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Nuclear Export in Plants: Use of Geminivirus Movement Proteins for a Cell-Based Export Assay

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The nuclear export of proteins and RNAs has been studied in heterokaryons or by microinjecting test substrates into nuclei of HeLa cells or Xenopus oocytes. We have previously shown that the two movement proteins BR1 and BL1 encoded by the plant pathogenic squash leaf curl virus act in a coordinated manner to facilitate virus cell-to-cell movement and that one of these (BR1) is a nuclear shuttle protein. By using a novel in vivo cell-based assay for nuclear export in which nuclear-localized BR1 is trapped by BL1 and redirected to the cortical cytoplasm, we demonstrate that residues 177 to 198 of BR1 contain a leucine-rich nuclear export signal (NES) of the type found in the Rev protein encoded by the human immunodeficiency virus and in Xenopus TFIIIA. We further show that the TFIIIA NES can functionally replace the NES of BR1 in both nuclear export and viral infectivity. These findings suggest that this basic pathway for nuclear export is highly conserved among plant and animal cells and in yeast.

INTRODUCTION

A fundamental aspect of cellular control is the regulation of bidirectional trafficking of macromolecules between the nucleus and cytoplasm that occurs through nuclear pore complexes (NPCs) (Forbes, 1992; Davis, 1995). Most substrates (proteins and nucleoprotein complexes) are transported across these channels by an active process that is saturable and involves the recognition of specific signal sequences on the cargo being transported (Görlich and Mattaj, 1996; Corbett and Silver, 1997; Görlich, 1997). The inhibition of both nuclear import and export by monoclonal antibodies against NPC components when microinjected into Xenopus oocytes demonstrates that the same NPCs function for nuclear import and export (Featherstone et al., 1988).

Nuclear import, which has been well studied in animal cells and yeast, is now being biochemically dissected in plant cells, and the fundamental import machinery appears to be highly conserved (Hicks et al., 1995, 1996; Merkle et al., 1996; Corbett and Silver, 1997; Haizel et al., 1997). This process is mediated by nuclear localization signals (NLSs) within the import substrate, which are recognized by soluble factors that dock the substrate to the NPC. The best characterized NLSs are the classic single and bipartite basic amino acid targeting sequences first described in simian virus 40 T antigen and nucleoplasmin, respectively (Görlich, 1997). A third type of NLS composed of basic and hydrophobic amino acids occurs in the yeast mating factor MATα2 and the maize transcription factor R but appears not to function in mammalian cells (Hicks and Raikhel, 1995). Despite their lack of similarity, competition binding studies using isolated plant nuclei suggest that both the MATα2-type NLS and the classic basic NLS bind to the same nuclear site(s) (Hicks et al., 1995).

Nuclear targeting of proteins that bear a classic basic NLS is mediated by the soluble importin α/β heterodimer (also termed karyopherin α/β) through NLS-dependent binding to the importin α subunit, followed by importin β–mediated docking of the complex at the NPC (Corbett and Silver, 1997; Görlich, 1997). Transport across the NPC then occurs in an energy-dependent process that in most cases requires a cycle of GTP hydrolysis mediated by the small Ras-like GTPase Ran and additional proteins that regulate the guanine nucleotide–bound state of Ran (Corbett and Silver, 1997; Görlich, 1997). The bifunctional M9 domain was first identified as a nuclear import signal in the A1 protein that is found in the complex of >20 proteins bound to pre-mRNA molecules in heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. This 38–amino acid domain lacks clusters of basic amino acids and bears no obvious resemblance to classic basic NLSs. Transportin, distantly related to importin β, acts to both bind the M9 domain within A1 and dock the A1 protein to the nuclear pore (Nakielny et al., 1996; Pollard et al., 1996; Fridell et al., 1997), with translocation across the

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NPC again requiring binding to Ran and GTP hydrolysis. This same M9 domain within A1 further functions in the export of mRNA from the nucleus to the cytoplasm (Nakielny et al., 1996; Pollard et al., 1996).

Our knowledge of the mechanism of nuclear export, and indeed, understanding whether export is an active energy-dependent process or simply involves masking of a nuclear retention signal, has lagged behind that of import due to the difficulty in demonstrating that a protein shuttles between the nucleus and cytoplasm and the arduous nature of the assays used. Nuclear shuttle proteins appear nuclear localized at equilibrium, apparently because the rate of import is more rapid than that of export. Export of these to the cytoplasm has been detected by either internuclear exchange of proteins in heterokaryons or the cytoplasmic accumulation of test substrates that were microinjected into nuclei of HeLa cells or Xenopus oocytes (Goldstein, 1958; Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b).

Recent molecular and classical genetic studies in animal cells and yeast have identified an essential nuclear export signal (NES) in proteins that rapidly shuttle between the nucleus and cytoplasm. First characterized in the human immunodeficiency virus (HIV) Rev protein, transcription factor IIIA (TFIIIA) from Xenopus, and inhibitor of protein kinase A (PKI) (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b), this NES is a leucine-rich hydrophobic sequence of 10 to 13 amino acids. Mutational studies show that the leucine residues are essential for function and that the NESs of PKI and TFIIIA can each functionally substitute for that in Rev (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b). Although these NESs are distinct in sequence from NLSs, including those such as the bifunctional M9 domain (Pollard et al., 1996), they resemble NLSs in being short stretches of amino acids that lack any apparent secondary structure because, when fused to a test substrate, they act in a position-independent manner to confer the ability to be exported from the nucleus (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996a, 1996b).

The bipartite geminivirus squash leaf curl virus (SqLCV) encodes the movement proteins BR1 and BL1, which cooperatively act to move the viral single-stranded DNA genome from its site of replication in the nucleus through the cytoplasm and across the cell wall for local cell-to-cell movement of the virus and eventual systemic infection of host plants (Noueiry et al., 1994; Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996; Ward et al., 1997). BR1 is a single-stranded DNA binding protein that has been shown to shuttle between the nucleus and cytoplasm in plant cells. It contains two basic NLSs within its N-terminal 100 residues, and at equilibrium, localizes to nuclei in systemic leaves from infected pumpkin plants and in transfected tobacco protoplasts (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996). However, this equilibrium is perturbed, and BR1 can be shown to shuttle from the nucleus to the cytoplasm when the second movement protein BL1 is present (Sanderfoot et al., 1996). BL1 is responsible for virus cell-to-cell movement (Noueiry et al., 1994; Ward et al., 1997). When coexpressed with BR1 in transfected tobacco protoplasts, BL1 traps BR1 in the cytoplasm and relocalizes it from the nucleus to the cell periphery (cortical cytoplasm), which is consistent with its proposed role in moving BR1–genome complexes to and across the cell wall (Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996). This specific interaction between the SqLCV movement proteins has been used to identify functional domains within BR1 and BL1 (Sanderfoot and Lazarowitz, 1996).

