The Plant Cell, Vol. 2, 987-998, October 1990 © 1990 American Society of Plant Physiologists

Nuclear Transport of Plant Potyviral Proteins

María A. Restrepo, Deon D. Freed, and James C. Carrington[1]

Department of Biology, Texas A&M University, College Station, Texas 77843

We have used immunoblotting, immunocytochemical, and gene fusion methods to examine the differential subcellular partitioning of tobacco etch potyvirus proteins that are potentially associated with RNA replication. From the earliest timepoints at which viral proteins could be detected, proteins NIa (49-kilodalton proteinase) and NIb (58-kilodalton polymerase) were localized primarily in the nucleus, whereas the 71-kilodalton cylindrical inclusion protein was identified in the cytoplasm. The NIa and NIb coding regions were fused to the β-glucuronidase (GUS) sequence in a plant expression vector, resulting in synthesis of chimeric proteins in transfected protoplasts and in transgenic plants. In situ localization of GUS activity revealed nuclear localization of the GUS-NIa and GUS-NIb fusion proteins and cytoplasmic localization of nonfused GUS. These results indicate that both NIa and NIb contain nuclear targeting signals, and that they may serve as useful models for studies of plant cell nuclear transport. A discussion of the general utility of the nuclear transport system described here, as well as the role of nuclear translocation of potyviral proteins, is presented.

INTRODUCTION

Many proteins undergo post-translational modifications and are targeted to different subcellular compartments. Transit into the secretory pathway (Klausner, 1989) and into some organelles, such as chloroplasts (Ellis, 1981) and mitochondria (Wickner and Lodish, 1985), is mediated by a sequence at the amino terminus of the protein. In these cases, the signal sequence is cleaved during or after transport of the protein. Entry of proteins into the nucleus, on the other hand, is mediated by a position-independent transport signal that is not cleaved upon translocation (Dingwall and Laskey, 1986). Many basic cellular processes, such as gene transcription, DNA replication, and cell division, obviously require proper nuclear localization of proteins. Additionally, switching of proteins between cytoplasmic and nuclear compartments has been recognized recently as a novel developmental and gene regulatory control mechanism (Hunt, 1989, and references therein). Although nuclear transport has been examined extensively in animal and lower eukaryotic systems, few studies have addressed nuclear translocation in plant cells.

Many plant viruses encode large quantities of nonstructural proteins that accumulate in different subcellular compartments during infection. All members of the potyvirus group form characteristic inclusions upon infection. The cylindrical inclusion (CI) protein associates with a cellular membrane early in infection and aggregates into pinwheel-shaped inclusions during later stages (Lawson and Hearon, 1971; Langenberg, 1986; Lesemann, 1988). Several potyviruses also induce nuclear inclusions composed of two distinct nonstructural proteins (NIa and NIb) present in equimolar ratios (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980b). In contrast to their animal virus counterparts, plant viral proteins have been underutilized as tools to study basic features of plant cell biology. Because the deduced amino acid sequences and subcellular sites of accumulation of numerous plant viral proteins are known, they would appear to be attractive models for analysis of plant cell protein transport.

Members of the potyvirus group possess flexuous, rod-shaped particles (Hollings and Brunt, 1981) composed of a positive-sense RNA molecule (about 10,000 nucleotides) and more than 2000 copies of a coat protein subunit (Allison et al., 1986; Dougherty and Carrington, 1988). The potyviral genome has a poly(A) sequence at the 3′ end (Hari et al., 1979) and is covalently bound at the 5′ end to a protein termed VPg (Hari, 1981; Siaw et al., 1985; Riechmann et al., 1989; Murphy et al., 1990). The tobacco etch potyvirus (TEV) genome nucleotide sequence has been determined (Allison et al., 1986) and contains a single open reading frame that is translated into a large polyprotein, as shown in Figure 1 (Allison et al., 1985). This initial translation product is proteolytically cleaved into eight mature proteins (Dougherty and Carrington, 1988). At least two of the mature TEV products (NIa and HC-Pro) function as proteinases that catalyze most of the polyprotein processing (Carrington and Dougherty, 1987; Carrington et al., 1989). At least three potyviral proteins (CI, NIa, NIb) have been postulated to function during RNA replication. Sequence comparisons with picornaviral protein 2C suggest that CI may be the membrane-binding component of the

[1] To whom correspondence should be addressed.

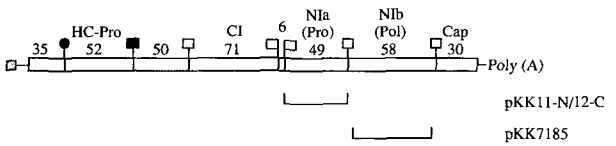


Figure 1. Genetic Map of Tobacco Etch Virus.

The polyprotein coding region is indicated by the long rectangular box. Positions coding for the polyprotein cleavage sites are shown by the vertical marks. The proteolytic enzymes responsible for processing are NIa (open boxes), HC-Pro (filled box), and a third proteinase that has not been mapped (filled circle). The genome-linked VPg protein is indicated by the shaded box at the far left. The approximate molecular masses (in kilodaltons) of the mature products are given. Complementary DNA representing those genome segments shown by the brackets were inserted into the nonfusion expression vector pKK233-2 for overexpression in *E. coli*. The names of the resulting recombinant plasmids are given to the right. Abbreviations: HC-Pro, helper component-proteinase; CI, cylindrical inclusion protein; NIa and NIb, nuclear inclusion protein a and b, respectively; Pro, proteinase; Pol, polymerase; Cap, capsid protein.

replicase complex (Domier et al., 1987; Dougherty and Carrington, 1988). Likewise, NIb contains extensive sequence similarity with RNA-dependent RNA polymerases encoded by all positive-strand RNA viruses (Allison et al., 1986; Domier et al., 1987). Besides functioning as a proteinase, the NIa protein appears to be involved with RNA replication because it is linked covalently to genomic RNA as the VPg (Murphy et al., 1990). Although CI, NIa, and NIb are believed to participate in RNA replication, they partition to distinct subcellular compartments.

To initiate a study of the roles of differential subcellular localization of potyviral proteins, we have investigated the nuclear accumulation and transport of TEV nonstructural proteins NIa and NIb. Both proteins were localized predominantly in the nucleus at all stages of infection that were examined. We have employed a protein-fusion reporter system using β-glucuronidase (GUS) to follow the movement of NIa and NIb into the nucleus in transient assays and in transgenic plants. Fusion of either NIa or NIb to GUS was sufficient to direct the reporter protein activity to the nucleus. This system should have broad application to the investigation of nuclear transport signals of plant and plant viral proteins, as well as to the analysis of events and interactions that regulate nuclear localization.

