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A Viral Movement Protein as a Nuclear Shuttle[1]

The Geminivirus BR1 Movement Protein Contains Domains Essential for Interaction with BL1 and Nuclear Localization

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For the nuclear replicating bipartite geminiviruses such as squash leaf curl to systemically infect the host requires the active participation of two virus-encoded movement proteins, BR1 and BL1. These act in a cooperative manner to transport the viral single-stranded DNA genome from its site of replication in the nucleus to the cell periphery (A.A. Sanderfoot, S.G. Lazarowitz [1995] Plant Cell 7: 1185–1194). We have proposed that BR1 functions as a nuclear shuttle protein, transporting the viral single-stranded DNA to and from the nucleus as a complex that is recognized by BL1 for movement to adjacent cells. To further investigate this, we expressed BR1 mutants known to affect viral infectivity in *Spodoptera frugiperda* insect cells and *Nicotiana tabacum* L. cv Xanthi protoplasts and found these to be defective in either their nuclear targeting or their ability to be redirected to the cell periphery when co-expressed with BL1. Translational fusions to β-glucuronidase and alanine-scanning mutagenesis further demonstrated that the C-terminal 86 amino acids of BR1 contains a domain(s) essential for its interaction with BL1 and identified two nuclear localization signals within the N-terminal 113 residues of BR1. These nuclear localization signals were precisely located within distinct 16- and 22-peptide segments of BR1. These studies support and extend our model for squash leaf curl virus movement, showing that BR1 has a domain structure, with an N-terminal region required for nuclear targeting and a C-terminal region required for its interaction with BL1.

Regulated trafficking across the nuclear membrane is an essential component of many signal transduction pathways in eukaryotic cells and a fundamental aspect of infection by animal and plant viruses that replicate in the nucleus. The transport of proteins across the nuclear membrane is an energy-requiring process mediated by proteins associated with nuclear pore complexes (Newmeyer and Forbes, 1988). Although nuclear pores have an effective size-exclusion limit of approximately 60 kD, proteins smaller than this appear not to diffuse efficiently across the nuclear membrane (reviewed by Silver, 1991). Karyophilic proteins contain NLSs, short basic regions of approximately 10 to 20 amino acids (reviewed by Raikhel, 1992) that interact with components of the nuclear pore complex for transport into the nucleus. Three NLS consensus motifs have been described: (a) an SV40-type NLS, first identified in SV40 T-antigen (Kalderon et al., 1984), which consists of a short sequence of predominantly basic amino acids flanked on either end by a helix-breaking Pro or Gly; (b) a bipartite NLS, originally characterized in *Xenopus laevis* nucleoplasmin (Robbins et al., 1991), consisting of two short sequences of basic amino acids separated by approximately 10 residues; and (c) a yeast MATα-2 NLS, which contains one or more basic amino acids interspersed with hydrophobic residues but not having a clear consensus sequence (Raikhel, 1992). NLSs have been best characterized in animal cells and yeast and more recently in plants. In particular, functional plant cell NLSs have been identified in maize in both the anthocyanin regulatory proteins Opaque2 and R (Varagona et al., 1992; Shieh et al., 1993) and the *Ac* transposase (Boehm et al., 1995), in auxin-inducible proteins in pea (Abel and Theologis, 1995), and in the GT box-binding transcription factor GT-2 from Arabidopsis (Dehesh et al., 1995). They have also been described in karyophilic proteins encoded by plant pathogens, namely, the NIa protein encoded by tobacco etch virus (Carrington et al., 1991) and the VirD2 and VirE2 proteins encoded by the Ti plasmid of *Agrobacterium tumefaciens* (Citovsky et al., 1992; Howard et al., 1992). Analysis of C1 and R further demonstrate that all three consensus NLSs are used in plants (Hicks et al., 1995).

The mechanism of nuclear import has received more attention than that of nuclear export. Thus, not surprisingly, the majority of karyophilic proteins characterized are those that function within or target complexes to the nucleus such as cellular transcription factors, virus-encoded replication or transactivator proteins such as SV40 T-antigen and herpes simplex virus VP16, or nucleic acid-binding and/or encapsidation proteins such as

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Abbreviations: CP, coat protein (AR1); MP, movement protein; NLS, nuclear localization signal; Sf9, *Spodoptera frugiperda* cell line; SqLCV, squash leaf curl virus; ssDNA, single-stranded DNA; SV40, simian virus 40; TexasRed, sulforhodamine 101; X-gluc, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid; Xanthi, *Nicotiana tabacum* L. cv Xanthi.

