)-Oxnard, but not from most other strains, confers the ability to move long distances in *Nicotiana tabacum* cultivar "V-20." This led to the hypothesis that all or part of NIa may interact with one or more cellular factors. To identify cellular proteins that interact with NIa in a host- or strain-specific manner, a yeast two-hybrid search of a tomato cDNA library was done. Ten proteins that interacted with NIa were recovered, with translation initiation factor eIF4E being by far the most common protein identified. Interaction of eIF4E with NIa was shown to be TEV strain-specific. eIF4E from both tomato and tobacco interacted well with NIa from the HAT strain, but not from the Oxnard strain. However, using chimeric NIa proteins, the determinant for systemic infection of V20 plants was found to be genetically distinct from the determinant controlling eIF4E interaction. In TEV-eIF4E coexpression experiments, evidence suggesting that eIF4E provides a positive effect on genome amplification was obtained.

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INTRODUCTION

The multifunctional NIa protein of potyviruses is required at several points in the virus infection cycle. The N-terminal half of NIa serves as the VPg during initiation of RNA synthesis (Murphy *et al.*, 1990a,b; Shahabuddin *et al.*, 1988). The C-terminal half is a picornavirus 3C-like proteinase that catalyzes most of the cleavage reactions of the viral polyprotein (Dougherty and Semler, 1993). The two domains are separated by a suboptimal NIa cleavage site that in the case of tobacco etch virus (TEV) NIa, is processed slowly *in vivo* (Carrington *et al.*, 1993). The VPg domain also contains signals for nuclear transport in infected cells (Carrington *et al.*, 1991; Restrepo *et al.*, 1990). In addition, NIa interacts with NIb, the virus-encoded RNA polymerase (Daròs *et al.*, 1999; Fellers *et al.*, 1998; Hong *et al.*, 1995; Li *et al.*, 1997). A model for how TEV NIa integrates several replicative functions has been proposed (Daròs *et al.*, 1999; Schaad *et al.*, 1997a).

The NIa protein of several potyviruses has also been shown to confer host genotype-specific cell-to-cell or long-distance movement functions in plants (Keller *et al.*, 1998; Nicolas *et al.*, 1997; Schaad *et al.*, 1997b). Strains of TEV (for example, TEV-HAT), with the exception of the Oxnard strain, are defective in their ability to systemically infect *Nicotiana tabacum* cv "V20" (Christie *et al.*, 1974). Restricted TEV strains are able to replicate in V20 cells and move cell to cell in inoculated leaves, but are unable

to move long distances via the phloem (Schaad and Carrington, 1996). Immunocytochemical analysis revealed that both TEV-HAT and TEV-Oxnard could reach phloem companion cells in inoculated V20 leaves (Schaad and Carrington, 1996). The restriction in V20 plants is conditioned by at least two recessive loci (Schaad and Carrington, 1996). The viral determinant in TEV-Oxnard that confers the ability to move long distances in V20 maps to a 67-nucleotide segment of the VPg domain within the NIa coding sequence (Schaad *et al.*, 1997b). Two possibilities for the role of TEV NIa in host genotype-specific long-distance movement have been presented (Schaad *et al.*, 1997b). The NIa protein, possibly as a free protein or linked to RNA as VPg, may interact with cellular factors to facilitate passage through the phloem transport pathway. Alternatively, NIa or free VPg domain may function to suppress one or more defense responses that specifically limit long-distance movement. In either case, NIa or VPg domain is proposed to interact with cellular factors to directly or indirectly promote long-distance movement.