RESULTS

Accumulation of TEV-Encoded Proteins

Because TEV proteins are encoded by a single translational unit that spans most of the genome, all proteins presumably are synthesized in equimolar ratios. To characterize the accumulation rates of TEV-encoded proteins in intact leaf tissue, immunoblot analyses were conducted with protein extracts from infected tobacco plantlets using antisera specific for NIa, NIb, CI, helper component-proteinase, and capsid proteins. Small tobacco plants (four-leaf stage) were inoculated with TEV, and SDS-soluble protein extracts were prepared after 0 days, 2 days, 3 days, 4 days, 5 days, and 7 days post-inoculation (p.i.). Immunoblots were prepared using the five monospecific antisera and preimmune serum. Accumulation of each protein was first detected 3 days p.i., as shown in Figure 2. Based on densitometric scanning, maximal accumulation levels for each protein were achieved 4 days to 5 days p.i. and remained at relatively constant or slightly decreased levels for an additional 3 days (data not shown). Immunocytochemical analysis of TEV-infected tissue revealed virus activity in most cells by 3 days p.i. (see below), suggesting that accumulation between days 3 and 5 was due to increases in actual concentrations within infected cells rather than increases in the numbers of infected cells. Each TEV-encoded protein appears to accumulate at approximately the same rate, suggesting that temporal mechanisms to differentially regulate the relative abundance of structural and nonstructural proteins may not exist.

Immunolocalization of TEV-Encoded Nonstructural Proteins

Nuclear inclusions composed of NIa and NIb have been identified in TEV-infected cells (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980a; Baunoch et al., 1988), whereas the CI protein has been found in association with the cellular membrane and in an aggregated form in the cytoplasm (Lawson and Hearon, 1971; Langenberg, 1986; Baunoch et al., 1988). Most published immunolocalization studies have been conducted with infected tissue several weeks post-inoculation. To determine whether pools of NIa and NIb also exist in the cytoplasm and to determine whether differences exist in the subcellular distribution of these proteins at different stages of infection, tobacco tissue was embedded in LR Gold resin at various times post-inoculation, and serial sections were made and subjected to immunogold labeling using anti-NIa, anti-NIb, anti-CI, and preimmune sera. The immunogold label was amplified by silver enhancement. The tissue samples were derived from TEV-infected plants at 0 days, 2 days, 3 days, 4 days, 5 days, and 7 days p.i.

No immunolabeling was detected when probing sections from tissue 4 days and 5 days p.i. with preimmune serum, (as shown in Figure 3, panels D4 and D5) or when analyzing sections from tissue 0 days (Figure 3, panels A0, B0, and C0) and 2 days (data not shown) p.i. with any of the sera. Proteins NIa and NIb were localized primarily to the nucleus at all timepoints between days 3 and 7 p.i.; photomicrographs are presented only for the timepoints at

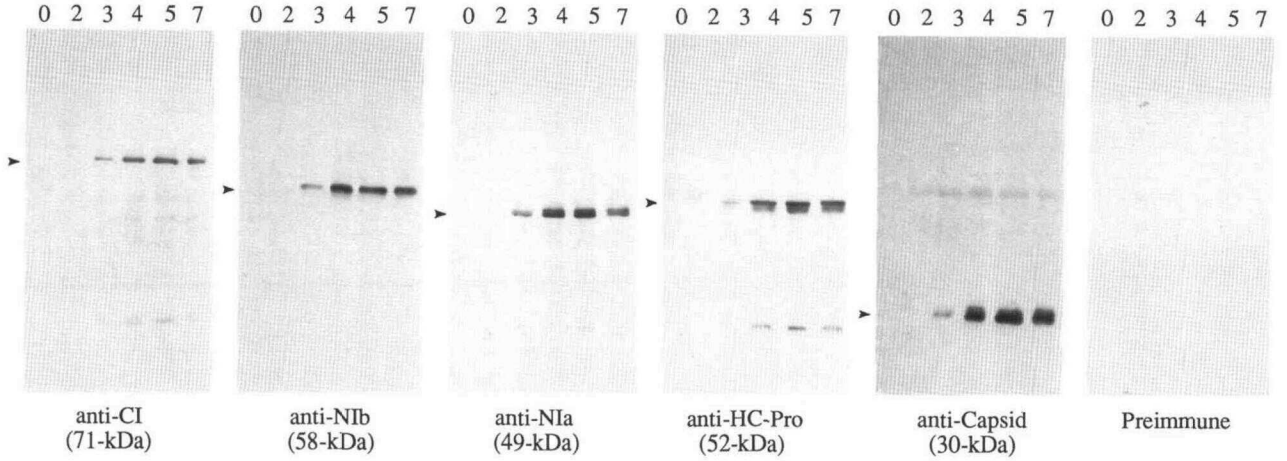


Figure 2. Accumulation of CI, NIb, NIa, HC-Pro, and Capsid Protein in TEV-Infected Tobacco Tissue.

Immunoblot analysis was conducted using a series of monospecific antisera and SDS-soluble protein extracts from noninfected plants (0) or plants at 2 days, 3 days, 4 days, 5 days, or 7 days post-inoculation, as indicated at the top of the figure. The antiserum used as primary antibody for each blot is given below each panel, as are the approximate molecular masses of the CI, NIb, NIa, HC-Pro, and capsid proteins. Anti-CI and anti-HC-Pro sera react slightly with low-molecular-weight proteins that we presume to be degradation products. Anti-capsid serum exhibits a weak reaction with the large subunit of ribulose bisphosphate carboxylase (52 kDa).

days 0, 3, and 7 p.i. (Figure 3). However, a small fraction of the anti-NIa-specific immunolabeling was observed consistently at all timepoints between 3 days and 7 days p.i. in the cytoplasmic region of cells. The CI protein was localized in the cytoplasm at all timepoints beginning at day 3 p.i. (Figure 3, panels C3 and C7). Immunoreaction patterns indicating aggregation of the CI protein were evident by 5 days to 7 days p.i. Although systemically infected tissue was used for these studies, the timing of infection of cells was surprisingly uniform. Virtually all cells were infected by 3 days p.i., as revealed by the proportion that reacted positively with antibodies (Figure 3, panel C3).

Nuclear Transport of NIa and NIb

Immunocytochemical analyses reveal that NIa and NIb accumulate primarily in the nucleus. What factors regulate differential subcellular accumulation patterns for various replication-associated potyviral proteins? β-Glucuronidase (GUS) fusions were constructed, resulting in linkage of NIa or NIb to the amino terminus (pTL7SN.3-NIa/GUS and pTL7SN.3-NIb/GUS) or the carboxy terminus (pTL7SN.3-GUS/NIa and pTL7SN.3-GUS/NIb) of GUS after in vitro transcription and translation, as shown in Figure 4A). Cell-free translation of transcripts derived from each plasmid revealed that they encoded fusion proteins of the anticipated sizes. Transcripts from the GUS-NIa and GUS-NIb fusion constructs directed synthesis of proteins of approximately 115 kD and 125 kD, respectively, whereas transcripts from pTL7SN.3-GUS, containing the nonfused GUS sequence, encoded a product of 68 kD (Figure 4B).

The coding sequences for the four GUS fusion proteins were transferred to the plant expression vector pRTL2 for transient expression in tobacco protoplasts. Plasmid DNA was introduced into protoplasts by the polyethylene glycol-mediated transfection procedure. Analysis of GUS activity in protoplasts 44 hr after transfection revealed that each GUS fusion protein exhibited enzymatic activity, but at a reduced level compared with the nonfused GUS control. This is shown in Table 1. Carboxy-terminal GUS fusions were severalfold more active than were amino-terminal fusions and, therefore, were used in subsequent experiments. To confirm that the GUS activity was due to the fusion proteins, the sizes of the enzymatically active proteins were determined by assaying GUS activity directly in SDS-polyacrylamide gels (Kavanagh et al., 1988). Whereas enzymatic activity in protoplasts containing pRTL2-GUS was associated with a 68-kD protein (the size of the GUS monomer), activity from protoplasts containing the fusions was associated with higher molecular weight proteins (data not shown).