VirD2 and VirE2 and NP of influenza virus (Martin and Helenius, 1991a; Citovsky et al., 1992; Howard et al., 1992). There is increasing evidence for the importance in intracellular trafficking of so-called nuclear shuttle proteins that cycle between nuclear and cytoplasmic compartments (Laskey and Dingwall, 1993). Such proteins include the major nucleolar proteins nucleolin and No38 (Borer et al., 1989; Laskey and Dingwall, 1993; Schmidt-Zachmann et al., 1993), members of the 70-kD family of heat-shock proteins (Mandel and Feldherr, 1990), the heterogeneous nuclear RNA-packaging protein A1 (Pinol-Roma and Dreyfuss, 1992), splicing factor U1A (Kambach and Mattaj, 1992), the nucleolar protein Nopp140 (Meier and Blobel, 1992), the progesterone receptor (Guichon-Mantel et al., 1991), and the influenza M1 matrix protein (Martin and Helenius, 1991b). Because the rate of nuclear import far exceeds that of nuclear export, these proteins characteristically accumulate in nuclei, and shuttling is usually demonstrated by the redistribution of nuclear proteins in heterokaryons or in microinjection studies (Bataille et al., 1990; Schmidt-Zachmann et al., 1993; Guichon-Mantel et al., 1994). Studies demonstrate that masking of nuclear retention signals, as well as the presence of an intact NLS, is required for a karyophilic protein to exit to the cytoplasm and thus function as a shuttle protein, and they have further suggested that most nuclear-localized proteins may in fact potentially be shuttle proteins (Schmidt-Zachmann et al., 1993; Guichon-Mantel et al., 1994).

We have recently reported that the BR1 MP of the bipartite geminivirus SqLCV appears to be a nuclear shuttle protein required for the transport of the replicated viral ssDNA genome to and from the nucleus in infected cells (Sanderfoot and Lazarowitz, 1995). The MPs BR1 and BL1 encoded by the bipartite geminiviruses are nonstructural proteins that are not required for replication or encapsidation but are essential for local cell to cell and long distance movement via phloem sieve elements to systemically infect the host plant (Brough et al., 1988; Etessami et al., 1988). BR1 is a ssDNA-binding protein that, like other known shuttle proteins, accumulates in the cell nucleus (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995). Its properties as a shuttle protein are demonstrated by transient expression assays in Sf9 insect cells and Xanthi protoplasts in which co-expression with BL1 specifically relocalizes BR1 from the nucleus to the cell periphery, the site of BL1 accumulation. BL1 does not similarly relocalize the SqLCV-encoded nuclear proteins AL2 (a transcription factor) or CP, both of which remain in the nucleus when co-expressed with BL1 (Sanderfoot and Lazarowitz, 1995). This unique property of BR1 relies on its ability to interact with BL1 and suggests that these two MPs must at least transiently co-exist in the same subcellular compartment, namely, the cytoplasm. Based on these findings we have proposed a model in which BR1 binds the replicated SqLCV ssDNA genomes in the nucleus and shuttles these into and out of the nucleus. Directionality for viral movement is imposed by BL1 trapping BR1-ssDNA complexes in the cytoplasm and guiding these to the cell periphery, where BL1 acts to direct these BR1-containing complexes across the cell plasma membrane and wall to adjacent uninfected cells (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995).

