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Bipartite Signal Sequence Mediates Nuclear Translocation of the Plant Potyviral NIa Protein

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The NIa protein of certain plant potyviruses localizes to the nucleus of infected cells. Previous studies have shown that linkage of NIa to reporter protein β-glucuronidase (GUS) is sufficient to direct GUS to the nucleus in transfected protoplasts and in cells of transgenic plants. In this study, we mapped sequences in NIa that confer karyophilic properties. A quantitative transport assay using transfected protoplasts, as well as an in situ localization technique using epidermal cells from transgenic plants, were employed. Two domains within NIa, one between amino acid residues 1 to 11 (signal domain I) and the other between residues 43 to 72 (signal domain II), were found to function additively for efficient localization of fusion proteins to the nucleus, although either region independently could facilitate a low level of translocation. Like signals from animal cells, both nuclear transport domains of NIa contain a high concentration of basic (arginine and lysine) residues. Nuclear transport signal domain II overlaps or is very near Tyr^{62}, which is the residue that mediates covalent attachment of a subset of NIa molecules to the 5′ terminus of viral RNA within infected cells. The nature of the NIa nuclear transport signal and the possibility for regulation of NIa translocation are discussed.

INTRODUCTION

The transport of proteins to the nucleus of eukaryotic cells is a tightly regulated process. Most proteins that are destined to the nuclear compartment contain specific signal sequences that are recognized by the import machinery (Roberts, 1989; Garcia-Bustos et al., 1991; Silver, 1991). Entry of proteins into the nucleus appears to involve at least two steps: binding to a nuclear pore receptor or complex and energy-dependent uptake through the pore (Newmeyer and Forbes, 1988; Richardson et al., 1988). Most of our knowledge concerning the requirements for nuclear translocation of proteins derives from studies of animal cells and cell extracts. Transport sequences and cellular factors involved in nuclear translocation of plant proteins are understood poorly. Experimental systems to examine this transport pathway in plant cells have been developed only recently (Restrepo et al., 1990; Varagona et al., 1991).

Several potyviruses, including tobacco etch virus (TEV), encode RNA replication-associated proteins that are sequestered in distinct subcellular compartments (Dougherty and Carrington, 1988). The potyviruses are positive-sense RNA-containing viruses that are serious pathogens in numerous crop species (Milne, 1988). The potyviral genome of approximately 10,000 nucleotides encodes a large polyprotein precursor that is proteolytically processed cotranslationally and posttranslationally into eight or more mature products and is linked covalently to a virus-encoded protein referred to as VPg (Dougherty and Carrington, 1988). The cytoplasmic CI protein has been demonstrated by Lain et al. (1990) to possess RNA helicase activity in vitro (using plum pox potyvirus), suggesting a role in RNA replication or template preparation. Two proteins, NIa and NIb, are localized predominantly in the nucleus (Knuhtsen et al., 1974; Baunoch et al., 1988; Restrepo et al., 1990). The multifunctional NIa protein catalyzes proteolytic processing of the viral polyprotein at five positions (Carrington and Dougherty, 1987a, 1987b; Carrington et al., 1988) and functions also as the genome-linked VPg (Shahabuddin et al., 1988; Murphy et al., 1990). The proteolytic domain of NIa is confined to the C-terminal half, whereas the VPg domain is located near the N terminus. Although the majority of NIa molecules are transported to the nucleus, virus particles containing genome-linked NIa (VPg) protein are found within the cytoplasm (Lesemann, 1988). Based on similarities with other positive-sense RNA viruses, the NIb protein probably represents the core RNA polymerase subunit that catalyzes chain elongation during viral RNA synthesis (Allison et al., 1986; Domier et al., 1987). Because the subcellular site(s) of potyviral RNA synthesis has not been identified, the role

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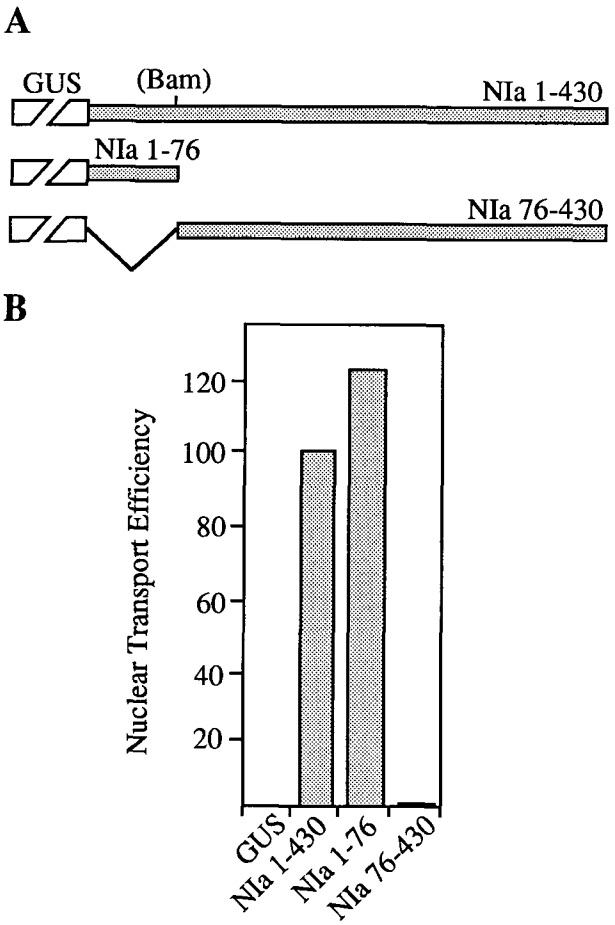

Figure 1. Nuclear Transport of GUS/NIa Fusion Proteins.

(A) Diagrammatic representation of GUS/NIa fusion proteins encoded by three plasmids. The amino acid residues from NIa are indicated above each diagram, as is the position that corresponds to the BamHI site in the NIa coding sequence.