To determine the subcellular site of accumulation of the GUS/NIa or GUS/NIb chimeric proteins, transfected protoplasts were incubated with the chromogenic GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc). The insoluble reaction product of GUS serves as an accurate colorimetric marker for the subcellular distribution of enzymatic activity. Protoplasts transfected in the presence of water exhibited no reaction product accumulation, as seen in Figure 5F. Nonfused GUS encoded by pRTL2-GUS was found uniformly distributed throughout the cytoplasm of transfected protoplasts (Figures 5A and 5B).

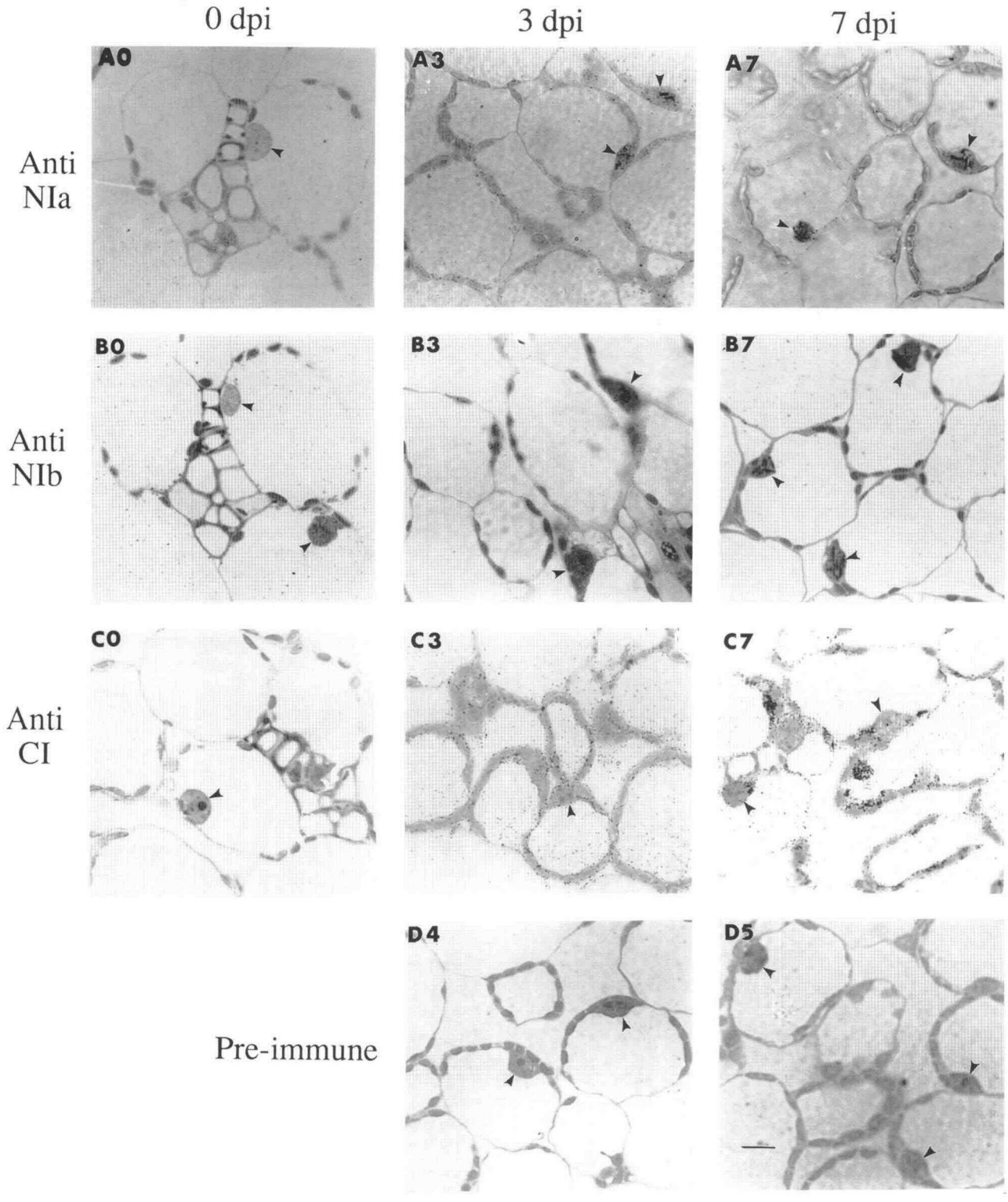
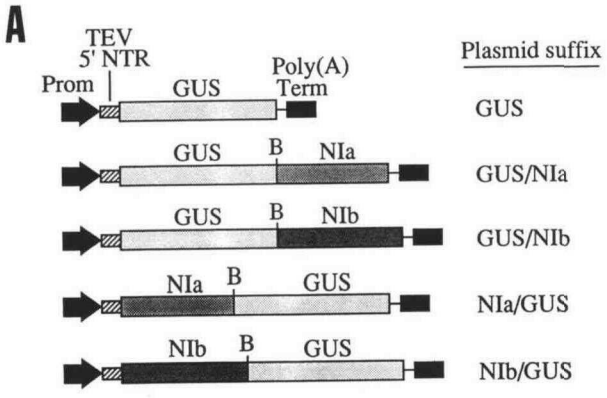


Figure 3. Immunolocalization of NIa, NIb, and CI Proteins in Semi-Thin Sections of TEV-Infected Tobacco Tissue.

Noninfected (0) or TEV-infected tissue at 3 days or 7 days post-inoculation (dpi) was embedded in LR Gold resin and subjected to immunogold labeling and silver enhancement as indicated in Methods. The antiserum used for each series is indicated at the left. Sections were viewed by light microscopy using bright-field optics. Positive immunolabeling is indicated by the dark grains. The arrowheads point to nuclei in each photomicrograph. Bar = 10 μm.



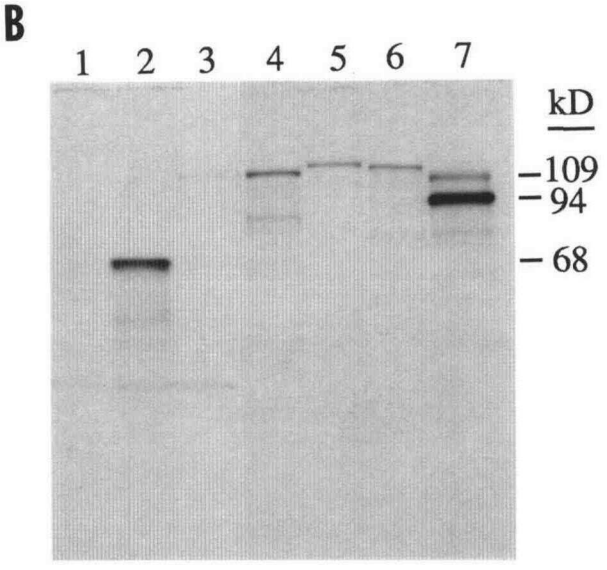


Figure 4. Construction and in Vitro Expression of GUS Fusion Plasmids.