Current investigations in animal cells and yeast suggest that multiple pathways for nuclear import and export exist (Lewis et al., 1996; Deane et al., 1997; Görlich, 1997; Schlenstedt et al., 1997). Although studies in animal cells and yeast continue to identify new signals for nuclear import and export and are beginning to identify export receptors (Fornerod et al., 1997; Schlenstedt et al., 1997; Stade et al., 1997; Arts et al., 1998; Senger et al., 1998; Truant et al., 1998), to date no studies on the mechanism of nuclear shuttling in plants cells have been reported, nor has a plant nuclear export signal been identified. Using the cooperative interaction of the two movement proteins encoded by SqLCV, we report here the identification of a nuclear export signal in BR1 that resembles the NES in HIV Rev, TFIIIA, and PKI. We further show that the Xenopus TFIIIA NES can functionally substitute for this plant NES in both nuclear export and viral infectivity, suggesting that the basic export machinery is highly conserved between animals, plants, and yeast.

RESULTS

Leucine Residues at Positions 189, 190, and 193 in BR1 Are Essential for Viral Infectivity

Inspection of the predicted amino acid sequence of BR1 revealed that the residues located between positions 184 and 193 resembled the NES found in HIV Rev, TFIIIA, and several other rapidly shuttling nuclear proteins (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b). As shown in Table 1, this sequence, located just upstream from the BL1 interactive domain (amino acids 199 to 256) within BR1, is hydrophobic in character and contains four leucine residues. Site-directed mutational studies have shown that the leucine residues within the NES of HIV Rev and PKI are essential for its function in export (Table 1) (Fischer et al., 1995; Fridell et al., 1996b). Thus, to examine the importance of this leucine-rich region for BR1 function, we mutated the leucine residues at positions 189, 190, and 193 to alanine residues and tested the resulting SqLCV mutant *BR1*$^{L189A/L190A/L193A}$ for infectivity.

As shown in Table 2, *BR1*$^{L189A/L190A/L193A}$ was null for infectivity in pumpkin plants. This suggested that this region of BR1, and specifically the leucine residues within this hydrophobic region, were important for BR1 function.

Table 1. Comparison of Known Leucine-Rich Nuclear Export Signals and BR1

Protein	NES[a]
HIV-1 Rev	LPPLERLTL
HIV-2 Rev	IQHLQGLTIQ
TFIIIA (*X. laevis*)	KPSGTETNGS*LVLDKLTI*Q
TFIIIA (*Bufo americanus*)	QPDASKADPLPVLENLTLK
Protein kinase A inhibitor	LALKLAGLDI
SqLCV BR1	VTKRVVSLEKDTLLIDLHGTTQL[b]

[a] These peptides are demonstrated to function as nuclear export signals when fused to a test substrate. Underlined are residues that mutational studies have shown to be essential for export function. Italicized in Xenopus TFIIIA are the residues shown to function in nuclear export in this study (see text for details).
[b] Residues 177 to 199 of BR1.

A Sequence Located between Amino Acids 177 and 199 of BR1 Functions in Nuclear Export

Previous studies have shown that when residues 110 to 256 of BR1 are fused to β-glucuronidase (GUS) and this fusion protein is coexpressed with BL1 in transfected tobacco protoplasts, the fusion protein GUS–BR1$^{110–256}$ interacts with BL1 (Sanderfoot et al., 1996). This was concluded from the finding that GUS–BR1$^{110–256}$, detected by indirect immunofluorescence staining with anti-GUS antibodies, is uniformly distributed throughout the cytoplasm of the protoplasts when expressed by itself but is relocalized to the cortical cytoplasm in the presence of BL1. These same studies showed that the two NLSs in BR1 are located in the N-terminal half (residues 1 to 113) of the protein. We used these results as the basis for an in vivo protoplast-based assay to identify the nuclear export signal within BR1.

Having shown that cytoplasmically localized GUS–BR1$^{110–256}$ with its intact interactive domain is relocalized to the cortical cytoplasm when coexpressed with BL1, we reasoned that such a fusion protein when targeted to the nucleus could only interact with BL1 and be relocalized to the cortical cytoplasm if it also contained an intact nuclear export signal that would allow it to exit from the nucleus. Thus, as shown in Figure 1, a nested series of four C-terminal fragments of BR1—BR1$^{149–256}$, BR1$^{177–256}$, BR1$^{199–256}$, and BR1$^{223–256}$—were fused in-frame to GUS and expressed from the cauliflower mosaic virus 35S promoter contained in the expression vector pRTL2–GUS/NIa (Restrepo et al., 1990). This series was chosen to potentially separate the putative NES (amino acids 184 to 193) from the BL1 interactive domain (amino acids 200 to 256). The putative NES is present in BR1$^{149–256}$ and BR1$^{177–256}$ but absent from BR1$^{199–256}$ and BR1$^{223–256}$. We also inserted the NLS from the maize transcription factor OPAQUE2 (O2) (Varagona et al., 1992) into each fusion in-frame between GUS and the BR1 fragment to construct GUS–BR1$^{149–256NLS}$, GUS–BR1$^{177–256NLS}$, GUS–BR1$^{199–256NLS}$, and GUS–BR1$^{233–256NLS}$ (Figure 1). Each fusion protein was expressed in transfected *Nicotiana tabacum* cv Xanthi protoplasts either by itself or together with BL1.

As shown in Figures 2A to 2D and Table 3, when expressed individually and assayed by indirect immunofluorescence staining with anti-GUS antibodies, GUS–BR1$^{149–256}$, GUS–BR1$^{177–256}$, GUS–BR1$^{199–256}$, and GUS–BR1$^{223–256}$ each stably accumulated throughout the cytoplasm of Xanthi protoplasts. When coexpressed with BL1, GUS–BR1$^{149–256}$, GUS–BR1$^{177–256}$, and GUS–BR1$^{199–256}$ were each relocalized to the cortical cytoplasm; however, GUS–BR1$^{223–256}$ was not relocalized by BL1 and remained throughout the cytoplasm (Figures 2E to 2H and Table 3). Thus, C-terminal fragments BR1$^{149–256}$, BR1$^{177–256}$, and BR1$^{199–256}$ each contained the intact BL1 interactive domain, but this domain was disrupted in BR1$^{223–256}$. These results demonstrated that residues 199 to 256 of BR1 were sufficient for interaction with BL1, consistent with our site-directed mutational studies (Sanderfoot et al., 1996).

When GUS–BR1$^{149–256NLS}$, GUS–BR1$^{177–256NLS}$, GUS–BR1$^{199–256NLS}$, and GUS–BR1$^{233–256NLS}$ were individually expressed in protoplasts, each stably accumulated in the nucleus (Figures 2I and 2J, and Table 3). When these same fusion proteins were each coexpressed with BL1, only GUS–BR1$^{149–256NLS}$ and GUS–BR1$^{177–256NLS}$, the two fusion proteins that contained the putative NES, were redirected to the cortical cytoplasm (Figure 2M and Table 3). GUS–BR1$^{199–256NLS}$ and GUS–BR1$^{233–256NLS}$ were retained in the nucleus in the presence of BL1 (Figure 2N and Table 3). This result was expected for GUS–BR1$^{233–256NLS}$ because it lacked the BL1 interactive domain. The nuclear retention of GUS–BR1$^{199–256NLS}$, which contains an interactive domain (Figure 1), was consistent with this fusion protein lacking an NES and suggested that the NES was between residues 177 and 199 of BR1.