Our studies of the subcellular targeting of BL1 and its ability to interact with BR1 have identified a central domain in BL1 required for this interaction and specific N- and C-terminal amino acids required for the correct subcellular targeting of BL1 to the cell periphery (Sanderfoot and Lazarowitz, 1995). These studies have utilized an extensive collection of missense and deletion mutants in *BL1* (Ingham et al., 1995), and the relevance of these domains to the function of BL1 in vivo is demonstrated by the direct correlation between the defects observed in these transient assays and those defects in the infectivity and host range properties of these mutants that we observe in inoculated plants (Sanderfoot and Lazarowitz, 1995). Utilizing the same transient expression assays in Sf9 cells and Xanthi protoplasts, and our collection of missense and deletion mutants in *BR1* (Ingham et al., 1995), we report here the identification and characterization of those domains in BR1 required for its ability to specifically interact with BL1 and for its correct nuclear targeting. As described below, we identified a specific C-terminal domain of BR1 required for its ability to interact with BL1 and, by translational fusions to GUS, found that the C-terminal 146 amino acids of BR1 alone were necessary and sufficient to confer on these GUS-BR1 fusions the ability to interact with BL1. Mutational analyses further localized this interactive domain to the C-terminal 86 residues of BR1. Using site-directed mutagenesis and translational fusions of segments of BR1 to GUS (Jefferson et al., 1987; Carrington et al., 1991), we also identified two NLSs in the N-terminal region of BR1: an SV40-type consensus at amino acids 87 to 95 that is essential for nuclear targeting of BR1 and a bipartite-type NLS at amino acids 25 to 39. We further showed that the former NLS was contained within the 16-residue peptide consisting of amino acids 81 to 96 and the latter in the 22-residue peptide from amino acids 21 to 42 of BR1.

MATERIALS AND METHODS

Expression Vectors

Vectors for expressing wild-type *BR1*, *BL1*, or *BL1* mutants in Sf9 insect cells and *Nicotiana tabacum* L. cv Xanthi protoplasts were described previously (Sanderfoot and Lazarowitz, 1995). Expression vectors for *BR1* mutants were constructed and CsCl gradient purified for transfection studies as previously described (Sanderfoot and Lazarowitz, 1995). In brief, blunt-ended fragments of the *BR1* coding sequence that contained the mutations of interest were cloned as transcriptional fusions to the baculovirus *Autographica californica* nuclear polyhedrosis virus gp64 promoter in the expression vector p166B-10 (G. Blissard, unpublished data) or the cauliflower mosaic virus 35S promoter contained in the expression vector p35S derived from pRTL2-GUS (Restrepo et al., 1990). These were transfected into Sf9 cells using the $CaPO_4$ precipitation method or electroporated into Xanthi protoplasts for transient

expression assays, respectively (Sanderfoot and Lazarowitz, 1995).

Translational fusions of BR1 peptides to the C terminus of GUS were constructed by PCR amplification of the appropriate coding sequences from pEBR1 (a plasmid containing the SqLCV-E BR1 coding sequence cloned utilizing introduced *Hin*dIII and *Xho*I sites, nucleotides 691 and 1597, respectively). Amplified fragments were used to make in-frame fusions to GUS at the *Bgl*II site in the expression vector pRTL2-GUS (Restrepo et al., 1990). For PCR amplification of BR1 fragments, each upstream oligonucleotide primer was synthesized to create a *Bgl*II or *Bam*HI site in-frame with the *Bgl*II site at the C terminus of GUS. The upstream (Forward) primers were named according to the numbered position of the initial amino acid codon sequence of BR1 included in the amplified fragment: 1F-*Bgl*II 5′-GGAGATCTATGTATTCGACG-3′, 1F-*Bam*HI 5′-GGGGATCCATGTATTCGACG-3′, 21F-*Bam*HI 5′-GGGGATCCCGTACAGGTGTC-3′, 57F-*Bgl*II 5′-GGAGATCTCAAGAGAACCAG-3′, 81F-*Bam*HI 5′-GGGGATCCAGCTATGTTAAG-3′, 110F-*Bgl*II 5′-GGAGATCTGGACAAGGTGAC-3′. Each downstream oligonucleotide primer was synthesized to create an *Xba*I site for cloning into the *Xba*I site of pRTL2-GUS. The downstream (Reverse) primers were named according to the last amino acid codon sequence of BR1 included in the amplified fragment: 42R-*Xba*I 5′-GGTCTAGAATACCACGTTGG-3′, 65R-*Xba*I 5′-GGTCTAGACAAATTCTGGGC-3′, 96R-*Xba*I 5′-GGTCTAGATCAATTTTATATACG-3′, 113R-*Xba*I 5′-GGTCTAGATGTCACCTTGTC-3′. The pUC-M13 reverse primer (GIBCO-BRL), which anneals downstream of the 3′ end of BR1 in pEBR1, was used to amplify the $BR1^{110-256}$ fragment; amplification with this primer resulted in inclusion of an *Xba*I site from the pEBR1 polylinker. Following PCR amplification, DNA fragments were digested with either *Bgl*II or *Bam*HI and with *Xba*I (New England Biolabs) and cloned into *Bgl*II/*Xba*I-digested pRTL2-GUS. For all constructs, the nucleotide sequence of the PCR-amplified fragments of BR1 were confirmed by sequencing using the dideoxy chain termination method (Sanger et al., 1977).