(A) Diagrammatic representation of relevant portions of plasmids encoding GUS fusion proteins. The fusions were assembled in the in vitro expression vector pTL7SN.3 (containing an SP6 transcriptional promoter) and in the plant expression vector pRTL2 (containing the CaMV 35S promoter). Coding sequences for GUS and NIa or NIb were ligated in-frame using the BglII (B) sites that were introduced by site-directed mutagenesis.

(B) Analysis of coding properties of the GUS fusion constructs using in vitro transcription and translation. ^{35}S-methionine-labeled translation products programmed by RNA transcripts from the constructs shown in **(A)** were resolved by SDS-PAGE and visualized by autoradiography. Translation reactions contained: lane 1, no added RNA; lane 2, pTL7SN.3-GUS transcripts; lane 3, pTL7SN.3-NIa/GUS transcripts; lane 4, pTL7SN.3-GUS/NIa transcripts; lane 5, pTL7SN.3-NIb/GUS transcripts; lane 6, pTL7SN.3-GUS/NIb transcripts; lane 7, brome mosaic virus RNA. The positions of the 109-kD and 94-kD brome mosaic virus RNA translation products, as well as the 68-kD GUS protein translated from pTL7SN.3-GUS, are given to the right.

Table 1. Relative Activities of GUS Fusion Proteins in Transfected Protoplasts

Construct	Relative Activity[a]
pRTL2-GUS	100%
pRTL2-NIa/GUS[b]	1.4% ± 0.22
pRTL2-NIb/GUS[b]	0.05% ± 0.04
pRTL2-GUS/NIa[c]	8.0% ± 1.9
pRTL2-GUS/NIb[c]	0.67% ± 0.26

[a] β-Glucuronidase activity from protoplasts transfected with the fusion plasmids was divided by activity from protoplasts transfected with pRTL2-GUS, which encodes a nonfused GUS protein.
[b] Encodes an amino-terminal GUS fusion protein.
[c] Encodes a carboxy-terminal GUS fusion protein.

However, the enzymatic activities of GUS/NIa and GUS/NIb fusion proteins were localized predominantly to the nucleus (Figures 5C, 5D, and 5E). In each case, the fusion protein activity appeared to accumulate within the nucleolar region, although we cannot assess whether this was due to actual activity in the nucleolus or to concentration of the indigo reaction product in the nucleolus. Nuclear localization of GUS fusion protein activity was corroborated by analysis of subcellular fractions isolated from transfected protoplasts. Compared with crude nuclear fractions from pRTL2-GUS-transfected protoplasts, GUS activities were approximately 15-fold and 35-fold higher in nuclear fractions from protoplasts transfected with pRTL2-GUS/NIa and pRTL2-GUS/NIb, respectively (data not shown).

The GUS, GUS/NIa, and GUS/NIb expression cassettes were inserted into the binary vector pGA482 (creating pGA-GUS, pGA-GUS/NIa, and pGA-GUS/NIb, respectively) and integrated into the tobacco genome using *Agrobacterium tumefaciens*. Transgenic plants that expressed substantial levels of GUS activity were identified only after transformation with pGA-GUS and pGA-GUS/NIa. Epidermal leaf strips from plants transformed with pGA482, pGA-GUS, and pGA-GUS/NIa were incubated with X-gluc and analyzed by light microscopy. No GUS activity was detected in pGA482-transformed leaf strips, as shown in Figures 6A and 6D). Activity was distributed uniformly throughout cells containing nonfused GUS encoded by pGA-GUS (Figures 6B and 6E). However, activity was detected in the nuclei of cells transformed with the GUS/NIa construct (Figures 6C and 6F).

DISCUSSION

Selective transport of proteins to the nucleus is believed to be a two-step process involving binding to the nuclear pore, followed by energy-dependent uptake into the nu-

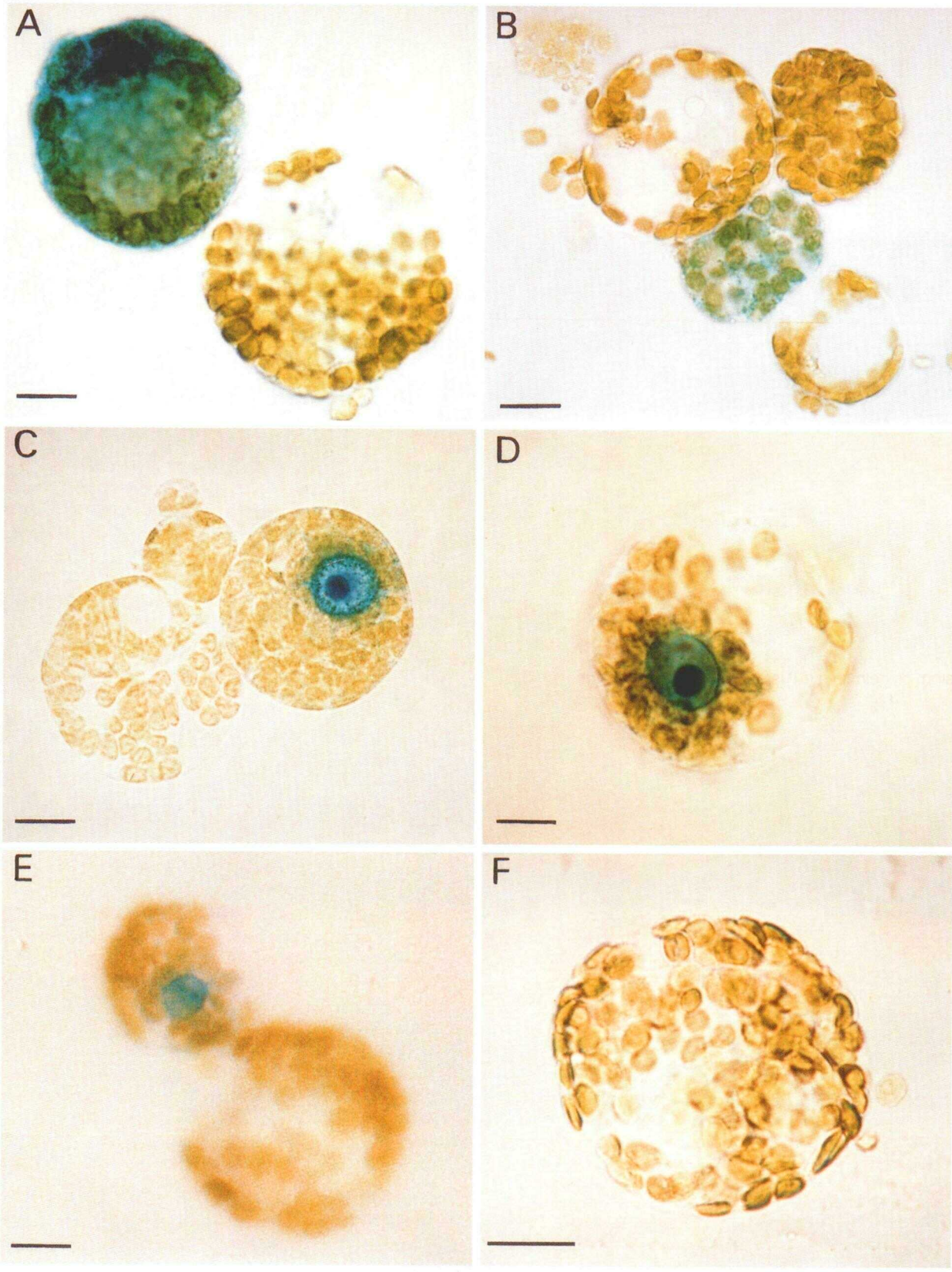


Figure 5. Nuclear Transport of GUS-NIa and GUS-NIb Fusion Proteins.