The TFIIIA NES Can Restore BR1 Relocalization to the Cortical Cytoplasm and SqLCV Infectivity

To further demonstrate that a defect in nuclear export was responsible for the nuclear retention of GUS–BR1$^{199–256NLS}$ when BL1 was present, the NES from Xenopus TFIIIA, contained between amino acids 326 and 344, was inserted into GUS–BR1$^{149–256NLS}$, GUS–BR1$^{177–256NLS}$, GUS–BR1$^{199–256NLS}$, and GUS–BR1$^{223–256NLS}$ in-frame between the O2 NLS and BR1 segment to produce GUS–BR1$^{149–256NLS/NES}$, GUS–BR1$^{177–256NLS/NES}$, GUS–BR1$^{199–256NLS/NES}$, and GUS–BR1$^{223–256NLS/NES}$ (Figure 1). Each of these four fusion proteins was found to stably accumulate in the nucleus when individually expressed in tobacco protoplasts (Figures 2K and 2L, and Table 3).

When GUS–BR1$^{177–256NLS/NES}$ was coexpressed with BL1, it was redirected to the cortical cytoplasm, as was GUS–BR1$^{177–256NLS}$ (Figures 2M and 2O, and Table 3), demonstrating that the TFIIIA NES did not interfere with interaction between and relocalization by BL1. In contrast to the nuclear

Table 2. Infectivity of SqLCV BR1 NES Mutants in Pumpkin

SqLCV Mutant	Infectivity (%)[a]
Wild type[b]	100
BR1$^{L189A/L190A/L193A}$	0
BR1$^{L189A/L190A/L193A/TFIIIA–NES}$	11

[a] Percentage of inoculated plants that developed systemic disease. The wild-type or mutant SqLCV B component was coinoculated with the wild-type viral A component. Shown are averages of two independent assays, with 16 (wild type) or 32 plants (each *BR1* mutant) inoculated in each assay. For *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$, three and four of the 32 inoculated plants in the two trials developed SqLCV symptoms and were shown to contain the appropriate viral DNA in systemically infected leaves (see text for details).
[b] B component encoding wild-type BR1.

retention of GUS–BR1$^{199–256NLS}$ when coexpressed in protoplasts with BL1, most, although not all of GUS–BR1$^{199–256NLS/NES}$ was now relocalized to the cortical cytoplasm by BL1 (Figure 2P and Table 3). GUS–BR1$^{223–256NLS/NES}$, which is unable to interact with BL1 (Figure 2H), remained in the nucleus when coexpressed with BL1 (Table 3), as did BR1$^{223–256NLS/NES}$. Hence, the nuclear export function of the TFIIIA NES partially restored the ability of GUS–BR1$^{199–256NLS}$ to be redirected from the nucleus to the cytoplasm by BL1.

These results demonstrated a role for nuclear export in enabling nuclear-localized forms of the GUS–BR1 fusion proteins to interact with BL1 in tobacco protoplasts. To further demonstrate the importance of nuclear export in BR1 function, we tested the ability of the TFIIIA NES to restore function to BR1$^{L189A/L190A/L193A}$ both in our protoplast interaction assay and in SqLCV infectivity.

We replaced residues 192 and 193 in BR1$^{L189A/L190A/L193A}$ with amino acids 336 to 343 from TFIIIA (see Table 1), which contain the NES, to construct BR1$^{L189A/L190A/L193A/TFIIIA–NES}$. Because of the partial relocalization of GUS–BR1$^{199–256NLS/NES}$ when coexpressed with BL1 in protoplasts, we used this smaller peptide containing the TFIIIA NES in an attempt to minimize disruption of BR1 structure. Nevertheless, the inserted TFIIIA peptide is distinct in its primary sequence from that of the NES-containing region of BR1 (Table 1). Both BR1$^{L189A/L190A/L193A}$ and BR1$^{L189A/L190A/L193A/TFIIIA–NES}$ stably accumulated in the nuclei of tobacco protoplasts in the absence of BL1 (Table 3). As shown in Figure 3A and Table 3, when BR1$^{L189A/L190A/L193A}$ was coexpressed with BL1 in tobacco protoplasts, it was retained in the nucleus, as we had found for GUS–BR1$^{199–256NLS}$ (Figure 2N and Table 3). In contrast, BR1$^{L189A/L190A/L193A/TFIIIA–NES}$ was relocalized to the cortical cytoplasm in the presence of BL1 (Figure 3B and Table 3), as is wild-type BR1 (Sanderfoot et al., 1996).

The ability of BR1$^{L189A/L190A/L193A/TFIIIA–NES}$ to interact with BL1 in our protoplast assay suggested that this mutated form of BR1 might also at least partially function in viral infectivity. To test this, we replaced wild-type *BR1* in the SqLCV B component with *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$, and this mutant B component was coinoculated with the wild-type viral A component onto pumpkin seedlings. As summarized in Table 2, in contrast to *BR1*$^{L189A/L190A/L193A}$, which was not infectious, *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$ was partially infectious in pumpkin plants, albeit at a low level.

To demonstrate that the inserted TFIIIA NES sequence was retained in *BR1*$^{L189A/L190A/L193A}$ and responsible for the restoration of viral infectivity, we used oligonucleotide primers specific for *BR1* sequences that flanked the inserted NES-coding sequence and the polymerase chain reaction (PCR) to amplify this region from viral DNA isolated from *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$ or wild-type SqLCV systemically infected leaves (see Methods). As expected, the fragment amplified from the *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$ symptomatic plant extracts was larger by ~20 nucleotides than that from the wild-type virus-infected plants (324 compared with 306 nucleotides). Furthermore, when resolved on DNA gel blots, this 324-nucleotide fragment amplified from *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$-infected plants was found to specifically hybridize with the Xenopus TFIIIA NES probe (TFIIIA-Btm), which itself did not hybridize with the SqLCV wild-type *BR1* sequence (data not shown). Thus, the TFIIIA NES was retained in and responsible for restoring infectivity to the *BR1*$^{L189A/L190A/L193A/TFIIIA–NES}$ mutant.

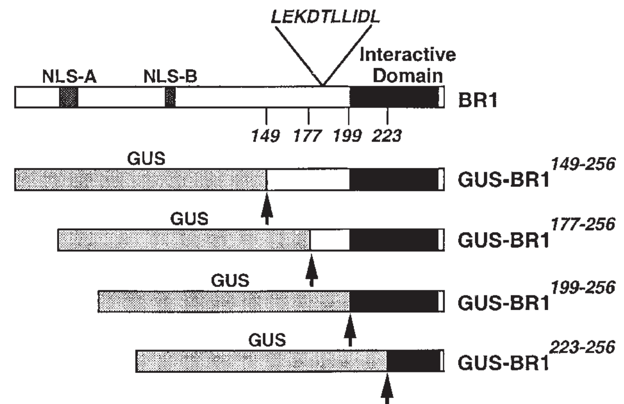

Figure 1. Schematic Diagram of BR1 and GUS–BR1 Fusion Constructs.

Shown within BR1 are the positions of the two NLSs (NLS-A and NLS-B) and the domain required for interaction with BL1 (Interactive Domain). The sequence above BR1 is that of the putative NES located between residues 184 to 193. Numbers indicate the amino acid positions at which BR1 C-terminal fragments were translationally fused to GUS. Arrows show where the NLS from O2, and subsequently the NES from TFIIIA, was inserted into the GUS–BR1 fusion proteins.