(B) Relative nuclear transport efficiency of GUS/NIa fusion proteins in protoplasts. Measurements of GUS activity in nuclear and non-nuclear fractions of lysed protoplasts were conducted. The levels of activity in the nuclear fractions from pRTL2-GUS (encoding a nonfused GUS protein) and pRTL2-GUS/NIa (encoding a GUS/NIa fusion protein) transfected protoplasts were used to set the 0% and 100% relative nuclear transport efficiency levels, respectively (see Methods). The transport efficiencies of the GUS/NIa 1-76 and GUS/NIa 76-430 fusion proteins were determined from the averages of three transfection experiments.

of nuclear/cytoplasmic segregation of potyviral proteins is not known.

We have initiated studies to understand the role of nuclear transport of NIa and NIb during the infection process and to identify signals and factors that regulate their import into the nucleus. Such studies are likely to illuminate not only the events involved in potyviral replication, but also the process of nuclear translocation of plant cellular proteins. We have shown previously that both NIa and NIb contain nuclear targeting signals because fusion of either protein to a reporter enzyme (β-glucuronidase, or GUS) results in localization of reporter enzyme activity to the nucleus (Restrepo et al., 1990). The present study employs the GUS fusion strategy, deletion mutagenesis, and transient and transgenic plant assays to determine sequences within NIa that are necessary for nuclear transport. Two short regions near or within the VPg domain of NIa have been found to contain karyophilic signals.

RESULTS

A Quantitative System To Measure Nuclear Transport in Plant Protoplasts

The NIa and NIb proteins encoded by the TEV genome contain nuclear targeting information in protoplasts and in transgenic plants, as revealed by analysis of translocation of fusion proteins containing the marker enzyme GUS (Restrepo et al., 1990). A quantitative protoplast-based nuclear transport assay, in which GUS activity was measured in the nuclear and non-nuclear fractions of transfected cells after lysis, was developed to enable comparisons of nuclear translocation efficiencies of modified fusion proteins. The most reproducible conditions for quantitative recovery of intact nuclei involved rapid lysis of cells by multiple passages through a 27-gauge needle in buffer containing 0.3% Nonidet P-40, followed by sedimentation through a Percoll step gradient. The percentage of GUS activity in the nuclear fraction from pRTL2-GUS/NIa-transfected cells (expressing a fusion protein containing the entire NIa sequence) was usually between 25% and 50% and was defined arbitrarily as the 100% nuclear transport efficiency level for a given experiment. The activity associated with the nuclear fraction from pRTL2-GUS-transfected cells (expressing nonfused GUS) was usually 1% to 3% and was defined as the 0% transport efficiency level. In some experiments, pRG/NIa 1-76 (expressing a fusion protein containing the N-terminal 76 residues of NIa) was used as the 100% efficiency control plasmid.

N-Terminal Region of NIa Contains a Nuclear Localization Signal

The N-terminal 76 and the C-terminal 355 amino acid residues of NIa were assayed independently for nuclear translocation activity using pRG/NIa 1-76- and pRG/NIa 76-430-transfected cells, respectively, as shown in Figure 1A. The fusion protein encoded by pRG/NIa 1-76 was

found to accumulate in the nuclear fraction with an efficiency comparable with the pRTL2-GUS/NIa-encoded protein (Figure 1B). In contrast, the protein encoded by pRG/NIa 76-430 accumulated primarily in the non-nuclear fraction, indicating little or no nuclear translocation activity.

Transgenic plants that produced pRG/NIa 1-76- and pRG/NIa 76-430-encoded fusion proteins were generated, and GUS activity was localized by histochemical analysis of epidermal leaf strips. Subcellular localization of GUS activity was carried out also with plants transformed with pRTL2-GUS (cytoplasmic accumulation control), pRTL2-GUS/NIa (nuclear accumulation control), and pGA482 (binary vector control). Histochemical assays from pRTL2-GUS/NIa-transformed and pRG/NIa 1-76-transformed plants revealed the presence of GUS activity predominantly in the nucleus of each cell, as illustrated in Figures 2C and 2D. In contrast, GUS activity was distributed uniformly throughout the cytoplasm of pRG/NIa 76-430-transformed epidermal cells (Figure 2E). This distribution resembled that of GUS activity in cells from plants transformed with pRTL2-GUS sequences (Figure 2B). No GUS activity was detected in cells from plants transformed with pGA482 (Figure 2A). The transient protoplast and transgenic plant results indicate clearly that the signal(s) for nuclear targeting of NIa reside within the N-terminal 76 amino acid residues.