RESULTS

Identification of host proteins that interact with NIa

A yeast two-hybrid search for NIa-interacting host proteins was done with the LexA interaction trap system (Gyuris *et al.*, 1993). The system uses the *LexAop-lacZ* reporter plasmid and the integrated *LexAop-LEU2* gene in yeast strain EGY48 as scorable and selectable markers for interaction. Interactors exhibited galactose-de-

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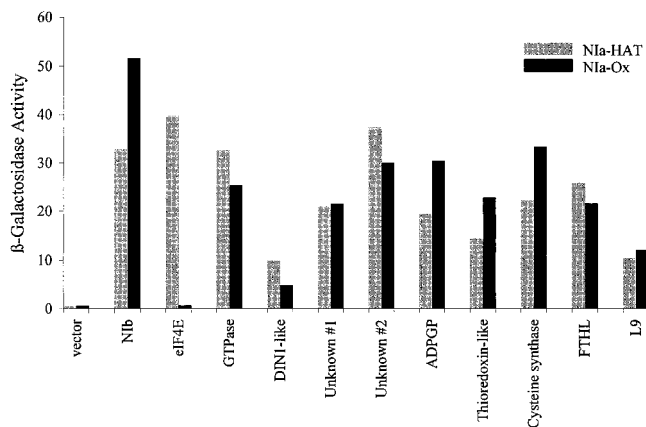


FIG. 1. β -Galactosidase assay for yeast two-hybrid interactions with N1a. Interaction in yeast was tested between N1a^{HAT} or N1a^{Ox} and TEV N1b, each of 10 tomato proteins identified in the two-hybrid search, or an empty vector. These are representative data from one of three experiments. ADPGP, ADP glucose pyrophosphorylase; FTHL, formate tetrahydrofolate ligase; L9, 60S ribosomal L9 precursor.

pendent growth on Leu⁻ medium and β -galactosidase activity. The TEV-HAT N1a (N1a^{HAT}) "bait" protein was constitutively expressed as a LexA DNA-binding domain fusion protein in plasmid pEG202 (Gyuris *et al.*, 1993). A cDNA library (a gift from Barbara Baker, University of California at Berkeley) prepared from total RNA from TMV-infected *Lycopersicon esculentum* cv VF36 leaves was constructed in the activation domain fusion plasmid, pJG4-5 (Gyuris *et al.*, 1993). The VF36 tomato cultivar is fully susceptible to all strains of TEV used in this study. The cDNA-encoded fusion proteins were conditionally expressed in yeast grown on galactose.

To verify that the N1a^{HAT} fusion protein was functional for protein-protein interaction, yeast cells containing pEG202-N1a^{HAT} were transformed with pJG4-5-N1b, which encodes a TEV-HAT N1b fusion protein, and interaction was tested by selective growth and β -galactosidase activity assays. Using a variety of other two-hybrid expression systems, N1a and N1b were shown to interact efficiently (Daròs *et al.*, 1999; Fellers *et al.*, 1998; Hong *et al.*,

1995; Li *et al.*, 1997). Interaction was detected in yeast expressing pEG202-N1a^{HAT} and pJG4-5-N1b using both assays (Fig. 1; and data not shown).

Approximately 30,000,000 yeast transformants containing pEG202-N1a^{HAT} and library plasmids were screened using a two-step procedure, which included a Leu⁻ growth selection step (Finley and Brent, 1996). Seven hundred thirty-eight potential interactors were identified after the initial selection. These candidate interactors were retested by selective growth and β -galactosidase activity assays, yielding 340 potential interactors. Each of the library plasmids from these colonies was recovered and subjected to specificity testing after retransformation into the N1a^{HAT} bait strain and strains containing *Drosophila* Bicoid^{HAT} or tomato Pto bait constructs. Fifty-nine of the 340 clones tested interacted specifically with N1a^{HAT} and not with control baits. Twenty-six clones resulted in β -galactosidase activity in excess of 10-fold (the arbitrary cut-off for advancement in the screen) over that induced by the empty pJG4-5 vector.