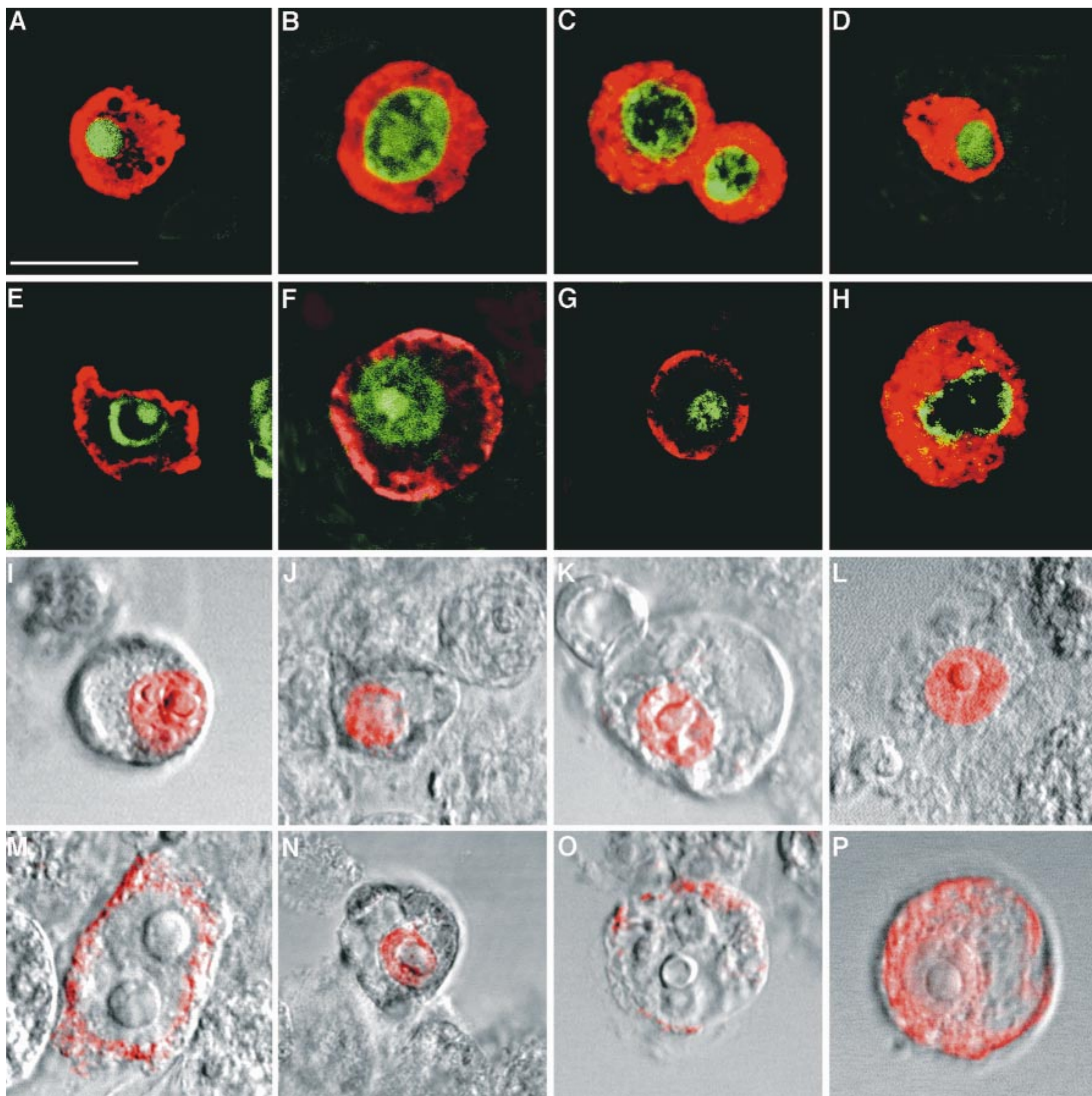

Figure 2. Indirect Immunofluorescence Staining of GUS–BR1 Fusion Protein Expressed in Tobacco Protoplasts.

Cells were stained with anti-GUS antiserum followed by Texas Red–conjugated goat anti–rabbit antibody and imaged by confocal microscopy. Green in **(A)** to **(H)** is chromomycin A staining (fluorescein channel) to show location of nuclei. In **(I)** to **(P)**, each immunofluorescence image has been superimposed on the bright-field image.

(A) to **(D)** and **(I)** to **(L)** Fusion protein expressed alone.

(E) to **(H)** and **(M)** to **(P)** Each fusion protein expressed together with BL1.

Fusion constructs are GUS–BR1$^{149–256}$ in **(A)** and **(E)**, GUS–BR1$^{177–256}$ in **(B)** and **(F)**, GUS–BR1$^{199–256}$ in **(C)** and **(G)**, GUS–BR1$^{223–256}$ in **(D)** and **(H)**, GUS–BR1$^{177–256NLS}$ in **(I)** and **(M)**, GUS–BR1$^{199–256NLS}$ in **(J)** and **(N)**, GUS–BR1$^{177–256NLS/NES}$ in **(K)** and **(O)**, and GUS–BR1$^{199–256NLS/NES}$ in **(L)** and **(P)**. Bar in **(A)** = 20 μm for **(A)** to **(P)**.

DISCUSSION

Regulating nucleocytoplasmic transport is a critical aspect of normal cell growth and differentiation. Following the demonstration that certain proteins rapidly shuttle between the nucleus and cytoplasm in animal cells, and the initial identification of a dedicated nuclear export signal within a few members of this group—specifically, HIV Rev, TFIIIA, and PKI—a number of animal cell and animal virus proteins have

been shown to contain a similar hydrophobic leucine-rich NES (Gerace, 1995; Görlich and Mattaj, 1996). The M9 and K domains represent two additional distinct bidirectional signals that function in both nuclear import and export (Michael et al., 1995, 1997). The finding that HIV Rev, and subsequently the adenovirus E1B 55-kD protein, can function to direct RNA export to the cytoplasm in yeast (Stutz and Rosbash, 1994; Liang et al., 1995; Stutz et al., 1995), taken together with biochemical studies on yeast importin α (Görlich et al., 1996) and genetic studies in yeast (Corbett and Silver, 1997), suggest that nuclear export pathways, like import pathways, are conserved in animals and yeast. Our identification and characterization of an NES in the SqLCV movement protein BR1 have now extended the study of nuclear export to plant cells.