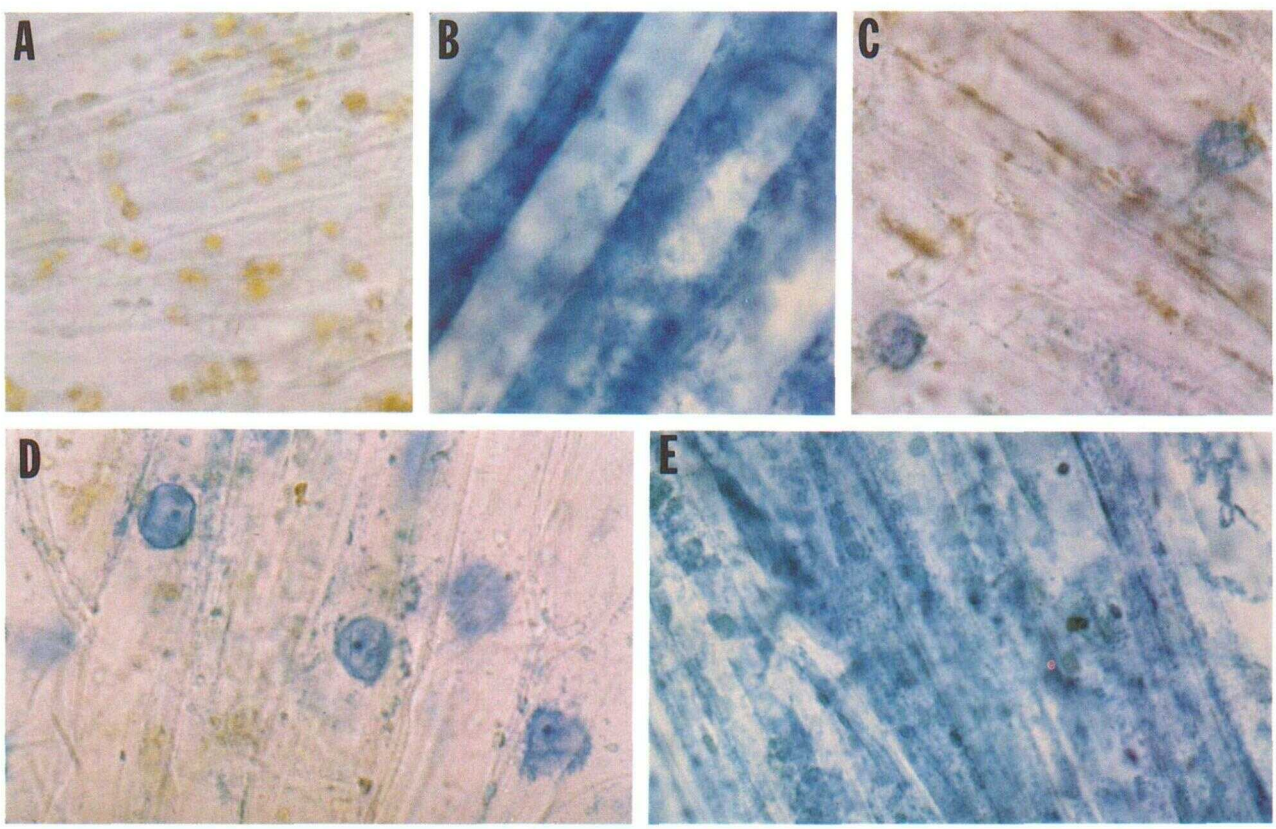

Figure 2. In Situ Localization of GUS Activity in Epidermal Leaf Cells of Transgenic Plants.

Leaf strips were incubated in the colorimetric substrate X-gluc and viewed under bright-field optics. One centimeter in the photographs equals approximately 20 μM.

(A) Leaf strip from pGA482-transformed tobacco. The plasmid pGA482, which does not contain the GUS coding sequence, was the binary vector used in all transgenic plant constructions.

(B) Leaf strip from pRTL2-GUS-transformed tobacco, expressing a nonfused GUS protein.

(C) Leaf strip from pRTL2-GUS/NIa-transformed tobacco.

(D) Leaf strip from pRG/NIa 1-76-transformed tobacco. Note the localization of GUS activity predominantly in the nuclear compartment.

(E) Leaf strip from pRG/NIa 76-430-transformed tobacco. Note that this fusion protein has no affinity for the nuclear compartment.

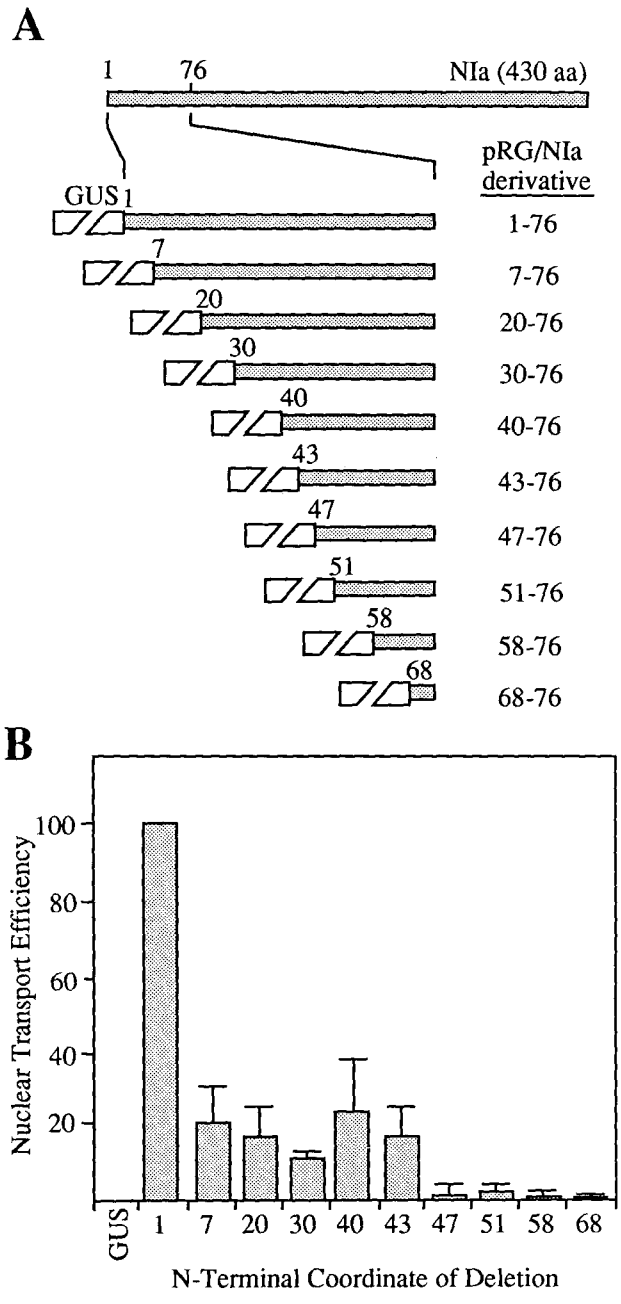

Figure 3. Relative Nuclear Transport Efficiencies of GUS/NIa Fusion Proteins Containing N-Terminal Deletions of NIa.

(A) Diagrammatic representation of GUS/NIa fusion proteins encoded by pRG/NIa deletion variants. The amino acid position at the N terminus of the NIa sequence is given above each diagram. **(B)** Nuclear transport efficiency of each fusion protein in transfected protoplasts was determined as described in Methods and in the legend to Figure 1. Nuclear transport activities of pRTL2-GUS- and pRG/NIa 1-76-encoded proteins were used as the 0% and 100% transport efficiency standards, respectively. The graph shows the averages and standard deviations from five to seven independent experiments.