cleoplasm (Newmeyer and Forbes, 1988; Richardson et al., 1988). Binding of nuclear proteins to the pore is mediated by interaction between a pore-associated receptor and a nuclear localization signal sequence within the transported protein (Dingwall and Laskey, 1986). Nuclear transport signals in animal cell and viral proteins generally consist of a short, highly basic amino acid sequence (7 to 13 residues), although there is considerable diversity among signal sequences from individual proteins. Heterologous reporter proteins or large macromolecules that are conjugated to nuclear transport signals, either by chemical means or by genetic manipulation, have been shown to localize to the nucleus (Dingwall and Laskey, 1986). Despite the interest in nuclear processes in plant cells, the requirements for nuclear localization of plant proteins have not been investigated previously, in part because of a lack of nuclear protein models.

Development of a Plant Nuclear Transport System

We have developed a system to study nuclear transport of proteins in isolated protoplasts after transfection and in transgenic plants. The potyviral proteins NIa and NIb appear to be useful tools because they are absent from normal plant cells, and they have been shown to transport a heterologous protein to the nucleus. Detection of the fusion protein in situ is facilitated by the high activity, specificity, and stability of GUS and by the use of a chromogenic substrate that produces an insoluble reaction product with limited diffusion capability (Jefferson, 1987). Nuclear transport of the fusion protein can be analyzed also by subcellular fractionation and in vitro enzymatic activity assays.

The effects on enzymatic activity of appending NIa and NIb to the amino and carboxy termini of GUS were determined. Although numerous reports have described the use of GUS as a reporter for transcriptional fusions, relatively few studies have employed GUS as a tool to probe protein function and transport. Carboxy-terminal fusion proteins clearly were more enzymatically viable than were amino-terminal fusions, although even the most active chimera possessed less than 10% activity compared with nonfused GUS. The debilitation of enzymatic function presumably was due to interference with proper protein folding or to inhibition of oligomeric assembly of the enzyme.

The nuclear transport assay described here should facilitate identification of specific targeting signals within NIa and NIb, as well as other plant nuclear proteins. Besides their value in understanding fundamental aspects of cell biology, such signals may be useful for novel applications involving the need to redirect genetically modified proteins to the plant nucleus. This system may also prove useful for investigations into the regulation of nuclear transport in plant cells.

Nuclear Transport of NIa and NIb

Proteins NIa and NIb were found to accumulate predominantly in the nucleus of TEV-infected cells at all timepoints during which viral antigens could be detected, implying that they possess efficient nuclear transport signals. Tobacco etch virus nuclear inclusion bodies are composed of equimolar quantities of NIa and NIb (Knuhtsen et al., 1974), suggesting that they associate either before or during inclusion formation. If NIa and NIb associate before nuclear transport, it would be possible that only one protein actually harbors the nuclear targeting signal while the other is chaperoned into the nucleus. However, NIa-GUS and NIb-GUS fusions are both capable of nuclear transport, indicating that each protein contains a nuclear targeting signal sequence.

The locations of the nuclear transport signals in NIa and NIb have yet to be identified. Nearly all animal nuclear localization signals contain at least 3 basic (arginine or lysine) residues within a 5-amino acid stretch, as well as a few additional charged or noncharged residues in flanking positions (Dingwall and Laskey, 1986; Roberts, 1989). A search for such sequences was conducted using NIa and NIb. Seven locally basic regions (defined as 3 basic residues within 5 positions) are present within NIa, with all but one occurring within the amino-terminal one-third of the protein. Only three basic regions occur in NIb. The heterogeneity among nuclear transport signals in animals, however, precludes identification of the signals within the potyviral proteins based on sequence similarity. Systematic deletion analysis of NIa-GUS and NIb-GUS fusion constructs is underway to map precisely the sequences required for transport.

In addition to a nuclear transport function, two activities have been attributed to NIa based on biochemical analyses. The carboxy-terminal one-half forms a proteolytic

Figure 5. (continued).

Tobacco protoplasts were transfected in the presence of supercoiled plasmid DNA or water, cultured for 44 hr, and incubated with the GUS substrate X-gluc. β-Glucuronidase activity results in conversion of the substrate to an insoluble indigo product. Protoplasts were viewed using bright-field optics. Note that not all cells exhibit X-gluc staining. Bar = 20 μM.

(A) and **(B)** Protoplasts transfected with pRTL2-GUS, which encodes a nonfused GUS protein.

(C) and **(D)** Protoplasts transfected with pRTL2-GUS/NIa, which encodes a GUS-NIa fusion protein.

(E) Protoplasts transfected with pRTL2-GUS/NIb, which encodes a GUS-NIb fusion protein.

(F) Protoplast transfected in the presence of water (no DNA).