Among the 26 N1a-interactors that passed through the two-hybrid screen, 11 were identified as the translation initiation factor eIF4E based on nucleotide sequence analysis and BLAST searches (Table 1 and Fig. 1). The tomato eIF4E (GenBank Accession No. AF259801) exhibited 76 and 53% amino acid identity with the eIF4E (p26) and the eIF4E isoforms (p28) from rice, respectively. eIF4E provides the 5' cap-binding function during formation of initiation complexes on most eukaryotic mRNAs (Shatkin, 1976). It has also been shown to be involved in control of cell growth and development, as overexpression of murine eIF4E in mouse NIH3T3 or Rat-2 cell lines results in oncogenic transformation (Lazaris-Karatzas *et al.*, 1990). Also, eIF4E can cooperate with nuclear oncogene proteins like c-myc or E1A to transform primary cells (Lazaris-Karatzas and Sonenberg, 1992). It has been reported that a fraction of eIF4E localizes to the nucleus in mammalian cells, suggesting that eIF4E may be involved in nuclear functions such as transcription, splicing, and nucleocytoplasmic transport (Lejbkiewicz *et*

TABLE 1

Summary of N1a-Interacting Proteins Recovered from the Two-Hybrid Screen

Probable protein or function	Most similar protein (Accession No.)	P value	Isolations
eIF4E	Rice eIF4E (1352440)	5.8×10^{-101}	11
GTPase	<i>Schizosaccharomyces pombe</i> GTPase (1935000)	4.6×10^{-51}	2
DIN1-like	Radish DIN1 (2190012)	3.5×10^{-31}	2
Unknown 1	<i>Arabidopsis</i> uridylyl transferase-like protein (2829923)	1.1×10^{-19}	2
Unknown 2	<i>Arabidopsis</i> hypothetical protein (2464895)	2.7×10^{-42}	1
ADP-glucose pyrophosphorylase	Tomato ADP-glucose pyrophosphorylase (1840116)	4.4×10^{-66}	2
Thioredoxin-like	<i>Porphyra yezoensis</i> chloroplast thioredoxin (1729949)	6.0×10^{-9}	1
Cysteine synthase	Potato cysteine synthase (3290022)	2.9×10^{-80}	2
Formate tetrahydrofolate ligase	Spinach formate tetrahydrofolate ligase (2507455)	1.3×10^{-70}	2
Ribosomal protein L9	Yeast mitochondrial 60S ribosomal L9 precursor (1710598)	1.7×10^{-32}	1

al., 1992). Importantly, the VPg domain of turnip mosaic potyvirus NIa was shown to interact with the *Arabidopsis* eIF4E isoform in yeast two-hybrid and *in vitro* assays (Wittmann *et al.*, 1997).

Each of six proteins, including those with a high level of similarity to GTPases, the DIN1 protein from radish, an unknown protein with uridylyl transferase-like motifs, ADP-glucose pyrophosphorylase, and formate tetrahydrofolate ligase, was identified on two occasions (Table 1 and Fig. 1). The DIN1 protein is strongly induced by dark or ethylene treatment of radish cotyledons, although the biochemical function of DIN1 is not clear (Azumi and Watanabe, 1991). Proteins with clear similarities to an *Arabidopsis* hypothetical protein of unknown function, chloroplast thioredoxin, and a ribosomal L9 precursor protein were identified on one occasion each (Table 1 and Fig. 1).

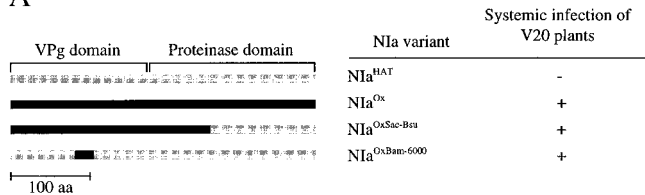
Strain-specific interaction between NIa and eIF4E

To determine whether any of the NIa-interacting proteins exhibit TEV strain-specificity, the 10 host proteins were tested in the two-hybrid assay using NIa^{HAT} and NIa from TEV-Oxnard (NIa^{Ox}). In all experiments, one of which is presented in Fig. 1, both NIa^{HAT} and NIa^{Ox} interacted well with N1b from the HAT strain. Among the host proteins, only eIF4E was shown to interact differentially with NIa^{HAT} and NIa^{Ox}. In the β -galactosidase assay, eIF4E interacted only with NIa^{HAT}. No activity above background was detected in strains carrying eIF4E and NIa^{Ox}. Because the objective of this study was to identify host proteins with strain-specific interaction characteristics with NIa, only eIF4E was investigated further.