Deletion Mapping of the NIa Nuclear Localization Signal

To map further the boundaries of the nuclear targeting signal, 5′-terminal and 3′-terminal deletions were introduced into the NIa coding sequence in pRG/NIa 1-76. Nuclear transport of each GUS/truncated-NIa fusion protein was assayed in transfected protoplasts using the pRG/NIa 1-76-derived fusion protein as the 100% transport efficiency standard (some constructs were analyzed also in transgenic plants). Nine deletions that resulted in removal of coding sequence for 6 to 67 amino acid residues from the N terminus of NIa were prepared, as shown in Figure 3A. GUS/NIa 7-76 protein, missing the N-terminal 6 residues of NIa, was transported to the nucleus with 20% efficiency (Figure 3B). Similarly, proteins encoded by pRG/NIa 20-76, pRG/NIa 30-76, pRG/NIa 40-76, and pRG/NIa 43-76 were transported with intermediate efficiencies ranging between 10% and 23%. Fusion proteins derived from pRG/NIa 47-76, pRG/NIa 51-76, pRG/NIa 58-76, and pRG/NIa 68-76, on the other hand, possessed little or no nuclear transport activity (1% to 3% efficiency). These data imply the existence of two regions that influence nuclear transport. One region is disrupted by deletion of the N-terminal 6 amino acids and the other is impaired by deletion of the N-terminal 46 residues.

The 3′-terminal deletion series resulted in plasmids encoding nine C-terminal truncated variants, as depicted in Figure 4A. Fusion proteins composed of NIa amino acids 1 to 76 and 1 to 72 were transported to the nucleus with comparable high efficiencies (Figure 4B). Protein containing amino acids 1 to 67, however, was transported with an efficiency of approximately 10% relative to the control (pRG/NIa 1-76-derived protein). Each of the other seven C-terminal deletion variants, the shortest of which possessed only the N-terminal 11 residues of NIa, was transported with intermediate efficiency. These results again suggest a bipartite organization of the NIa nuclear transport signal, with one region being interrupted by removal of all sequence beyond position 67, and the other being affected by deletion of the N-terminal 11 residues.

Transgenic plants expressing the GUS/NIa C-terminal deletion variants encoded by pRG/NIa 1-72, pRG/NIa 1-47, pRG/NIa 1-42, and pRG/NIa 1-18 were generated. Protein gel blot analysis using anti-GUS serum (provided by D. Bergey and T. McKnight, Texas A&M University) indicated that each fusion protein was present in extracts from these plants and that their apparent molecular masses were slightly larger than that of nonfused GUS (68 kD) (data not shown). Both the GUS/NIa 1-76 and GUS/NIa 1-72 proteins were identified predominantly in the nucleus of epidermal cells, as shown in Figures 5A and 5B. Fusion proteins encoded by pRG/NIa 1-42 and pRG/NIa 1-18 were localized primarily to the cytoplasm (Figures 5D and 5E), although a low level of accumulation was detected usually in the nucleus in both cases. These

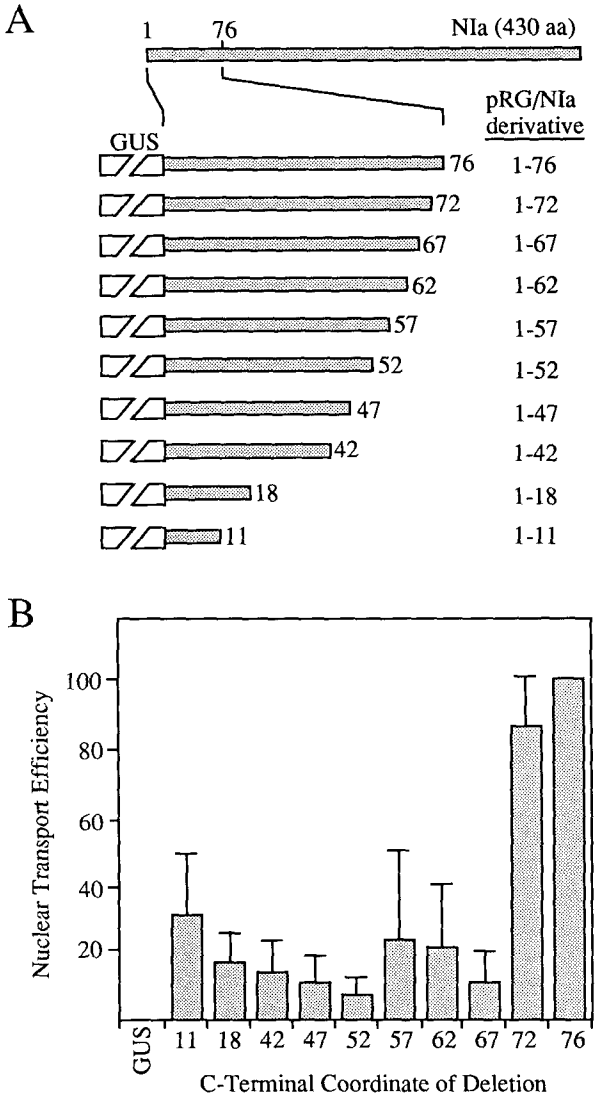

Figure 4. Relative Nuclear Transport Efficiencies of GUS/NIa Fusion Proteins Containing C-Terminal Deletions of NIa.

(A) Diagrammatic representation of GUS/NIa fusion proteins encoded by pRG/NIa deletion variants. The amino acid position at the C terminus of the NIa sequence is given beside each diagram. **(B)** Nuclear transport efficiency of each fusion protein in transfected protoplasts was determined as described in Methods and in the legend to Figure 1. Nuclear transport activities of pRTL2-GUS- and pRG/NIa 1-76-encoded proteins were used as the 0% and 100% transport efficiency standards, respectively. The graph shows the averages and standard deviations from five to 16 independent experiments.

distribution patterns were consistent with the results of the protoplast transient assays. The fusion protein encoded by pRG/NIa 1-47 was identified almost exclusively within the nuclear compartment of transgenic plant cells (Figure 5C). Because the transient assay data suggested that this protein should have been localized predominantly to the cytoplasm, this result was surprising.