Our studies identified this NES between residues 177 to 199 of BR1. The leucine-rich core of this 22–amino acid peptide (LEDKTLLIDLH) is strikingly similar in overall character to the NES found in Rev, TFIIIA, and PKI but distinct in its primary sequence. As found for these NESs, the leucine residues within the BR1 NES are essential for the nuclear export of BR1, as assayed in tobacco protoplasts. These leucine residues are also essential for SqLCV infectivity. Importantly, insertion of a peptide containing the Xenopus TFIIIA NES (LVLDKLTI) restored the ability of GUS–BR1$^{199–256NLS}$ and BR1$^{L189A/L190A/L193A}$ to be relocalized to the cortical cytoplasm by BL1, but not GUS–BR1$^{223–256NLS}$, which lacks the BL1 interactive domain. Thus, two distinct functions—a BL1 interactive domain and a nuclear export signal—are needed for BR1 to be relocalized from the nucleus to the cortical cytoplasm in the presence of BL1. That GUS–BR1$^{177–199NLS/TFIIIA–NES}$, with an intact BR1 NES, was completely redirected out of the nucleus when coexpressed with BL1 shows that the TFIIIA NES does not interfere with function of the native BR1 NES or interactive domain. Insertion of the TFIIIA NES into BR1$^{L189A/L190A/L193A}$ also partially restored SqLCV infectivity in pumpkin. Thus, nuclear export mediated by this NES is essential for the in vivo function of BR1, and it appears that this NES between amino acids 177 to 199 is the only one within BR1. In addition, these findings more precisely localize the NES in Xenopus TFIIIA to the short eight-residue peptide LVLDKLTI.

That many rapidly shuttling proteins—the retroviral Rev protein, adenovirus E4 34-kD protein, and herpes simplex virus ICP27, each of which contains a hydrophobic leucine-rich NES (Fischer et al., 1995; Dobbelstein et al., 1997; Sandri-Goldin, 1998), or the influenza virus M1 matrix protein and nucleocapsid protein NP, each of which appears to contain a bidirectional signal (Whittaker et al., 1996)—are encoded by viruses is not surprising. The replication cycle of each of these viruses involves nuclear events in which the virus must compete for export with host mRNAs and viral transcripts, and/or the viral genome must be exported to the cytoplasm in a regulated manner. In retroviruses such as HIV, the viral genome is an unspliced mRNA replicated from an inserted chromosomal proviral copy. At late times in infection, HIV Rev acts to promote the nuclear export of the viral genome and other unspliced HIV transcripts that encode the viral structural proteins (Fields et al., 1996). Influenza virus replicates in the nucleus because it requires host RNA cap structures to initiate viral transcription and host-splicing enzymes to process certain viral transcripts. NP and M1 promote the nuclear export of the encapsidated viral negative-sense RNA genome for assembly into enveloped virions at the plasma membrane (Whittaker et al., 1996). Adenovirus and herpes simplex virus are both double-stranded DNA viruses that replicate in the nucleus, as do most eukaryotic DNA viruses, so as to utilize host DNA replicating and modifying enzymes (Fields et al., 1996). Each of these viruses inhibits host protein synthesis and promotes viral protein synthesis through the action of ICP27 or E4 34-kD (the latter complexed with the E1B 55-kD protein) to inhibit the nuclear export of host mRNAs and to promote the export of viral mRNAs (Dobbelstein et al., 1997; Sandri-Goldin, 1998).

Perhaps most analogous to influenza virus NP and M1, SqLCV BR1 acts to export the viral genome (single-stranded DNA) from the nucleus to the cytoplasm, where the cooperative interaction of BR1 with BL1 will direct the BR1–genome complex to the cortical cytoplasm and across the cell wall into adjacent uninfected cells to propagate infection (Sanderfoot et al., 1996; Sanderfoot and Lazarowitz, 1996). M1 acts to prevent reimport of influenza virus NP1–genome RNA complexes into the nucleus (Martin and Helenius, 1991; Whittaker et al., 1996). Following interaction with BL1, BR1 targets to the perinuclear region of the cytoplasm and does not reenter the nucleus. Thus, it appears that following release

Table 3. Subcellular Localization of BR1 Fusion and Mutant Proteins

Protein	Subcellular Localization[a]	
	−BL1[b]	+BL1[b]
GUS–BR1$^{149–256}$	C	P
GUS–BR1$^{177–256}$	C	P
GUS–BR1$^{199–256}$	C	P
GUS–BR1$^{223–256}$	C	C
GUS–BR1$^{149–256NLS}$	N	P
GUS–BR1$^{177–256NLS}$	N	P
GUS–BR1$^{199–256NLS}$	N	N
GUS–BR1$^{223–256NLS}$	N	N
GUS–BR1$^{149–256NLS/NES}$	N	P
GUS–BR1$^{177–256NLS/NES}$	N	P
GUS–BR1$^{199–256NLS/NES}$	N	P/N[c]
GUS–BR1$^{223–256NLS/NES}$	N	N
BR1$^{L189A/L190A/L193A}$	N	N
BR1$^{L189A/L190A/L193A/TFIIIA–NES}$	N	P

[a] C, cytoplasm; N, nucleus; P, cell periphery (cortical cytoplasm).
[b] BR1 proteins were expressed in the absence (−BL1) or presence (+BL1) of BL1.
[c] Approximately 80 to 90% relocalized to the cortical cytoplasm (see Figure 2 and text).

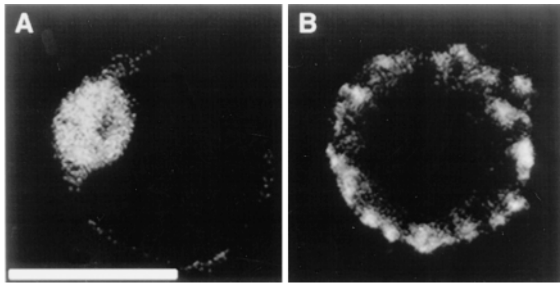

Figure 3. Indirect Immunofluorescence Staining of BR1 NES Mutants Coexpressed with BL1 in Tobacco Protoplasts.

Cells were stained with anti-BR1 antiserum followed by Texas Red–conjugated goat anti–rabbit antibody and imaged by confocal microscopy.
(A) BR1$^{L189A/L190A/L193A}$ coexpressed with BL1.
(B) BR1$^{L189A/L190A/L193A/TFIIIA-NES}$ coexpressed with BL1.
Bar in **(A)** = 20 μm for **(A)** and **(B)**.

from BL1 in uninfected cells, a post-translational modification of BR1, possibly in its phosphorylation state, may act to prevent reimport of BR1 into the nucleus together with the viral genome (Sanderfoot and Lazarowitz, 1996). From the perspective of exporting the viral genome from the nucleus, HIV Rev can be viewed as performing a function analogous to BR1 in exporting unspliced viral transcripts, one of which is the HIV genome, to the cytoplasm.

Studies of nuclear import in plants suggest that the basic import machinery has been highly conserved in plant cells, and in animal cells and yeast (Hicks and Raikhel, 1995). Our identification of an NES in BR1 and demonstration that the NES from TFIIIA can at least in part substitute for function of this BR1 NES in nuclear export and in viral infectivity suggest that the basic mechanisms of nuclear export have also been similarly conserved. This is also suggested by the fact that BR1 can be redirected by BL1 from the nucleus to the cell periphery in Sf9 insect cells (Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996). Thus, animal cells possess the machinery to recognize the NES in BR1 and export BR1 from the nucleus.