It was possible that the differential interaction of eIF4E with the two NIa proteins was an artifact of using eIF4E from tomato. To determine whether eIF4E from tobacco interacted differentially with NIa^{HAT} and NIa^{Ox}, cDNA clones were isolated from three *N. tabacum* cultivars and tested for interaction with the two NIa proteins. The cDNA for eIF4E homologues from three tobacco cultivars (Xanthi nc, Havana 425 and V20) was PCR-amplified with tobacco-specific primers (unpublished sequence kindly provided by Jonathan Combe and David Twell, University of Leicester, UK) and cloned into the yeast library vector, pJG4-5. Nucleotide sequence analysis revealed that the eIF4E sequence from each of the cultivars was identical (data not shown). Both Xanthi nc and Havana425 are fully susceptible to both TEV-HAT and TEV-Oxnard, whereas V20 is fully susceptible only to TEV-Oxnard. Interaction with NIa^{HAT} was detected in yeast carrying the eIF4E clones from each tobacco cultivar, although the level of β -galactosidase induced upon interaction was generally less than that induced with tomato eIF4E (Fig. 2). No interaction above background levels was detected between NIa^{Ox} and any of the three tobacco eIF4E proteins.

The ability of TEV-Oxnard to move long distances in

A



B

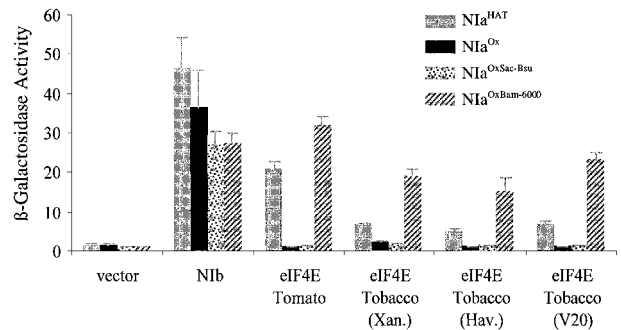


FIG. 2. β -Galactosidase assay for yeast two-hybrid interactions between NIa and eIF4E. (A) Diagrammatic representation of NIa^{HAT}, NIa^{Ox}, NIa^{OxSac-Bsu}, and NIa^{OxBam-6000}. The ability of each NIa protein to confer systemic infection capability to TEV in V20 tobacco plants is shown based on previous data (Schaad *et al.*, 1997b). (B) Interaction was tested between each of the four NIa variants and eIF4E isolated from tomato and three cultivars of tobacco. Data represent the mean \pm SD ($n = 3$) from a representative experiment. Xan., Xanthi nc; Hav., Havana 425.

the V20 cultivar was mapped previously to a 67-nucleotide segment spanning codons 78–99 within the VPg domain of the NIa coding sequence (Schaad *et al.*, 1997b). There are five substitutions resulting in amino acid changes between TEV-HAT and TEV-Oxnard in this region. To determine whether or not this same region of the VPg domain determines the strain-specific interaction of eIF4E with NIa, interaction of each tobacco eIF4E was tested with NIa^{HAT}, NIa^{Ox}, and two chimeric NIa proteins (Fig. 2A). The NIa^{OxSac-Bsu} protein contains TEV-Oxnard amino acid sequences between positions 1 and 277, which includes the entire VPg domain, and TEV-HAT sequence between positions 278 and 430. NIa^{OxBam-6000} is predominantly derived from TEV-HAT, except for 27 amino acid residues in the VPg domain between positions 78 and 105. Viruses containing either NIa^{OxSac-Bsu} or NIa^{OxBam-6000} are able to move long distances in V20; NIa^{OxBam-6000} contains the minimal TEV-Oxnard sequence that was identified as necessary for V20 infection (Schaad *et al.*, 1997b). Yeast two-hybrid interaction analysis revealed that each NIa variant interacted well with N1b from TEV-HAT (Fig. 2), indicating that each protein was active for protein–protein interaction. However, only NIa^{HAT} and NIa^{OxBam-6000} interacted with the eIF4E proteins. In fact, NIa^{OxBam-6000} interacted with eIF4E from tomato and tobacco better than did NIa^{HAT}. Little or no interaction above background levels was detected using NIa^{Ox} or NIa^{OxSac-Bsu} and eIF4E from either tomato or tobacco.