Nuclear transport signals within animal and yeast proteins are characterized by high concentrations of Arg and Lys residues within a short polypeptide segment (Roberts, 1989; Silver, 1991). Two highly basic regions are located within the N-terminal 76 residues of NIa (Allison et al., 1986). The amino acid sequence between positions 2 and 10 contains 5 Lys residues, whereas the sequence between position 41 to 45 contains 3 Lys and 1 Arg residue. Two deletions that affected these regions were introduced into the NIa coding sequence of pRTL2-GUS/NIa, as given in Figure 6A, and their effects were determined in the protoplast transient assay. One deletion construct encoded a fusion protein lacking NIa amino acid residues 2 to 10 (pRTL2-GUS/NIaΔ2-10). The other construct encoded a protein lacking amino acid residues 43 to 46 (pRTL2-GUS/NIaΔ43-46). Both of the resulting fusion proteins were transported to the nucleus, but with intermediate efficiencies (Figure 6B). The Δ2-10 variant also was expressed in transgenic plants. In situ localization of GUS activity revealed that this protein was present in both nuclear and cytoplasmic compartments (data not shown). These data suggest that two regions near the N terminus of NIa function to promote efficient nuclear transport.

DISCUSSION

We have investigated the primary sequence requirements for nuclear translocation of the NIa protein encoded by TEV. This study was facilitated by the development of a quantitative nuclear transport assay, as well as a transgenic plant in situ localization technique. These methods have several notable features that should permit their widespread application. First, the GUS fusion strategy obviates the requirement for immunological techniques for the localization of modified proteins. Fluorescently tagged antibody probes often are limited by high levels of plant cell autofluorescence, high levels of cross- or nonspecific reactivity, or the presence of the nuclear protein under study as a normal constituent of the cell (Knox, 1982). Second, highly sensitive quantitative and qualitative assays have been developed to analyze GUS reporter activity in vitro and in situ (Jefferson, 1987). And third, the transient protoplast assay and subcellular fractionation protocols are rapid, thus allowing the analysis of numerous protein derivatives that result from site-directed mutations and deletions. Potential complications of the transient assay, however, involve the possibility for extraction or leakage

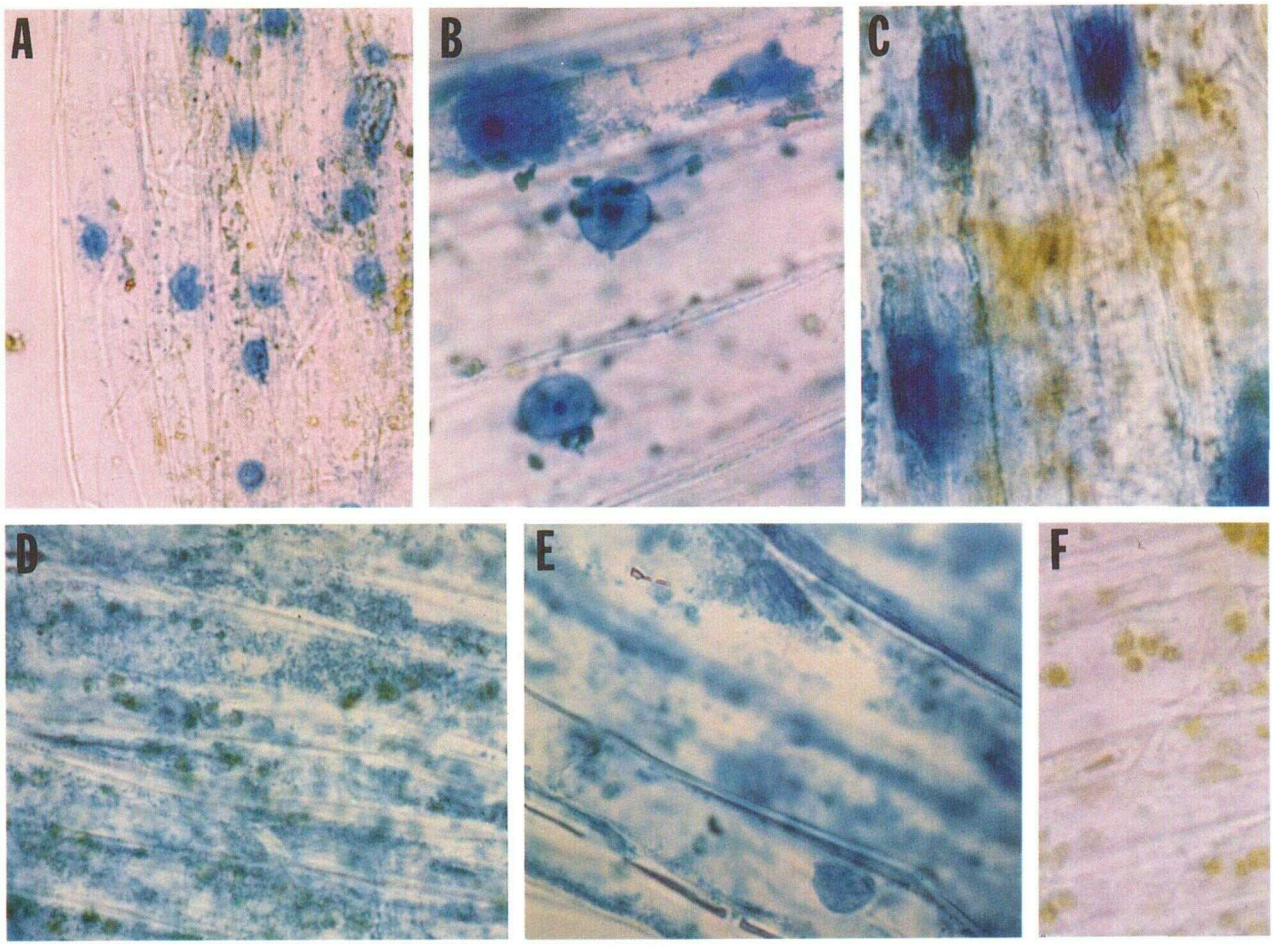

Figure 5. In Situ Localization of GUS Activity in Epidermal Leaf Cells of Transgenic Plants Containing GUS/NIa Deletion Variants.