Construction and in Vivo Characterization of *BR1* Mutants

To construct mutants $BR1^{F148A/D149A}$, $BR1^{D170A/R171A}$, $BR1^{D212A}$, $BR1^{R215A/D216A}$, and $BR1^{K227A/N228A}$, Ala substitutions (Ala scanning, Cunningham and Wells, 1989) were introduced into BR1 by site-directed mutagenesis using synthetic oligonucleotide primers, as described previously (Ingham et al., 1995). These were each tested for their ability to infect pumpkin following agroinoculation, as described previously (Lazarowitz and Lazdins, 1991). The construction of all other *BR1* mutants used in this study and the characterization of their infectivity, host range, and pathogenic properties have been described (Ingham et al., 1995).

Transient Expression Assays in Sf9 Insect Cells and Xanthi Protoplasts

BR1 or BL1 was detected in transfected Sf9 cells or Xanthi protoplasts by indirect immunofluorescent staining and confocal microscopy, as described previously (Sanderfoot and Lazarowitz, 1995). In brief, cells taken at varying times following transfection were fixed in either −20°C ethanol (Sf9) or paraformaldehyde (Xanthi) and incubated with rabbit polyclonal anti-BR1 or anti-BL1 antisera (Pascal et al., 1993) followed by trimethylrhodamine- or TexasRed-conjugated goat anti-rabbit secondary antibodies (Jackson Labs, Bar Harbor, ME; Molecular Probes, Eugene, OR). Reproducibly 20 to 30% of transfected or co-transfected Xanthi protoplasts or Sf9 cells maximally expressed the appropriate MP(s) by 24 or 48 h posttransfection, respectively. MPs were expressed at sufficiently high levels to be detected on western blots. To visualize nuclei, cells were counterstained with chromomycin A (Sigma), which is detected at fluorescein wavelengths, as described previously (Sanderfoot and Lazarowitz, 1995). Stained cells were mounted in PBS containing 50% glycerol and visualized using a Bio-Rad MRC-1000 krypton/argon dual-laser confocal system attached to an Optiphot microscope (Nikon) at a final magnification of 1500× for Sf9 cells and 1000× for Xanthi protoplasts.

To study the ability of GUS-BR1 fusions to interact with BL1, Xanthi protoplasts were co-transfected with plasmids expressing each fusion construct (see above) and p35S-BL1E (Sanderfoot and Lazarowitz, 1995). Localization of GUS was detected by indirect immunofluorescent staining as described above, using rabbit polyclonal anti-β-glucuronidase antibodies (Molecular Probes) followed by TexasRed-conjugated goat anti-rabbit secondary antibodies.

Histochemical Assay for GUS

Twenty micrograms of each *BR1-GUS* fusion were electroporated into Xanthi protoplasts as described by Sanderfoot and Lazarowitz (1995). Histochemical staining was done 24 h posttransfection as described by Howard et al. (1992). Briefly, approximately 1×10^5 cells were placed in GUS assay buffer (4.3 g/L Murashige-Skoog salts [GIBCO-BRL], 50 mM KPO_4 [pH 6.8], 300 mM mannitol, 10 mM EDTA, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 2 mM X-gluc [Molecular Probes]) and incubated at room temperature until the indigo color developed (approximately 1 h). Following the development of sufficient color, the unfixed cells were photographed under phase contrast optics at 400× using a Nikon Optiphot microscope. Localization of the GUS-specific staining was qualitatively determined based on the appearance of the stained cell.