Although the TFIIIA NES fully restored function in nuclear export to BR1$^{L189A/L190A/L193A}$ as assayed in tobacco protoplasts, it did not fully restore SqLCV infectivity. Perhaps the recombinant protein that contained the inserted TFIIIA NES did not fully refold into a native configuration. The nuclear export receptor that interacted with TFIIIA in plant cells might also differ from the one that recognizes TFIIIA in animal cells. Competition studies suggest that as in nuclear import, there are a number of distinct pathways that function in the nuclear export of tRNAs, mRNAs, U snRNAs (small nuclear RNAs), and NES-bearing cargo (Gerace, 1995; Görlich and Mattaj, 1996; Görlich et al., 1996; Weis, 1998). Nuclear export receptors for export of mRNAs, tRNAs, or NES-containing proteins have just begun to be identified (Fornerod et al., 1997; Stade et al., 1997; Arts et al., 1998; Snay-Hodge et al., 1998; Tseng et al., 1998), and it has been suggested that this may be the tip of the iceberg (Görlich and Mattaj, 1996; Izaurralde and Adam, 1998; Weis, 1998). Although a number of hydrophobic leucine-rich NESs have been identified, it remains to be seen whether all will be recognized by the same export receptor. As in nuclear import, a variety of related adapter proteins may be involved in nuclear export (Görlich and Mattaj, 1996; Corbett and Silver, 1997).

Nuclear export in animal cells has been studied by the formation of heterokaryons or the microinjection of test substrates into the nuclei of HeLa cells or Xenopus oocytes, and in genetic studies in yeast (Fischer et al., 1995; Wen et al., 1995; Corbett et al., 1996; Fridell et al., 1996a; Whittaker et al., 1996; Fornerod et al., 1997; Stade et al., 1997). Here, we have used an in vivo cell-based assay to investigate nuclear export in plant cells, identifying an NES in BR1 based on its specific interaction with and entrapment in the cytoplasm by BL1. The biochemical identification and characterization of nuclear export receptors and other components of the export machinery are essential for understanding the details of the export mechanism and how this trafficking of macromolecules across NPCs is regulated.

Our understanding of the mechanism of nuclear import has been significantly advanced through the development of permeabilized animal cell assays that allowed for the specific depletion of factors and reconstitution of import activity (Newmeyer and Forbes, 1988; Adams et al., 1990; Melchoir et al., 1993; Dingwall and Palacios, 1998). The development of a permeabilized tobacco protoplast system competent for nuclear import now provides the opportunity for similar studies in plants (Hicks et al., 1996; Merkle et al., 1996). Recently, digitonin-permeabilized animal cell systems have been described for investigating nuclear export (Yang et al., 1997; Kehlenbach et al., 1998). These were derived from cell lines that expressed glucocorticoid receptors or a green fluorescent protein–tagged form of the transcriptional activator NFAT. Our demonstration of the utility of BR1–BL1 interaction for investigating nuclear export now provides the basis for the development of a permeabilized cell-based assay for biochemically dissecting nuclear export in plant cells, and it can also be applied to similar studies in animal cells.

METHODS

Construction of Expression Vectors

The following oligonucleotides were used in this study: 149-F, 5′-GGGGAAGATCTGATGAGTTG-3′; 177-F, 5′-GGGGAAGATCTGTTACGAAGCGTGTG-3′; 199-F, 5′-GGGGAAGATCTCTGTCTAACAAGCG-3′; 223-F, 5′-GGGGAAGATCTGGTAACATAACCAAG-3′; O2-F, 5′-CAGGATCCAAGATGCCTACCGAG-3′; O2-R, 5′-CCAGATCTAGCT**AGGCCT**TCCAGTTCTTTCAG-3′; TF3A-F, 5′-T**AGATCT**CCAATTGCTAAGCCCTCTGG-3′; TF3A-R, 5′-C**AGATCT**ACCATGGGCTTGTATAGTTAATTTATC-3′; NESKO-R, 5′-AGATCTA**CCATG*G***GCATCAAT*TG*CAGCCGTGTCCTTTTCAA-3′; TF3A-Top, 5′-AAT-

TTTGGTTCTAGATAAATTAACTATA-3′; and TF3A-Btm, 5′-CATGTA-TAGTTAATTTATCTAGAACCAA-3′.

To substitute an alanine for a leucine at positions 189, 190, and 193 in BR1 and insert an MfeI site, we amplified the HindIII-NcoI fragment of *BR1* (nucleotides 691 to 1288) encoding residues 1 to 196 from the plasmid pGEM–BR1 (Pascal et al., 1993, 1994) by using the polymerase chain reaction (PCR), using the antisense oligonucleotide NESKO-R (nucleotide changes that inserted alanine residues into BR1 are shown in italics above; the MfeI site is underlined; and the NcoI site is in boldface) and the SP6 primer (Promega). The SalI-NcoI fragment that encompassed these changes was then subcloned between the SalI and NcoI sites of *BR1* to create $BR1^{L189A/L190A/L193A}$. For expression in tobacco protoplasts, $BR1^{L189A/L190A/L193A}$ was inserted into the expression vector pRTL2–GUS/NIa (Restrepo et al., 1990), as previously described (Sanderfoot and Lazarowitz, 1995). The sequence of all amplified and subcloned DNA constructs was confirmed by DNA sequence analysis at the Genetic Engineering Facility of the University of Illinois.

Forward primers 149-F, 177-F, 199-F, and 223-F, each with the T7 primer (Promega), were used to PCR amplify the fragments encoding the four C-terminal truncations of BR1 from pGEM–BR1 (Ingham et al., 1995), with an inserted BglII site (underlined) immediately upstream of the designated residue (149, 177, 199, and 223). To create the fusion constructs $GUS–BR1^{149–256}$, $GUS–BR1^{177–256}$, $GUS–BR1^{199–256}$, and $GUS–BR1^{223–256}$, we ligated the BglII-XhoI fragment from each amplified fragment (XhoI, located in the *BR1* 3′ untranslated region, was blunted) into the expression vector pRTL2–GUS/NIa (Restrepo et al., 1990), which had been digested with BglII and XbaI, with the XbaI site blunted. To insert the OPAQUE2 (O2) NLS into each GUS–BR1 fusion construct, we PCR amplified the sequence encoding residues 228 to 247 of O2 to contain a 5′-BamHI site and 3′-BglII and StuI sites by using the forward primer O2-F and the reverse primer O2-R (the BglII site is underlined; the StuI site is in boldface). The amplified fragment was cloned as a BamHI-BglII fragment in-frame at the BglII site between the GUS and BR1 coding sequences in each fusion expression vector. The orientation of each fragment was confirmed by sequencing. To insert the TFIIIA nuclear export signal (NES) into each GUS–BR1 fusion construct, we PCR amplified the sequence encoding residues 326 to 344 of the *Xenopus laevis* TFIIIA from pSPTF15 (Vrana et al., 1988) by using the forward primer TF3A-F (the BglII site is in boldface; the MfeI site is underlined) and the reverse primer TF3A-R (the BglII site is in boldface; the NcoI site is underlined). The MfeI-BglII fragment (5′-3′) from this was cloned (MfeI site blunted) in-frame between the GUS and BR1 coding sequences by using the StuI and BglII sites, and the orientation was confirmed by sequencing.

Oligonucleotides TF3A-Top and TF3A-Btm were annealed together to create a double-stranded DNA with a MfeI and a NcoI overhang compatible for ligation (underlined). This was ligated into $BR1^{L189A/L190A/L193A}$ that had been digested with NcoI and MfeI to construct $BR1^{L189A/L190A/L193A/TFIIIA–NES}$. For expression in tobacco protoplasts, $BR1^{L189A/L190A/L193A/TFIIIA–NES}$ was inserted into the expression vector pRTL2–GUS/NIa (Restrepo et al., 1990), as previously described (Sanderfoot and Lazarowitz, 1995).