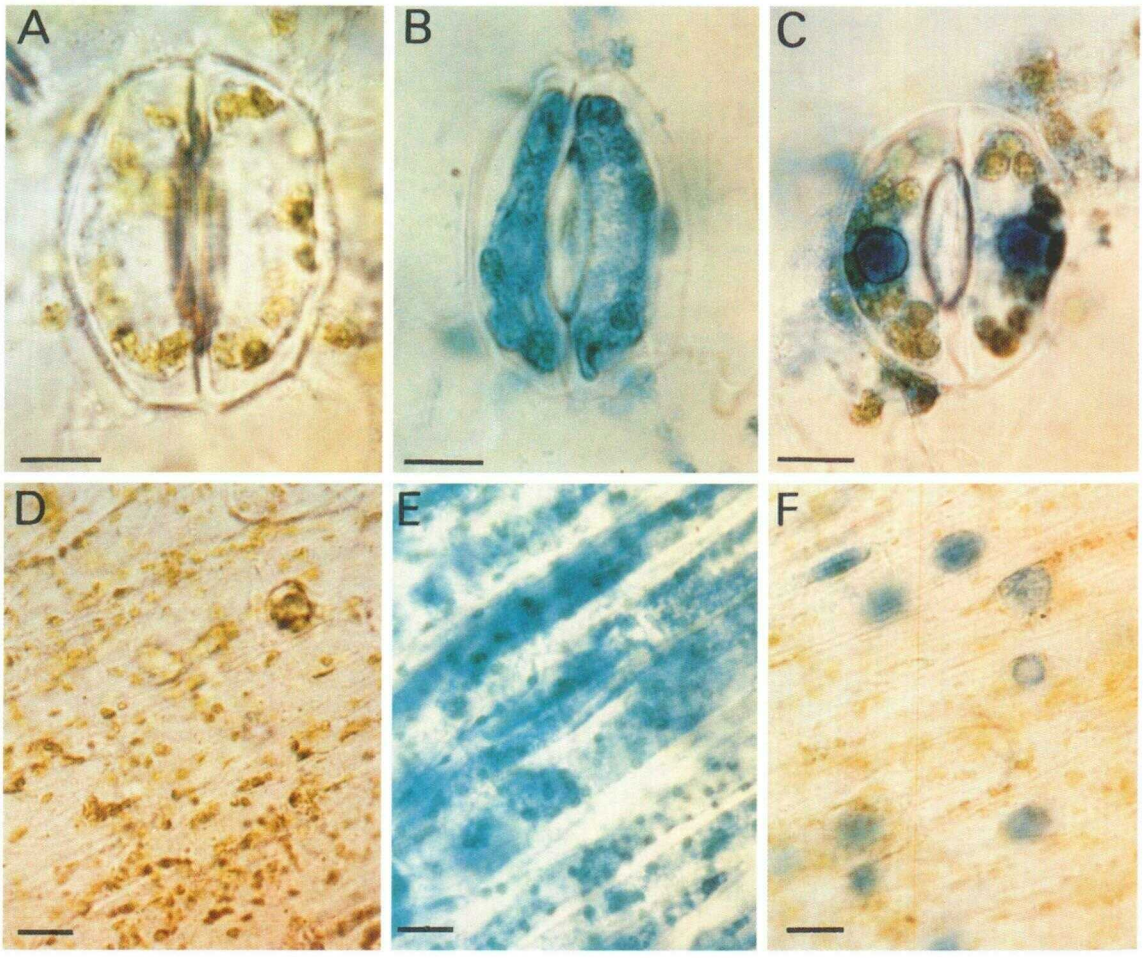


Figure 6. Nuclear Transport of GUS-NIa Fusion Protein in Transgenic Plants.

Transgenic plants were generated, and epidermal leaf strips were removed and treated with X-gluc. The leaf strips were viewed under bright-field optics. Bar = 20 μM.

(A) and **(D)** Stomatal and epidermal cells, respectively, from control (pGA482) transgenic plants.

(B) and **(E)** Stomatal and epidermal cells, respectively, from pGA-GUS-transformed plants. Note that the X-gluc reaction product is distributed uniformly throughout the cells.

(C) and **(F)** Stomatal and epidermal cells, respectively, from pGA-GUS/NIa-transformed plants. Note that the reaction product is localized primarily to the nuclei.

domain that catalyzes cleavage of the potyviral polyprotein at five sites (Carrington and Dougherty, 1987). The amino-terminal region contains the VPg domain, which is involved in covalently attaching NIa to the 5′ terminus of genomic RNA (Shahabuddin et al., 1988; Murphy et al., 1990). The VPg may function to prime potyviral RNA synthesis and/or to process RNA replication intermediates, although direct experimental proof for either role is lacking.

It is interesting to note that two pools of NIa have been identified from infected plant cells. The majority of NIa is localized to the nucleus along with NIb. However, genome-linked NIa (VPg) accumulates in virus particles that are located within the cytoplasm (Lesemann, 1988). The weak, cytoplasmic signal that was detected during the immunogold localization experiments (Figure 3) may have been due to the genome-linked pool of NIa protein. If NIa possesses a nuclear targeting signal, how does a subpopulation of NIa (as VPg) localize to the cytoplasm? Two possibilities are worth considering. First, linkage of NIa to genomic RNA may conceal or destroy the nuclear transport signal. Covalent attachment of RNA may occur at an amino acid residue situated within the signal sequence, or at a nearby position, causing a disruption or distortion of the signal. If the nuclear transport sequence contains a

highly basic character, it conceivably could form a genomic RNA-protein complex that blankets the signal sequence. Second, genomic RNA may serve as an anchor that restricts movement of NIa into the nucleus. Association of RNA with replication complexes, the translational apparatus, and capsid protein may effectively inhibit movement of NIa to and through the nuclear envelope.

The role of nuclear transport of NIa and NIb during the potyviral replication cycle has not been determined. We suspect that the nucleus does not represent the site of RNA synthesis because the CI protein, a putative factor necessary for viral RNA replication, accumulates in association with cellular membranes and aggregates in the cytoplasm. Most positive-strand RNA virus replicase complexes are membrane associated (Hall et al., 1982). The CI protein exhibits amino acid sequence similarity to the membrane-bound, replication-associated 2C protein of picornaviruses (Domier et al., 1987). If potyviral RNA synthesis occurs in a membrane-associated complex with CI protein, NIa (VPg), and NIb (RNA-dependent RNA polymerase), the fraction of NIa and NIb localized to the nucleus may represent accumulation in excess of that needed for replicase formation. Timing of specific events (e.g., translation, RNA synthesis, encapsidation) in the replication cycle may be controlled, at least partially, by the levels of nonstructural proteins present within the cytoplasm. Because all TEV proteins are synthesized at the same rate (as part of a polyprotein), their effective concentrations in the cytoplasm may be governed by spatial control mechanisms. It should not be discounted, however, that NIa and NIb perform additional functions in the nucleus, or that one or both interact with nuclear components to effect host cell metabolism.

Finally, it should be pointed out that several positive-strand RNA viruses that replicate within the cytoplasm have been shown to encode proteins that accumulate within the nucleus (Faaberg and Peebles, 1988; Lyles et al., 1988; Tadano et al., 1989; Peränen et al., 1990). Approximately 50% of the Semliki Forest virus (an alphavirus) nonstructural protein nsP2, for example, localizes to the nucleus (Peränen et al., 1990). Interestingly, nsP2 functions as a proteinase to process a viral polyprotein and is required also for viral RNA synthesis.

METHODS

Virus and Antisera

The highly aphid-transmitted strain of TEV was used for these studies and was obtained from Dr. W. G. Dougherty (Oregon State University). Virus was propagated in tobacco (*Nicotiana tabacum* cv Xanthi nc), and was purified by the method of Dougherty and Hiebert (1980b). Monospecific antisera were raised in New Zealand White rabbits against NIa, NIb, and HC-Pro proteins that were overproduced in *Escherichia coli* and against CI and capsid proteins isolated from infected plants (Dougherty and Hiebert, 1980a) and virions, respectively, using the method of Harlow and Lane (1988). Anti-HC-Pro serum has been described (Carrington et al., 1990).

Plasmid Construction and Protein Overexpression

Complementary DNA representing TEV protein coding regions were inserted into the nonfusion expression vector pKK233-2 (Amann and Brosius, 1985) to facilitate overproduction of NIa and NIb in *E. coli*. The NIa coding sequence was excised from pTL-5473/11-N/12-C (Carrington et al., 1988) by digestion with SmaI, and was inserted into pKK233-2 that had been cut with NcoI and treated with Klenow fragment to generate blunt ends. A DraI-DraI DNA fragment encoding a 52-kD segment of NIb was isolated from pTL-5495 (Carrington and Dougherty, 1987) and inserted into NcoI-cut, Klenow-treated pKK233-2. The NIa and NIb proteins were isolated in the form of insoluble granules from cultures (3 hr post-induction) containing the recombinant plasmids as described previously (Carrington et al., 1990). The granules were dissolved in SDS (0.1%) before emulsification with adjuvant and injection into rabbits.