RESULTS

BR1 Mutants Exhibit Defects in Timing and Subcellular Targeting

Ala scanning and deletion mutations in BR1 when inoculated onto the permissive hosts pumpkin (*Cucurbita maxima* Duch. var Big Max), squash (*Cucurbita pepo* L. var Early Prolific), or *Nicotiana benthamiana* Domin. have been found to affect the infectivity and host range properties of SqLCV but not viral pathogenicity (Ingham et al., 1995). These mutations could be globally misfolded or defective in their

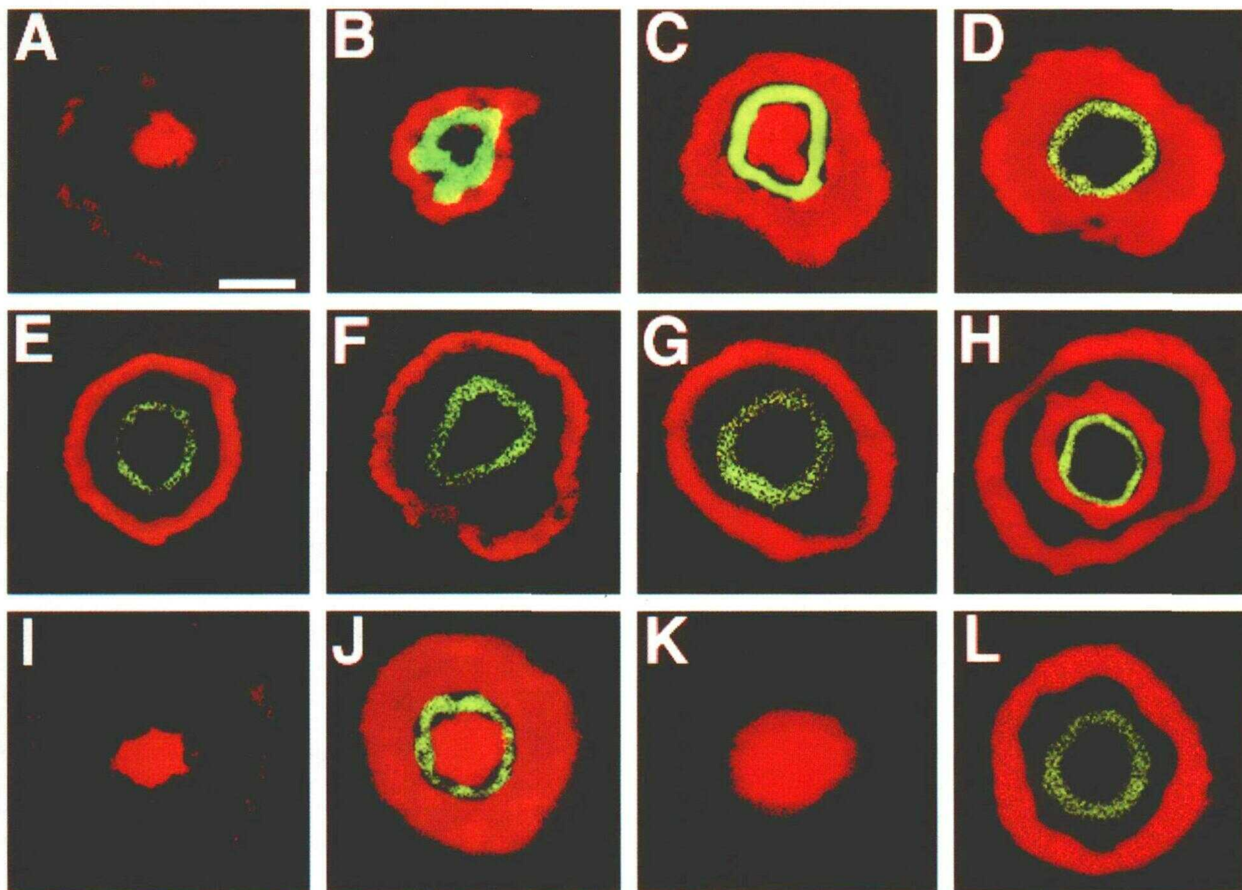

Figure 1. Immunofluorescent staining of mutant BR1 proteins and GUS-BR1 fusions in Xanthi protoplasts when expressed alone (A–D) or co-transfected with BL1 (E–L). Cells were stained with either anti-BR1 rabbit polyclonal antibodies (A–J) or anti-GUS rabbit polyclonal antibodies (K and L), followed by TexasRed-conjugated goat anti-rabbit secondary antibodies. Green fluorescence is chromomycin A staining of DNA to show location of nuclei. Under the fixation conditions used, chromatin is found as a ring under the nuclear envelope. All cells are shown 24 h posttransfection, unless otherwise noted. A, BR1$^{R36A/R37A/R38A}$; B, BR1$^{N201A/K202A/R203A}$; C, BR1^{N219A}; D, BR1$^{R89/R91}$; E, BR1$^{R36A/R37A/R38A}$ co-transfected with BL1; F, BR1$^{R89A/R91A}$ co-transfected with BL1; G, BR1$^{R215A/D216A}$ co-transfected with BL1; H, BR1$^{N201A/K202A/R203A}$ co-transfected with BL1 48 h posttransfection; I, BR1$^{N201A/K202A/R203A}$ co-transfected with BL1 120 h posttransfection; J, BR1^{N219A} co-transfected with BL1 120 h posttransfection; K, GUS-BR1$^{1-113}$ co-transfected with BL1; L, GUS-BR1$^{110-256}$ co-transfected with BL1. Bar in A = 10 μm. All panels are shown at the same magnification.

nuclear targeting, interaction with BL1, or ability to bind to nucleic acids. Transient expression assays in Sf9 cells, in which BL1 or BR1 is expressed as transcriptional fusions to the strong baculovirus gp64 early promoter, and in Xanthi protoplasts, in which gene expression is driven from the cauliflower mosaic virus 35S promoter, allow us to test BR1 and BL1 mutants for their correct subcellular targeting and mutual interactions (Sanderfoot