Thus, the regions within NIa that confer the ability to move long distances in V20 and to interact efficiently with eIF4E are genetically distinct. Presumably, efficient interaction with eIF4E requires TEV-HAT sequences encoding NIa residues 1–77 and/or 99–277.

Coexpression of eIF4E in the TEV genome

Interaction of eIF4E with NIa could serve to either directly or indirectly promote infection by TEV. This could conceivably occur by a number of mechanisms. For example, the interaction could suppress eIF4E function and lead to down-regulation of translation of capped (cellular) mRNAs. This would lead to both increased availability of the translational apparatus for expression of the TEV genome and suppression of defense responses that require translation. The interaction might also be involved directly in TEV genome replication or may play a role in translational efficiency, although TEV RNA appears to be translated efficiently by m7GpppG cap-independent mechanisms *in vivo* and *in vitro* (Carrington and Freed, 1990). Alternatively, interaction of eIF4E with NIa could lead to defensive processes that limit infection by TEV.

In initial experiments to shed light on these possibilities, tobacco eIF4E forms were expressed from within the TEV genome (Fig. 3A). Coexpression would result in stoichiometric synthesis of ectopic eIF4E and TEV-encoded proteins in infected cells. An enhancement of RNA amplification was predicted if the interaction of NIa with eIF4E provides a direct stimulatory function. In contrast, a decrease in amplification was predicted if the interaction promotes a TEV-limiting host defense response or if free eIF4E suppresses (either directly or indirectly) TEV replication. A potential complication in interpretation of results arises, however, because the additional sequences added to the TEV genome might have inherent debilitating effects on replication. To overcome this complication, three types of mutations affecting different functions of eIF4E were introduced into the tobacco eIF4E sequence. The Trp-115 to Leu (W115L) equivalent mutation was shown to abolish cap binding in mouse eIF4E (Marcotrigiano *et al.*, 1997; Morino *et al.*, 1996; Ueda *et al.*, 1991). The G124D mutation was shown to inhibit the interaction of eIF4E with another translation initiation factor, eIF4G (Altmann and Trachsel, 1989; Tarun and Sachs, 1997). A cluster of substitutions (V82A, E83A, and W86A, termed cluster 1 mutations) was shown to affect surface-accessible residues identified as part of the 4E-BP (a translation initiation complex repressor protein)-binding site in yeast eIF4E (Matsuo *et al.*, 1997). If one or more of these mutations resulted in debilitation of RNA amplification in comparison to the nonmutant eIF4E-containing virus, this would suggest that the wild-type eIF4E was providing a positive effect on replication. If, on the other hand, the eIF4E mutations enhanced