Transient Expression and Subcellular Localization in Xanthi Protoplasts

All vectors used for transient expression assays were purified on CsCl gradients. Purified vectors were electroporated into protoplasts prepared from *Nicotiana tabacum* cv Xanthi suspension cells. Protoplasts were fixed 48 to 72 hr after transfection, and proteins were detected by indirect immunofluorescence staining using Texas Red–conjugated goat anti–rabbit antibody (Molecular Probes, Eugene, OR), as previously described (Pascal et al., 1994), with the following exceptions: fixed protoplasts were centrifuged onto lysine-coated slides prior to permeabilization and staining, and stained samples were mounted in 90% (v/v) glycerol, 10% (v/v) sodium borate, pH 9.0, with 3% (w/v) *n*-propyl gallate added as an antifade agent. Chromomycin A (Sigma) staining of DNA was used to visualize nuclei (fluorescein channel). A Bio-Rad MRC-1024 Kyypton/Argon dual laser confocal system attached to an optiphot microscope (Nikon, Melville, NY) was used to detect and image the fluorescent signals. Captured images were colorized and overlaid using Photoshop Version 4.0.1 (Adobe Systems, Inc., San Jose, CA).

Infectivity Assays

Squash leaf curl virus (SqLCV) B components containing either $BR1^{L189A/L190A/L193A}$ or $BR1^{L189A/L190A/L193A/TFIIIA–NES}$ in place of wild-type *BR1* were constructed as previously described (Ingham et al., 1995). For infectivity assays, each B component, cloned as a tandem direct repeat in pMON505, was introduced into *Agrobacterium tumefaciens* and inoculated onto pumpkin seedlings using the agroinoculation procedure (Lazarowitz and Lazdins, 1991; Ward et al., 1997). To verify that the inserted TFIIIA NES coding sequence within BR1 was responsible for the restoration of infectivity observed for $BR1^{L189A/L190A/L193A/TFIIIA–NES}$, DNA was extracted with cetyltrimethylammonium bromide from systemically infected leaves of plants coinoculated with SqLCV A and either $BR1^{L189A/L190A/L193A/TFIIIA–NES}$ or the wild-type SqLCV B component, and 10 μL (of 100 μL total) was used as substrate for PCR analysis (McGarvey and Kaper, 1991). We used the following *BR1*-specific primers, which flanked the region of the inserted NES coding sequence: BR1–seq574 (forward primer), 5′-ATCTGTCGTACCTGCATTG-3′, located 64 nucleotides upstream of the TFIIIA insertion site at nucleotides 486 to 512 of *BR1*; and EBR1–256R (reverse primer), 5′-GGGAATTCTATTATTCTTAATCA-3′, which starts 19 nucleotides downstream of the *BR1* stop codon (TGA). These were predicted to amplify a 306- and 324-bp fragment from wild-type *BR1* and $BR1^{L189A}$/*L190A*/*L193A*/TFIIIA–NES, respectively. PCR products were resolved on 1.5% agarose gels. DNA gel blots of these were hybridized with ^{32}P-labeled TF3A-Btm as probe at 55°C in 0.5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). Under these stringent conditions, the TF3A-Btm (melting temperature of 70°C) oligonucleotide should hybridize only with its cognate sequence. Given the distinct sequences, calculations show that had a sufficient number of changes in $BR1^{L189A/L190A/L193A}$ occurred to even partially revert the TFIIIA NES to the primary sequence of the BR1 NES, the TF3A-Btm probe would not have hybridized with the isolated viral DNA under these conditions.

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REFERENCES

Adams, S.A., Sterne-Marr, R., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. **111,** 807–816.

Arts, G.J., Fornerod, M., and Mattaj, I.W. (1998). Identification of a nuclear export receptor for tRNA. Curr. Biol. **8,** 305–314.

Corbett, A., Ferrigno, P., Henry, M., Kahana, J., Koepp, D., Lee, M., Nguyen, L., Schlenstedt, G., Seedorf, M., Shen, E., Taura, T., Wong, D., and Silver, P. (1996). Genetic analysis of macromolecular transport across the nuclear envelope. Exp. Cell. Res. **229,** 212–216.

Corbett, A.H., and Silver, P.A. (1997). Nucleocytoplasmic transport of macromolecules. Microbiol. Mol. Biol. Rev. **61,** 193–211.

Davis, L.I. (1995). The nuclear pore complex. Annu. Rev. Biochem. **64,** 865–896.

Deane, R., Schafer, W., Zimmermann, H.P., Mueller, L., Görlich, D., Prehn, S., Ponstingl, H., and Bischoff, F.R. (1997). Ran-binding protein 5 (RanBP5) is related to the nuclear transport factor importin-beta but interacts differently with RanBP1. Mol. Cell. Biol. **17,** 5087–5096.

Dingwall, C., and Palacios, I. (1998). In vitro systems for the reconstitution of snRNP and protein nuclear import. Methods Cell Biol. **53,** 517–543.

Dobbelstein, M., Roth, J., Kimberly, W.T., Levine, A.J., and Shenk, T. (1997). Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a Rev-like signal sequence. EMBO J. **16,** 4276–4284.

Featherstone, C., Darby, M.K., and Gerace, L. (1988). A monoclonal antibody against the nuclear port complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. J. Cell Biol. **107,** 1289–1297.

Fields, B.N., Knipe, D.M., and Howley, P.M., eds (1996). Virology. (New York: Lippincott-Raven).

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82,** 475–483.

Forbes, D.J. (1992). Structure and function of the nuclear pore complex. Annu. Rev. Cell Biol. **8,** 495–527.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90,** 1051–1060.

Fridell, R.A., Bogerd, H.P., and Cullen, B.R. (1996a). Nuclear export of late HIV-1 mRNAs occurs via a cellular protein export pathway. Proc. Natl. Acad. Sci. USA **93,** 4421–4424.

Fridell, R.A., Fischer, U., Luhrmann, R., Meyer, B.E., Meinkoth, J.L., Malim, M.H., and Cullen, B.R. (1996b). Amphibian transcription factor IIIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 Rev. Proc. Natl. Acad. Sci. USA **93,** 2936–2940.

Fridell, R.A., Truant, R., Thorne, L., Benson, R.E., and Cullen, B.R. (1997). Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. J. Cell Sci. **110,** 1325–1331.

Gerace, L. (1995). Nuclear export signals and the fast track to the cytoplasm. Cell **82,** 341–344.

Goldstein, L. (1958). Localization of nucleus-specific protein as shown by transplantation experiments in *Amoeba proteus*. Exp. Cell Res. **15,** 635–637.

Görlich, D. (1997). Nuclear protein import. Curr. Opin. Cell Biol. **9,** 412–419.

Görlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science **271,** 1513–1518.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R.A., Mattaj, I.W., and Izaurralde, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell **87,** 21–32.

Haizel, T., Merkle, T., Pay, A., Fejes, E., and Nagy, F. (1997). Characterization of proteins that interact with the GTP-bound form of the regulatory GTPase Ran in *Arabidopsis*. Plant J. **11,** 93–103.