and Lazarowitz, 1995). Thus, to obtain information about specific lesions in our defective BR1 mutants, these were each tested in these transient expression assays for their nuclear targeting and ability to be relocalized from the nucleus to the cell periphery when co-expressed with BL1. As found previously (Sanderfoot and Lazarowitz, 1995), 20 to 30% of Xanthi protoplasts or Sf9 cells maximally expressed BR1 or BL1 by 24 or 48 h posttransfection, respectively.

Of particular interest, inspection of the BR1 coding sequence identified two potential NLSs within the N-terminal region of BR1: a potential bipartite NLS located between residues 25 and 39 (KRSYGAARGDDRRRP) and a potential SV40-type NLS located between residues 87 to 95 (PNRTRTYIK). These potential NLSs were specifically targeted in Ala-scanning mutants BR1$^{K25A/R26A}$, BR1$^{R36A/R37A/R38A}$, BR1$^{R89A/R91A}$, and BR1$^{K97A/R98A/R100A}$. When individually expressed in Sf9 cells or in Xanthi protoplasts, mutants BR1$^{R36A/R37A/R38A}$ and BR1$^{K97A/R98A/R100A}$ were correctly targeted to the nucleus, their timing of appearance in the nucleus and intensity of immunofluorescent staining being indistinguishable from wild-type BR1 (Fig. 1A; Table I; Sanderfoot and Lazarowitz, 1995). Both of these mutants are partially defective mutants (class II, Ingham et al., 1995) that have greatly reduced infectivities of <20% in pumpkin and squash and are no longer infectious for *N. benthamiana*.

Mutants BR1$^{K25A/R26A}$ (targeting the potential bipartite NLS), BR1$^{N201A/K202A/R203A}$, BR1^{D212A}, and BR1$^{R215A/D216A}$

Table I. *Phenotypes of BR1 mutants: subcellular location and ability to be relocalized by wild-type or mutant BL1*

BR1 Mutant[a]	Subcellular Localization[b]		Interaction[c]	
	Sf9 cells	Xanthi cells	BL1	BL1$^{W208A/K211}$
Wild-type	N	N	+[c]	+
K25A/R26A	n (2)	n (2)	+	+
R36A/R37A/R38A	N	N	+	+
R89A/R91A	c (2)	c (2)	+	+
K97A/R98A/R100A	N	N	+	+
F148A/D149A	nd	N	+	nd
D170A/R171A	nd	N	+	nd
N201A/K202A/R203A	n (5)	n (5)	−[d]	−[d]
D212A	nd	n (5)	−[d]	nd
R215A/D216A	nd	n (5)	+	nd
N219A	c (5)	n + c (5)	−[d]	−[d]
N224A	c (5)	c (5)	−	−
K227A/N228A	nd	c (5)	−	nd
Δ195–256	c (5)	nd	−	−