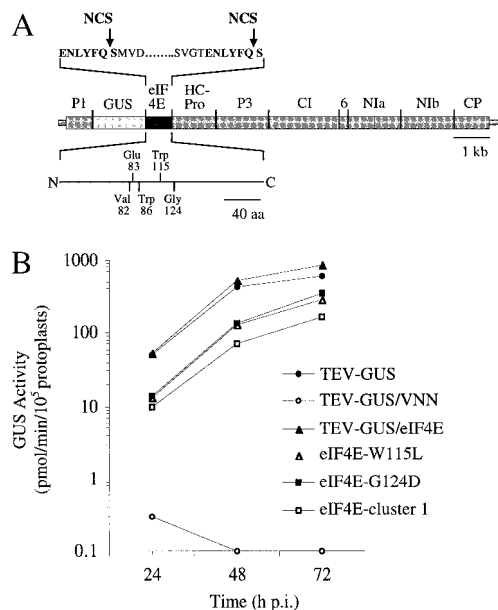


FIG. 3. (A) Diagrammatic representation of the TEV-GUS genome and recombinant genomes. The sequences coding for viral proteins (P1, HC-Pro, etc.) and GUS are indicated on the genome map. The N- and C-terminal sequences of eIF4E, flanked by the sequences comprising the NIa cleavage sites (NCS, boldface type), are shown above the genome map. An enlarged diagram showing relative positions of sites targeted by mutagenesis in eIF4E is shown below the genome map. (B) Amplification of TEV-GUS genomes containing eIF4E forms from tobacco. GUS activity (picomoles of substrate cleaved per minute per 10^5 protoplasts) in all experiments was measured in samples taken at 24, 48, and 72 h p.i. Each data point represents the mean from three independent transfections using the same batch of protoplasts. TEV-GUS/VNN encodes a nonfunctional Nib polymerase and was used to show baseline GUS activity values induced by an amplification-defective genome.

amplification of the recombinant viruses above the level detected with viruses containing wild-type eIF4E, this would suggest that wild-type eIF4E was conferring a negative effect on replication. In all cases, recombinant viruses were constructed such that synthetic NIa proteolytic cleavage sites flanked the eIF4E sequence (Fig. 3A). This resulted in the addition of one and six residues to the N- and C-termini, respectively, of all eIF4E variants. The eIF4E sequences were inserted into TEV-GUS, a virus that expresses the β -glucuronidase (GUS) reporter. Genome amplification in protoplasts was measured using GUS activity as a surrogate marker as in previous studies.

Infectious transcripts corresponding to each of the recombinant genomes were introduced into tobacco protoplasts (Fig. 3B). The tobacco eIF4E-expressing virus (TEV-GUS/eIF4E) amplified to levels comparable to those of the parental virus, TEV-GUS (Fig. 3B). In contrast, each of the tobacco eIF4E mutant viruses accumulated to levels approximately four- to sevenfold lower ($P < 0.01$) than that of TEV-GUS/eIF4E. These results indicate that viruses coexpressing the mutagenized forms of eIF4E

are relatively debilitated compared to TEV-GUS/eIF4E and are consistent with a model in which the presence of tobacco eIF4E has a stimulatory effect on TEV replication.

DISCUSSION

A yeast two-hybrid search using a tomato cDNA library revealed 10 host proteins that interacted with the TEV NIa protein. Proteins with clear similarity were identified in the sequence databases for each NIa interactor, although functions for some could not be inferred. It is likely that at least a few additional interactors could be identified with additional screening, although nearly 90% of the interacting colonies contained cDNAs that were recovered on at least two occasions. It is also likely that many or most of the NIa interactors identified are not physiologically relevant in the context of TEV infection, even though the proteins interacted specifically with NIa in the yeast system. This underscores a significant weakness in the two-hybrid strategy when applied to experimental systems for which information about functionality of interactions is not available.

Among the NIa interactors identified, eIF4E was interesting for several reasons. It was recovered on 11 occasions from numerous independently transformed batches of yeast, accounting for 42% of the positive interactors that advanced through the screens. Also, the *Arabidopsis* eIF4E isoform was shown to interact with the turnip mosaic potyvirus NIa and VPg domain (Wittmann *et al.*, 1997). The identification of similar proteins with NIa-binding activity in multiple potyvirus–host combinations suggests that the interaction may have significance. The TuMV NIa protein interacted with eIF4E isoform both in yeast two-hybrid and *in vitro* assays. In several experiments using soluble and matrix-bound forms of tobacco and tomato eIF4E fused to glutathione S-transferase, no interaction with ³⁵S-labeled NIa^{HAT}, NIa^{Ox}, or NIa^{OxBam-6000} was detected *in vitro* (data not shown). However, TEV NIa^{HAT} also failed to interact with the NIb protein *in vitro*, even though these two proteins interact in plants cells and in yeast (data not shown). The basis for this lack of interaction activity with TEV NIa protein *in vitro* is not clear.