The coding sequences for NIa, NIb, and bacterial GUS were inserted into the in vitro expression vector pTL7SN.3. The TEV 5′-nontranslated region (NTR) has been incorporated into pTL7SN.3, resulting in cap-independent enhancement of translation of transcripts in vitro and in vivo (Carrington and Freed, 1990). Each plasmid (pTL7SN.3-NIa, pTL7SN.3-NIb, and pTL7SN.3-GUS) was used to produce two mutagenized derivatives—one that resulted in insertion of a BglII restriction enzyme site at the 5′ end of the open reading frame and one that resulted in a BglII site at the 3′ end. The coding sequences for NIa and NIb were then fused to either the 5′ end or 3′ end of the GUS gene by ligation of DNA fragments at the common BglII sites. Mutagenesis was carried out by the method of Taylor et al. (1985). The coding regions were maintained in-frame through each of the fusion junctions. Plasmids containing fusions in which the GUS gene was positioned 5′ relative to the NIa or NIb coding sequences were named pTL7SN.3-GUS/NIa or pTL7SN.3-GUS/NIb. Plasmids containing the GUS gene positioned to the 3′ side of the NIa or NIb sequences were named pTL7SN.3-NIa/GUS or pTL7SN.3-NIb/GUS.

The gene fusions were transferred to the plant expression vector pRTL2, which contains the cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region, the TEV 5′-NTR, a polylinker sequence for insertion of open reading frames, and the CaMV 35S polyadenylation signal. pTL7SN.3-GUS/NIa and pTL7SN.3-GUS/NIb were cut with NcoI, which cleaves the DNA at the 5′ end of the GUS sequence and at the 3′ end of the NIa or NIb sequence. The DNA fragment containing the gene fusion was isolated and inserted into pRTL2 at the unique NcoI site. The NcoI site covers the translation start codon (CCATGG) adjacent to the TEV 5′-NTR. The gene fusions within pTL7SN.3-NIa/GUS or pTL7SN.3-NIb/GUS were excised with NcoI and XbaI and inserted into pRTL2 between the unique NcoI and XbaI sites. These recombinant plasmids were assigned names that were similar to the pTL7SN.3-based plasmids, except the vector prefix was changed to pRTL2 (e.g., pRTL2-GUS/NIa).

Gene fusions and transcriptional control sequences were excised from the pRTL2-based plasmids using HindIII, which cleaves immediately upstream of the 35S promoter and immediately downstream from the 35S poly(A) signal, and inserted into the

unique HindIII site of the binary vector pGA482 (An, 1986). Recombinant plasmids were named using the pGA prefix (e.g., pGA-GUS/NIa).

Immunoblot Analysis

Small tobacco plants (four-leaf stage, 1.5 cm) were dusted with carborundum and inoculated by abrading the two bottom leaves with a cotton applicator saturated with a virus suspension (0.05 μg/μL in 20 mM Tris-HCl, pH 8.0). Total SDS-soluble protein extracts were prepared from leaf tissue (0.1 g) after 0 days to 7 days post-inoculation as described (Carrington et al., 1990), except that the samples were first frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The concentration of protein in each extract was measured using the Bio-Rad Protein Assay Kit with BSA as a concentration standard. The proteins were resolved by electrophoresis through a 12.5% SDS-polyacrylamide gel, blotted to nitrocellulose, and reacted with antiserum at 1:500 or 1:1,000 dilutions. The protein-antibody complexes were visualized by incubation with alkaline phosphatase-linked, goat anti-rabbit IgG, followed by reaction with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride substrate. Densitometric scanning, using a Bio-Rad model 620 video densitometer, was used to quantify the intensities of the reactions.

Immunolocalization of TEV Proteins

Leaf pieces (1 mm × 5 mm) from systemically infected tobacco plants were fixed in fresh 4% formaldehyde, 0.125% glutaraldehyde in 50 mM KPO_4, pH 7.2. Tissue samples were embedded in LR Gold by the method of McFadden et al. (1988), but with minor modifications. After fixation, dehydration, and infiltration, samples were incubated in LR Gold resin containing 0.2% benzoyl peroxide for 1 hr at 4°C. The samples were polymerized in LR Gold containing 0.5% benzoyl peroxide in the dark for at least 24 hr at room temperature. Blocks were sectioned (1 μm thickness) using an LKB Ultracut E ultramicrotome. The sections were adhered to poly-L-lysine-coated glass slides by floating on a drop of polyvinyl alcohol-vinyltriethoxysilane (Fink, 1987) and drying on a warm plate overnight at 42°C. Sections were incubated with blocking solution containing 5% goat serum, 0.1 M NaCl, 2.5 mM KCl, 10 mM dibasic sodium phosphate, 1.5 mM monobasic potassium phosphate, 0.05% Tween 20, 0.5% BSA, 0.5% gelatin, and 0.05% sodium azide. The sections were subsequently reacted with anti-NIa, anti-NIb, anti-CI, or pre-immune sera, followed by colloidal gold (5 nm)-linked goat anti-rabbit IgG as secondary antibody. Colloidal gold label was amplified by the silver enhancement technique (Danscher and Nörgaard, 1983) according to the manufacturer's (Janssen, Belgium) instructions. All antisera were used at 1:60 dilutions, with the exception of anti-NIb serum (1:120). Sections were counterstained with methylene blue and viewed under bright-field optics using a Zeiss Axiophot photomicroscope.

In Vitro Transcription and Translation

Methods for transcription by SP6 RNA polymerase and translation in a rabbit reticulocyte lysate have been described previously (Carrington and Dougherty, 1987). Plasmid DNA was digested with PvuII, which cuts within vector sequences, before transcription.

Protoplast Isolation and Transfection

N. tabacum cv Xanthi nc protoplasts were isolated as described (Carrington and Freed, 1990). Plasmid DNA (20 μg) was introduced into protoplasts (3 to 5 × 10^5) for transient expression using the polyethylene glycol-mediated transfection procedure (Negrutiu et al., 1987).

Generation of Transgenic Plants

Transgenic tobacco plants that expressed sequences from pGA482, pGA-GUS, pGA-GUS/NIa, and pGA-GUS-NIb were generated essentially as described (Carrington and Freed, 1990; Carrington et al., 1990).

GUS Assays in Protoplasts, Transgenic Plants, and In Vitro

β-Glucuronidase assays were conducted in situ using protoplasts 44 hr after transfection. The protoplast culture medium (Negrutiu et al., 1987) was replaced by fresh medium containing 1.2 mM X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 10 mM EDTA. Reaction of the X-gluc substrate yielded an insoluble indigo precipitate after 2 hr to 24 hr. Cells were photographed under bright-field optics using a Zeiss Axiophot photomicroscope.

Epidermal leaf strips were peeled from the underside of leaves from transgenic plants and floated on X-gluc solution (see above) for 1 hr to 24 hr at room temperature. Leaf strips were viewed by light microscopy under bright-field optics and photographed.

β-Glucuronidase assays in vitro were conducted as described previously (Jefferson, 1987).

ACKNOWLEDGMENTS

We thank Dr. Kate VandenBosch for valuable assistance with the embedding, immunogold labeling, and silver enhancement procedures. We are grateful to Kerri L. Braden for her technical assistance, to Ray Vonder Haar for rapid synthesis of mutagenic oligonucleotides, and to Ron Hartwig, Linda Guarino, Don Jarvis, Greg Pogue, Loren Marsh, and A.L.N. Rao for providing valuable comments on the manuscript. This work was supported by Grant AI27832 from the National Institute of Allergy and Infectious Disease.