Hicks, G.R., and Raikhel, N.V. (1995). Protein import into the nucleus: An integrated view. Annu. Rev. Cell Dev. Biol. **11,** 155–188.

Hicks, G.R., Smith, H.M.S., Shieh, M., and Raikhel, N.V. (1995). Three classes of nuclear import signals bind to plant nuclei. Plant Physiol. **107,** 1055–1058.

Hicks, G.R., Smith, H.M.S., Lobreaux, S., and Raikhel, N.V. (1996). Nuclear import in permeabilized protoplasts from higher plants has unique features. Plant Cell **8,** 1337–1352.

Ingham, D.J., Pascal, E., and Lazarowitz, S.G. (1995). Both geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. Virology **207,** 191–204.

Izaurralde, E., and Adam, S. (1998). Transport of macromolecules between the nucleus and the cytoplasm. RNA **4,** 351–364.

Kehlenbach, R.H., Dickmanns, A., and Gerace, L. (1998). Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro. J. Cell Biol. **141,** 863–874.

Lazarowitz, S.G., and Lazdins, I.B. (1991). Infectivity and complete nucleotide sequence of the cloned genomic components of a bipartite squash leaf curl geminivirus with a broad host range phenotype. Virology **180,** 58–69.

Lewis, J.D., Görlich, D., and Mattaj, I.W. (1996). A yeast cap binding protein complex (yCBC) acts at an early step in pre-mRNA splicing. Nucleic Acids Res. **24,** 3332–3336.

Liang, S., Hitomi, M., and Tartakoff, A.M. (1995). Adenoviral E1B—55-kDa protein inhibits yeast mRNA export and perturbs nuclear structure. Proc. Natl. Acad. Sci. USA **92,** 7372–7375.

Martin, K., and Helenius, A. (1991). Nuclear transport of influenza virus ribonucleoproteins: The viral matrix protein (M1) promotes export and inhibits import. Cell **67,** 117–130.

McGarvey, P., and Kaper, J.M. (1991). A simple and rapid method for screening transgenic plants using the PCR. BioTechniques **11,** 428–432.

Melchoir, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J. Cell Biol. **123**, 1649–1659.

Merkle, T., Leclerc, D., Marshallsay, C., and Nagy, F. (1996). A plant in vitro system for the nuclear import of proteins. Plant J. **10**, 1177–1186.

Michael, W.M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: A signal-mediated, temperature-dependent nuclear protein export pathway. Cell **83**, 415–422.

Michael, W.M., Eder, P.S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: A novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. **16**, 3587–3598.

Nakielny, S., Siomi, M.C., Siomi, H., Michael, W.M., Pollard, V., and Dreyfuss, G. (1996). Transportin: Nuclear transport receptor of a novel nuclear protein import pathway. Exp. Cell Res. **229**, 261–266.

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

Noueiry, A.O., Lucas, W.J., and Gilbertson, R.L. (1994). Two proteins of a plant DNA virus coordinate nuclear and plasmodesmatal transport. Cell **76**, 925–932.

Pascal, E., Goodlove, P.E., Wu, L.C., and Lazarowitz, S.G. (1993). Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. Plant Cell **5**, 795–807.

Pascal, E., Sanderfoot, A.A., Ward, B.M., Medville, R., Turgeon, R., and Lazarowitz, S.G. (1994). The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. Plant Cell **6**, 995–1006.

Pollard, V.W., Michael, W.M., Nakielny, S., Siomi, M.C., Wang, F., and Dreyfuss, G. (1996). A novel receptor-mediated nuclear protein import pathway. Cell **86**, 985–994.

Restrepo, M.A., Freed, D.D., and Carrington, J.C. (1990). Nuclear transport of plant potyviral proteins. Plant Cell **2**, 987–998.

Sanderfoot, A.A., and Lazarowitz, S.G. (1995). Cooperation in viral movement: The geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. Plant Cell **7**, 1185–1194.

Sanderfoot, A.A., and Lazarowitz, S.G. (1996). Getting it together in plant virus movement: Cooperative interactions between bipartite geminivirus movement proteins. Trends Cell Biol. **6**, 353–358.

Sanderfoot, A.A., Ingham, D.J., and Lazarowitz, S.G. (1996). A viral movement protein as a nuclear shuttle: The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. Plant Physiol. **110**, 23–33.

Sandri-Goldin, R.M. (1998). ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev. **12**, 868–879.

Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Görlich, D., Ponstingl, H., and Bischoff, F.R. (1997). Yrb4p, a yeast Ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. EMBO J. **16**, 6237–6249.

Senger, B., Simos, G., Bischoff, F.R., Podtelejnikov, A., Mann, M., and Hurt, E. (1998). Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. EMBO J. **17**, 2196–2207.

Snay-Hodge, C.A., Colot, H.V., Goldstein, A.L., and Cole, C.N. (1998). Dbp5p/Rat8p is a yeast nuclear pore–associated DEAD-box protein essential for RNA export. EMBO J. **17**, 2663–2676.

Stade, K., Ford, C.S., Guthrie, C., and Weis, K. (1997). Exportin 1 (CRM1p) is an essential nuclear export factor. Cell **90**, 1041–1050.

Stutz, F., and Rosbash, M. (1994). A functional interaction between Rev and yeast pre-mRNA is related to splicing complex formation. EMBO J. **13**, 4096–4104.

Stutz, F., Neville, J., and Rosbash, M. (1995). Identification of a novel nuclear pore–associated protein as a functional target of the HIV-1 Rev protein in yeast. Cell **82**, 495–506.

Truant, R., Fridell, R.A., Benson, R.E., Bogerd, H., and Cullen, B.R. (1998). Identification and functional characterization of a novel nuclear localization signal present in the yeast Nab2 poly(A)$^+$ RNA binding protein. Mol. Cell. Biol. **18**, 1449–1458.

Tseng, S.S., Weaver, P.L., Liu, Y., Hitomi, M., Tartakoff, A.M., and Chang, T.H. (1998). Dbp5p, a cytosolic RNA helicase, is required for poly(A)$^+$ RNA export. EMBO J. **17**, 2651–2662.

Varagona, M.J., Schmidt, R.J., and Raikhel, N.V. (1992). Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. Plant Cell **4**, 1213–1227.

Vrana, K.E., Churchill, M.E., Tullius, T.D., and Brown, D.D. (1988). Mapping functional regions of transcription factor TFIIIA. Mol. Cell. Biol. **8**, 1684–1696.

Ward, B.M., Medville, R., Lazarowitz, S.G., and Turgeon, R. (1997). The geminivirus BL1 movement protein is associated with endoplasmic reticulum–derived tubules in developing phloem cells. J. Virol. **71**, 3726–3733.

Weis, K. (1998). Importins and exportins: How to get in and out of the nucleus. Trends Biochem. Sci. **23**, 185–189.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell **82**, 463–473.

Whittaker, G., Bui, M., and Helenius, A. (1996). Nuclear trafficking of influenza virus ribonucleoproteins in heterokaryons. J. Virol. **70**, 2743–2756.

Yang, J., Liu, J., and DeFranco, D.B. (1997). Subnuclear trafficking of glucocorticoid receptors in vitro: Chromatin recycling and nuclear export. J. Cell Biol. **137**, 523–538.