Interaction was detected between eIF4E and NIa^{HAT}, but not NIa^{Ox}, in the yeast two-hybrid assay, although both forms of NIa interacted with NIb from TEV-HAT. This was particularly intriguing in view of the role of NIa in host genotype-specific infection by TEV and other potyviruses in plants (Keller *et al.*, 1998; Nicolas *et al.*, 1997; Schaad *et al.*, 1997b). However, using chimeric NIa variants, the eIF4E-interaction and host-specific functions of NIa were genetically separable, suggesting that differential eIF4E-interaction activity is not the basis for differential infection of tobacco plants by TEV strains. The

phenotypic consequence of inefficient interaction between NIa^{Ox} and eIF4E, therefore, is not readily apparent.

What role might interaction of NIa with eIF4E play during TEV infection? One possibility is that interaction of eIF4E with the NIa or VPg domain linked to the 5' end of viral RNA may stimulate translation of genomic RNA. This idea is at least superficially appealing, as it would explain how viral RNA is translated efficiently in the absence of a typical methylated cap. However, the 5' untranslated region of the TEV genome confers cap-independent translation function in the absence of NIa or VPg domain linked to the RNA (Carrington and Freed, 1990; Gallie *et al.*, 1995). If interaction with eIF4E serves to promote translation, the effect would likely be additive with other cap-independent translation initiation functions. An alternative role of eIF4E interaction with the NIa or VPg domain may be in viral RNA replication. The NIa protein, bound to endoplasmic reticulum-derived membranes in the form of a 6-NIa polypeptide, was postulated to both recruit NIb polymerase to replication complexes and prime RNA synthesis through the VPg activity (Schaad *et al.*, 1997a). Release of NIa RNA from the membrane would then occur by autocatalytic cleavage. Interaction of eIF4E with NIa might stimulate one of these NIa functions, or another function, during initiation of RNA synthesis. In support of a direct role for eIF4E in enhancing translation or replication are the results of coexpression of tobacco eIF4E forms with the TEV genome. Recombinant viruses containing each of three eIF4E mutant proteins were debilitated relative to the virus coexpressing the nonmutant eIF4E.

Additional possibilities for the role of interaction of NIa with eIF4E should be considered. Binding of NIa to eIF4E may act to suppress host cell functions, by inhibiting host cell translation or defense reactions. Interaction with NIa may interfere with, or inactivate, eIF4E in infected cells. This would indirectly suppress translation of the majority of cellular mRNAs, which depend on the cap-binding function of eIF4E for formation of initiation complexes. Inactivation of cellular mRNA translation in animal virus-infected cells can occur by a number of mechanisms, including debilitation of translation initiation and cap-binding factors (Sachs *et al.*, 1997). In addition, eIF4E appears to be involved in cellular processes other than translation initiation, such as RNA export from the nucleus (Lejbkiewicz *et al.*, 1992). Thus, inactivation of eIF4E could have wide-ranging effects on RNA transport, translation, and metabolism. In this context, it is worth noting that Wang and Maule (1995) have shown that pea seed-borne mosaic potyvirus transiently suppresses mRNA accumulation and translation in infected cotyledon cells of pea. Whether or not this is due to suppression of eIF4E or to interference with other factors remains to be determined.

MATERIALS AND METHODS

All media and protocols for yeast two-hybrid analysis were as described (Finley and Brent, 1996). Yeast containing the NIa^{HAT} "bait" plasmid (pEG202) and the lacZ reporter plasmid (pSH18-34) was transformed with a cDNA library (provided by Bing Wei Lu and Barbara Baker, University of California at Berkeley) prepared from total RNA from TMV-infected *Lycopersicon esculentum* cv VF36 leaves constructed in the plasmid pJG4-5 (Gyuris *et al.*, 1993). Transformants representing a total of approximately 30,000,000 independent clones were collected and frozen at -70°C . Transformants able to grow on galactose⁺ Leu⁻ medium were selected. Colonies were replica plated to test for galactose-dependent lacZ expression and growth on Leu⁻ medium.