Received June 26, 1990; accepted August 13, 1990.

REFERENCES

Allison, R.F., Sorenson, J.C., Kelly, M.E., Armstrong, F.B., and Dougherty, W.G. (1985). Sequence determination of the capsid

protein gene and flanking regions of tobacco etch virus: Evidence for synthesis and processing of a polyprotein in potyvirus genome expression. Proc. Natl. Acad. Sci. USA **82,** 3969–3972.

Allison, R., Johnston, R.E., and Dougherty, W.G. (1986). The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: Evidence for the synthesis of a single polyprotein. Virology **154,** 9–20.

Amann, E., and Brosius, J. (1985). 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40,** 183–190.

An, G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. **81,** 86–91.

Baunoch, D., Das, P., and Hari, V. (1988). Intracellular localization of TEV capsid and inclusion proteins by immunogold labeling. J. Ultrastruct. Mol. Struct. Res. **99,** 203–212.

Carrington, J.C., and Dougherty, W.G. (1987). Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. J. Virol. **61,** 2540–2548.

Carrington, J.C., and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. **64,** 1590–1597.

Carrington, J.C., Cary, S.M., and Dougherty, W.G. (1988). Mutational analysis of tobacco etch virus polyprotein processing: *cis* and *trans* proteolytic activities of polyproteins containing the 49-kilodalton proteinase. J. Virol. **62,** 2313–2320.

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.

Carrington, J.C., Freed, D.D., and Oh, C.-S. (1990). Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. EMBO J. **9,** 1347–1353.

Danscher, G., and Nörgaard, J.O.R. (1983). Light microscopic visualization of colloidal gold in resin-embedded tissue. J. Histochem. Cytochem. **31,** 1394–1398.

Dingwall, C., and Laskey, R.A. (1986). Protein import into the cell nucleus. Annu. Rev. Cell. Biol. **2,** 367–390.

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 Hiebert, E. (1980a). 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 **104,** 174–182.

Dougherty, W.G., and Hiebert, E. (1980b). Translation of potyvirus RNA in a rabbit reticulocyte lysate: Reaction conditions and identification of capsid protein as one of the products of in vitro translation of tobacco etch and pepper mottle viral RNAs. Virology **101,** 466–474.

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

Ellis, R.J. (1981). Chloroplast proteins: Synthesis, transport and assembly. Annu. Rev. Plant Physiol. **32,** 111–137.

Faaberg, K.S., and Peebles, M.E. (1988). Strain variation and nuclear association of Newcastle disease virus matrix protein. J. Virol. **62,** 586–593.

Fink, S. (1987). Some new methods for affixing sections to glass slides. I. Aqueous adhesives. Stain Technol. **62,** 27–33.

Hall, T.C., Miller, W.A., and Bujarski, J.J. (1982). Enzymes involved in the replication of plant viral RNAs. In Advances in Plant Pathology, Vol. 1, D. Ingram and P.A. Williams, eds (London: Academic Press), pp. 179–211.

Hari, V. (1981). The RNA of tobacco etch virus: Further characterization and detection of protein linked to RNA. Virology **112,** 391–399.

Hari, V., Siegel, A., Rozek, C., and Timberlake, W.E. (1979). The RNA of tobacco etch virus contains poly (A). Virology **92,** 568–571.

Harlow, E., and Lane, D. (1988). Immunizations. In Antibodies: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 53–138.

Hollings, M., and Brunt, A.A. (1981). Potyviruses. In Handbook of Plant Virus Infections and Comparative Diagnosis, E. Kurstak, ed (New York: Elsevier/North Holland Biomedical Press), pp. 732–777.

Hunt, T. (1989). Cytoplasmic anchoring proteins and the control of nuclear localization. Cell **59,** 949–951.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Kavanagh, T.A., Jefferson, R.A., and Bevan, M.W. (1988). Targeting a foreign protein to chloroplasts using fusions to the transit peptide of a chlorophyll *a/b* protein. Mol. Gen. Genet. **215,** 38–45.

Klausner, R.D. (1989). Sorting and traffic in the central vacuolar system. Cell **57,** 703–706.

Knuhtsen, H., Hiebert, E., and Purcifull, D.E. (1974). Partial purification and some properties of tobacco etch virus induced intranuclear inclusions. Virology **61,** 200–209.

Langenberg, W.G. (1986). Virus protein association with cylindrical inclusions of two viruses that infect wheat. J. Gen. Virol. **67,** 1161–1168.

Lawson, R.H., and Hearon, S.S. (1971). The association of pinwheel inclusions with plasmodesmata. Virology **44,** 454–456.

Lesemann, D.-E. (1988). Cytopathology. In The Plant Viruses—The Filamentous Plant Viruses, Vol. 4, R.G. Milne, ed (New York: Plenum Publishing Corporation), pp. 179–235.

Lyles, D.S., Puddington, L., and McCreedy, B.J., Jr. (1988). Vesicular stomatitis virus M protein in the nuclei of infected cells. J. Virol. **62,** 4387–4392.

McFadden, G.I., Bonig, I., Cornish, E.C., and Clarke, A.E. (1988). A simple fixation and embedding method for use in hybridization histochemistry on plant tissues. Histochem. J. **20,** 575–586.

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.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G., and Sala, F. (1987). Hybrid genes in the analysis of transformation conditions. 1. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol. Biol. **8,** 363–373.

Newmeyer, D.D., and Forbes, D.J. (1988). Nuclear import can be separated into distinct steps in vitro: Nuclear pore binding and translocation. Cell **52,** 641–653.

Peränen, J., Rikkonen, M., Liljeström, P., and Kääriäinen, L. (1990). Nuclear localization of semliki forest virus-specific nonstructural protein nsP2. J. Virol. **64,** 1888–1896.

Richardson, W.D., Mills, A.D., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1988). Nuclear protein migration involves two steps: Rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell **52,** 655–664.

Riechmann, J.L., Laín, S., and García, J.A. (1989). The genome-linked protein and 5′ end RNA sequence of plum pox potyvirus. J. Gen. Virol. **70,** 2785–2789.

Roberts, B. (1989). Nuclear localization signal-mediated protein transport. Biochim. Biophys. Acta **1008,** 263–280.

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

Siaw, M.F.E., Shahabuddin, M., Ballard, S., Shaw, J.G., and Rhoads, R.E. (1985). Identification of a protein covalently linked to the 5′ terminus of tobacco vein mottling virus RNA. Virology **142,** 134–143.

Tadano, M., Makino, Y., Fukanaga, T., Okuno, Y., and Fukai, K. (1989). Detection of dengue 4 virus core protein in the nucleus. I. A monoclonal antibody to dengue 4 virus reacts with the antigen in the nucleus and the cytoplasm. J. Gen. Virol. **70,** 1409–1415.

Taylor, J.W., Ott, J., and Eckstein, F. (1985). The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucl. Acids Res. **13,** 8765–8785.

Wickner, W.T., and Lodish, H.F. (1985). Multiple mechanisms of protein insertion into and across membranes. Science **230,** 400–407.