Leaf strips were incubated in the colorimetric substrate X-gluc and viewed under bright-field optics. One centimeter in the photographs equals approximately 20 μm, except in panel **(A)**, where 1 cm equals approximately 50 μm.
(A) Leaf strip from pRG/NIa 1-76-transformed tobacco. This sample was from the same transgenic plant that was used in Figure 2D.
(B) Leaf strip from pRG/NIa 1-72-transformed tobacco.
(C) Leaf strip from pRG/NIa 1-47-transformed tobacco.
(D) Leaf strip from pRG/NIa 1-42-transformed tobacco.
(E) Leaf strip from pRG/NIa 1-18-transformed tobacco.
(F) Leaf strip from pGA482-transformed tobacco.

of proteins from the nucleus during the isolation protocol, as well as disruption of nuclei during the lysis step. Hence, we advocate the use of the transient protoplast system and the transgenic plant in situ assay as complementary approaches for the analysis of plant nuclear translocation signals.

All of the analyses reported here have involved fusion of NIa sequences to the C terminus of GUS. We have found that appending NIa to either the N or C terminus of GUS results in fusion proteins that are transported to the nucleus with comparable efficiencies (J.C. Carrington, D.D. Freed, and M.A. Restrepo-Hartwig, unpublished data). We believe, therefore, that the NIa nuclear translocation signal functions in a position independent fashion. We employed the C-terminal GUS fusions in this study because the enzymatic activity of GUS is highest when NIa is linked in this manner (Restrepo et al., 1990).

The N-terminal and C-terminal deletion analysis using the protoplast transfection assay suggested that the NIa nuclear transport signal is divided among two distinct polypeptide regions. Deletion of the NIa sequence beyond position 72 had little or no effect on nuclear transport,

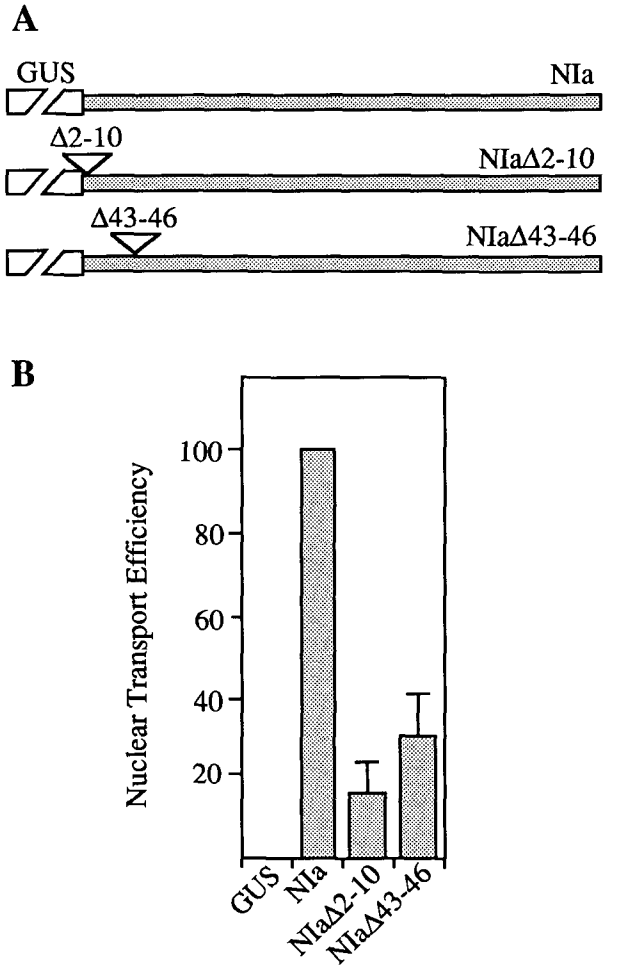

Figure 6. Nuclear Transport of GUS/NIa Fusion Proteins Containing Deletions in Putative Nuclear Transport Signal Sequences.

(A) Diagrammatic representation of GUS/NIa fusion proteins encoded by pRTL2-GUS/NIa, pRTL2-GUS/NIaΔ2-10, and pRTL2-GUS/NIaΔ43-46.

(B) Nuclear transport efficiency of each fusion protein in transfected protoplasts was determined as described in Methods and in the legend to Figure 1. Nuclear transport activities of pRTL2-GUS- and pRTL2-GUS/NIa-encoded proteins were used as the 0% and 100% transport efficiency standards, respectively. The graph shows the averages and standard deviations from four independent experiments.

indicating that karyophilic information is contained near the N terminus of the protein. Deletion of sequences past amino acid 67 lowered the fusion protein nuclear translocation efficiency to an intermediate level, as did deletions that resulted in elimination of sequences beyond positions 62, 57, 52, 47, 42, 18, and 11 (Figure 4). We propose that deletion of sequences through position 67 disrupts the C-terminal boundary of one domain of the nuclear transport signal and that sequences between residues 1 and 11 contain an additional, semiautonomous translocation sequence. Results of the N-terminal deletion series support this view. Deletion of the N-terminal 6, 19, 29, 39 or 42 amino acid residues of NIa lowered the transport efficiency to an intermediate level, whereas deletion of the N-terminal 46, 50, 57, or 67 residues nearly or completely eliminated transport activity (Figure 3). These results can be explained by proposing that deletion of the N-terminal 6 amino acid residues disrupts the functioning of one nuclear transport domain (signal domain I, between positions 1 and 11), whereas deletion beyond position 42 interrupts the second component (signal domain II, between positions 43 and 72) of the transport signal. Although the two domains appear to function with partial independence, optimal nuclear translocation clearly requires both sequences.