The library plasmids were isolated from yeast and used to transform *Escherichia coli* SURE cells (Stratagene). The plasmids were used to transform yeast bait strains containing TEV NIa^{HAT}, *Drosophila* Bicoid (provided by Marianne Dutton and Barbara Baker, University of California, Berkeley), or tomato Pto (provided by Greg Martin, Cornell University). Bicoid and Pto were used for specificity testing. The tomato Pti-1 protein expressed in the pJG4-5 plasmid served as a positive control for the Pto control bait (Zhou *et al.*, 1995). Clones that passed the specificity screen were sequenced. Quantitative β -galactosidase assays were done as described (Daròs *et al.*, 1999).

The eIF4E sequence was identified in the tomato cDNA library. The tobacco eIF4E homologue from three cultivars (Xanthi nc, Havana 425, and V20) was amplified using tobacco-specific primers (unpublished tobacco eIF4E sequence kindly provided by Jonathan Combe and David Twell) and inserted into the yeast library vector, pJG4-5. The tobacco eIF4E coding sequence was introduced into the genome of TEV-GUS by PCR amplification with *Pfu* polymerase (Stratagene) using 5' and 3' primers containing *KpnI* restriction sites. The 5' primer also contained the coding sequence for a TEV NIa proteinase cleavage site (Fig. 3A). The PCR product was first inserted into the *KpnI* site of the intermediate vector (p7SN.0823.KGUS \downarrow H) (Cronin *et al.*, 1995). The *KpnI* fragment was then subcloned into the full-length TEV vector pTEV7DAGUS \downarrow H K Δ H (Kasschau and Carrington, 1995). The resulting construct contained the eIF4E coding sequence immediately adjacent to coding sequences for NIa cleavage sites at both the 5' and the 3' ends of eIF4E. Three mutations—Trp-115 to Leu (W115L), Gly-124 to Asp (G124D), and a combination mutation containing substitutions of Ala for Val-82, Glu-83, and Trp-86 (cluster 1)—were introduced into the tobacco eIF4E sequence by PCR gene sewing. The coding sequence for each mutant eIF4E variant was sequenced and confirmed to be correct.

In vitro synthesis of infectious transcripts was done as

described (Schaad *et al.*, 1997b). The RNA was introduced into tobacco protoplasts by the polyethylene glycol-mediated transformation method (Negrutiu *et al.*, 1987). Protoplasts were harvested at 24, 48, and 72 h postinfection (p.i.), and GUS activity was measured by the fluorometric assay. Activity values were calculated as picomoles of substrate cleaved per minute per 10^5 protoplasts. In every experiment, transfections were done in triplicate for all transcripts and in parallel with parental TEV-GUS and TEV-GUS/VNN transcripts as positive and negative amplification controls, respectively (Li and Carrington, 1995). The TEV-GUS/VNN mutant contains a replication-debilitating mutation affecting the conserved GDD motif within the NIb polymerase. Statistical analysis of data was done using single-factor ANOVA in Microsoft Excel.

ACKNOWLEDGMENTS

We thank Barbara Baker and Bing Wei Lu for the tomato cDNA library, Marianne Dutton for the Bicoid bait plasmid, Greg Martin for providing the Pto bait plasmid and the Pti-1 library plasmid, and Roger Brent for the plasmids and yeast for the LexA system. We are grateful to David Twell and Jonathan Combe for providing us with the unpublished tobacco eIF4E sequence. We also thank Karen Browning for helpful discussions. This work was supported by grants from the National Institutes of Health (AI27832) and the United States Department of Agriculture (98-35303-6485).

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