The observed effects of the C-terminal deletions of NIa on fusion protein nuclear transport in transgenic plants were in agreement with the transient assay results, but with one exception. The GUS/NIa 1-47 fusion protein was found to localize primarily to the nucleus in transgenic plants (comparable with GUS/NIa 1-76 and GUS/NIa 1-72 proteins), whereas it was transported to the nucleus with intermediate efficiency (10%) in the protoplast transient assays. This could be due possibly to the presence of an essential localization signal between positions 43 and 47, which is sufficient for transport in transgenic plants, and a transport-enhancing sequence between positions 48 and 72. The latter element may be necessary for efficient localization in protoplasts where physiological conditions are suboptimal. It should be noted also that the protoplast incubation time after transfection was relatively short (20 hr) compared with the extended period during which protein transport could have occurred in transgenic plant cells. The idea of a modulator sequence that exerts a quantitative effect on a nuclear targeting signal is not without precedent. The ability of the nuclear targeting signal (Pro-Lys-Lys-Lys-Arg-Lys-Val) of simian virus 40 large T-antigen to direct a foreign protein to the nucleus is enhanced dramatically by the presence of 15 amino acid residues that precede the signal in the T-antigen sequence (Rihs and Peters, 1989).

The two regions of NIa that contribute to nuclear localization are characterized by a dense clustering of Lys and Arg residues, shown in Figure 7, a feature found in most or all nuclear localization signals from animal and yeast cells (Roberts, 1989). Also, several animal cell and viral nuclear proteins have been found to contain multiple translocation signals that function independently to transport heterologous proteins to the nucleus (Richardson et al., 1986; Picard and Yamamoto, 1987; Dang and Lee, 1988). The polyoma virus large T-antigen, for example, contains two signal sequences that are separated by approximately 90 amino acid residues and that function cooperatively to facilitate nuclear translocation (Richardson et al., 1986). Deletion of one signal lowers, but does not eliminate,

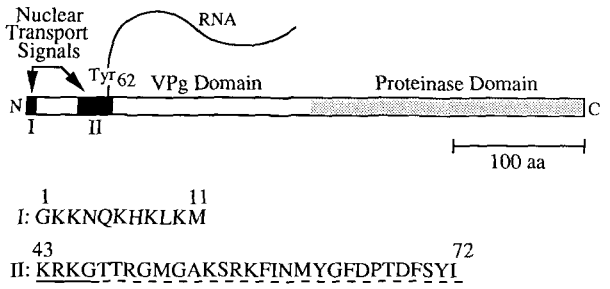

Figure 7. Diagrammatic Representation of the NIa Protein of TEV.

The various domains in the 430-amino acid protein are shown. The identification of Tyr^{62} as the VPg-RNA attachment site, as well as the identification of the boundaries of the proteinase domain, were determined previously by Murphy et al. (1991) and Carrington and Dougherty (1987b), respectively. The amino acid sequences of the two regions that contain nuclear translocation signals are shown below. The solid underline of sequence II indicates the region that, along with sequence I, is sufficient for near-complete nuclear localization of GUS/NIa fusion proteins in transgenic plants. The dashed underline indicates the additional sequences that are necessary for efficient localization of fusion proteins in transfected protoplasts. aa, amino acids.

nuclear transport efficiency. This division of nuclear transport information appears to resemble that found within the potyviral NIa protein, where the two signaling domains are separated by approximately 30 amino acid residues. Recently, a class of nuclear transport signals that consist of two interdependent basic domains separated by 10 to 12 residues has been identified. The nucleoplasmin transport signal, Lys-Arg-$(X)_{10}$-Lys-Lys-Lys-Lys, can tolerate certain amino acid substitutions within one of the basic regions and between the basic regions, but not within both basic regions simultaneously (Robbins et al., 1991). Interestingly, the sequence Lys-Arg-Lys-$(Xaa)_{10}$-Arg-Lys is found in the NIa signal domain II, between amino acid positions 43 and 57 (Figure 7). Until a higher resolution mutational analysis is conducted, however, the contribution of specific residues in the putative NIa transport signal is a matter for speculation.

The potyviral NIa protein performs two known functions. A proteinase domain within the C-terminal one-half of the molecule catalyzes proteolytic processing at five sites in the viral polyprotein (Carrington and Dougherty, 1987b), whereas a VPg domain that facilitates covalent linkage of NIa to the 5′ terminus of viral RNA is found near the N terminus (Murphy et al., 1991). Covalent attachment of NIa (or an N-terminal fragment of NIa) to RNA occurs through a phosphoester linkage with the hydroxyl group of Tyr at position 62 (Figure 7). Although the site of RNA replication has not been identified, mature virus particles accumulate in the cytoplasm of infected cells. If RNA synthesis occurs in the cytoplasm, as is the case with most or all well-characterized positive-strand RNA viruses, nuclear transport of NIa might be suppressed by interactions with viral RNA or with other replication-associated proteins in the cytoplasm. In addition, translocation of NIa may be affected by proteolytic processing of the viral polyprotein. Certain polyprotein processing intermediates containing NIa may be transported with decreased efficiency, providing a cytoplasmic molecule that could perform a necessary function during the virus life cycle. Given the importance of known regulatory mechanisms that control nuclear transport of proteins during growth and development of animal cells (Hunt, 1989), it is worth investigating possible mechanisms that may control NIa nuclear translocation. Such studies may reveal not only processes that affect potyviral replication, but also signal transduction mechanisms that operate to control plant cell growth, division, and differentiation.

METHODS

Bacterial Strains and Plants

Cloning of plasmids was performed usually in *Escherichia coli* TG1. Single-stranded DNA for sequence analysis was produced from plasmids after infection of cells with the defective helper phage M13K07. Single-stranded DNA for site-directed mutagenesis was generated in *E. coli* strain RZ1032 (*dut*$^-$ *ung*$^-$). *Agrobacterium tumefaciens* LBA4404 was used for leaf-disc transformation. Transgenic plant experiments were conducted using *Nicotiana tabacum* varieties Xanthi-nc and $Havana_{425}$. *Nicotiana tabacum* Xanthi-nc was used for isolation of protoplasts.

Vectors and Recombinant Plasmids

β-Glucuronidase/NIa gene fusions were assembled initially in the vector pRTL2, which contains the cauliflower mosaic virus 35S promoter with a duplication in the upstream regulatory sequence (between nucleotide −340 and −90 relative to the transcriptional start site), the TEV 5′ nontranslated sequence, and the cauliflower mosaic virus polyadenylation signal. The GUS coding sequence was introduced into pRTL2, forming pRTL2-GUS. Fusion of the 5′ terminus of the NIa coding region to the 3′ terminus of the (GUS) sequence was accomplished by ligation at a BglII restriction site, which was introduced into the relevant plasmids by site-directed mutagenesis. This initial construct, pRTL2-GUS/NIa, encoded a fusion protein of approximately 117 kD consisting of NIa appended to the C terminus of GUS. Each of the aforementioned plasmids has been described previously (Restrepo et al., 1990). All fusion proteins described in this work contained the NIa sequence adjacent to the C terminus of GUS. Mutations were introduced by the method of Kunkel et al. (1987). The nucleotide sequence around each mutation site was verified by dideoxynucleotide sequence analysis.

Several plasmids encoding GUS/NIa-deletion variants were generated in the vector pRTL2SK, which differs from pRTL2 by

possessing the M13 IG region as well as a series of stop codons in each reading frame at the beginning of the 3′ nontranslated region. pRTL2SK-derived plasmids encoding GUS/NIa fusions were assigned names with a prefix, pRG/NIa, followed by a designation of the boundaries of the encoded NIa amino acid sequence (e.g., 76-430). pRG/NIa 1-76 was produced by deleting the coding sequence for amino acid residues 77 to 430 (the C terminus of the protein) through removal of a restriction fragment bounded by BamHI sites. BamHI restriction sites are located within the NIa coding sequence (around codon 76) and immediately beyond the NIa sequence in the 3′ noncoding region. The reciprocal deletion construct, pRG/NIa 76-430, was generated by site-directed insertion of a BamHI restriction site at the 5′ end of the NIa coding sequence (immediately downstream from the GUS sequence), followed by removal of the resulting BamHI-BamHI restriction fragment containing codons 1 to 76.

The plasmid pRG/NIa 1-76 was used to derive additional NIa deletion constructs. Five plasmids (pRG/NIa 1-72, 1-47, 1-42, 1-30, and 1-18) that encoded GUS/NIa fusion proteins with truncations at the NIa C terminus were produced by the exonuclease III/S1 nuclease strategy (Henikoff, 1984). Five additional plasmids (pRG/NIa 1-67, 1-62, 1-57, 1-52, and 1-11) that encoded C-terminal deletion variants were constructed by first introducing BamHI restriction sites at various positions within the NIa coding sequence by site-directed mutagenesis and then removal of the coding sequence downstream from the introduced site by excision of the BamHI-BamHI restriction fragment. A series of plasmids (pRG/NIa 7-76, 20-76, 30-76, 40-76, 51-76, 58-76, and 68-76) that encoded GUS/NIa fusion proteins with progressive deletion of N-terminal amino acid residues of NIa was generated as follows. Site-directed insertion of BglII restriction sites at several positions was carried out. Because the GUS/NIa boundary within pRG/NIa 1-76 contains a BglII site, removal of the resulting BglII-BglII restriction fragment encompassing various lengths of the 5′ coding sequence was carried out.

The coding sequences for NIa amino acid residues 1-11 and 43-46 were deleted individually from pRTL2-GUS/NIa by site-directed "loop-out" mutagenesis (Kunkel et al., 1987). The resulting plasmids were named pRTL2-GUS/NIaΔ1-11 and pRTL2-GUS/NIaΔ43-46.

The expression cassette (35S promoter, TEV 5′ nontranslated sequence, coding sequence, and polyadenylation site) from several of the plasmids described above was excised using HindIII and inserted into the HindIII site of the binary vector pGA482 (An, 1986). The resulting recombinant plasmids were mobilized into *A. tumefaciens* by triparental mating (Ditta et al., 1980).

Analysis of Nuclear Transport of GUS/NIa Fusion Proteins in Protoplast Transient Assays

N. tabacum cv Xanthi-nc protoplasts were prepared, transfected with supercoiled plasmid DNA, and incubated in culture medium for approximately 20 hr as described previously (Restrepo et al., 1990). Cells (2 to 3 × 10^5) were harvested by centrifugation and resuspended in 250 μL of nuclear isolation buffer (NIB) consisting of 250 mM sucrose, 20 mM Tris-HCl, pH 6.8, 1.5 mM $MgCl_2$, 0.14 M NaCl, 10 mM β-mercaptoethanol, 5% glycerol, and 0.3% Triton X-100. To facilitate lysis, the cell suspension was passed immediately five to 10 times through a 27-gauge needle attached to a syringe. Half of the lysed sample (125 μL) was layered onto a 15%/50% Percoll (Pharmacia, diluted in NIB) step gradient (100 μL per step) in a 1.5-mL microcentrifuge tube. The gradients were placed in a swinging bucket rotor and spun at 2000*g* for 15 min at 10°C. The nuclear fraction was collected at the interface of the Percoll layers, whereas the cytosolic/lysed organelle fraction was recovered from above the 15% Percoll zone. The volumes of the two fractions were equalized by addition of NIB to the nuclear fraction. *N*-Lauryl sarcosine (1 mg/mL) was added to both fractions to dissociate protein aggregates and membranes, and GUS activity was measured as described (Carrington and Freed, 1990).

Analysis of Nuclear Transport of GUS/NIa Fusion Proteins in Transgenic Plants

A. tumefaciens-mediated leaf-disc transformation and plant regeneration were accomplished by methods published previously (Horsch et al., 1985; Carrington and Freed, 1990). Subcellular localization of GUS activity in epidermal cells using the colorimetric histochemical substrate X-gluc has been described (Restrepo et al., 1990). Cells were viewed and photographed using a Zeiss Axiophot photomicroscope with bright-field optics.

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