[a] Shown are amino acids mutated by Ala substitution or deleted (see text for details). [b] N, Nucleus; C, cytoplasm; n, retained in perinuclear region prior to targeting to the nucleus; c, retained in perinuclear region prior to targeting to the cytoplasm; n + c, retained in perinuclear region prior to partitioning between nucleus and cytoplasm. Numbers in parentheses indicate days posttransfection on which final targeting to the nucleus and/or cytoplasm was first observed. All mutants having wild-type localization in both cell types were independently tested twice; all other mutants were independently transfected three to five times. [c] Ability of BR1 protein indicated to be redirected from the nucleus to the cell periphery of Sf9 cells and/or Xanthi protoplasts when co-expressed with either BL1 or BL1$^{W208A/K211A}$. +, Relocalized to periphery; −, not relocalized, with BR1 having the same subcellular location as when expressed in the absence of BL1. nd, Not determined. All BR1 mutants that interacted with BL1 in a manner identical to wild-type BR1 were independently tested twice; all other BR1 mutants were independently co-transfected with BL1 three to five times. [d] BR1$^{N201A/K202A/R203A}$ and BR1^{N219A} were not relocalized by BL1 in Sf9 cells. In Xanthi protoplasts, each mutant was transiently relocalized by BL1 to the cell periphery for periods less than 72 h, after which period these mutants were found in the nucleus (see text for details).

were also found to eventually localize to the nucleus of transfected Xanthi protoplasts; however, each of these mutants exhibited a delay in nuclear targeting when compared to wild-type BR1. Whereas wild-type BR1 was localized to Xanthi nuclei by 24 h posttransfection (Sanderfoot and Lazarowitz, 1995), BR1$^{K25A/R26A}$ was not concentrated in Xanthi nuclei until 3 d, and BR1$^{N201A/K202A/R203A}$, BR1^{D212A}, and BR1$^{R215A/D216A}$ were not observed in Xanthi nuclei until 5 d posttransfection (Table I). Prior to their concentrating in the nucleus, each of these mutant BR1 proteins was found localized to the perinuclear region of the Xanthi protoplasts, detected as a ring of immunofluorescent staining around the nuclei (Fig. 1B). BR1$^{K25A/R26A}$ and BR1$^{N201A/K202A/R203A}$ when tested in Sf9 cells also exhibited a perinuclear localization prior to nuclear targeting, similar to that found in Xanthi protoplasts, with a 2- or 4-d delay, respectively, in nuclear targeting compared to wild-type BR1 (Table I). BR1^{N219A} was also found to localize to the perinuclear region up to 4 d posttransfection in both Sf9 cells and Xanthi protoplasts. By 5 d posttransfection this mutant was localized to the cytoplasm of Sf9 cells; however, in Xanthi protoplasts at this time it was partitioned between the nucleus and the cytoplasm, with visually equivalent staining detected in both compartments (Fig. 1C). *BR1*$^{K25A/R26A}$, *BR1*$^{N201A/K202A/R203A}$, and *BR1*^{N219} are those class I mutants for which the presence of viral CP compensates ("masks") for their lack of infectivity in cucurbits (Ingham et al., 1995). When CP is present, these three mutants all have wild-type levels of 100% infectivity in pumpkin. However, both *BR1*$^{K25A/R26A}$ and *BR1*$^{N201A/K202A/R203A}$ exhibit a null phenotype when co-inoculated onto pumpkin with *AR1* mutants that no longer express CP or encode a CP that is defective in binding to DNA; *BR1*^{N219A} infectivity for pumpkin is reduced to approximately 20% of wild-type levels with a delay of 5 d in appearance of symptoms when co-inoculated with these same *AR1* mutants (Ingham et al., 1995).

The remaining BR1 mutants BR1$^{R89A/R91A}$ (targeting the potential SV40-type NLS), BR1^{N244A}, BR1$^{K227A/N228A}$, and BR1$^{\Delta195-256}$ also localized to the perinuclear region of Sf9 cells and Xanthi protoplasts at early time points. However, all of these mutants eventually mislocalized to the cytoplasm, with BR1$^{R89A/R91A}$ being fully cytoplasmic by 3 d posttransfection and BR1^{N224}, BR1$^{K227A/N228A}$, and BR1$^{\Delta195-256}$ being localized to the cytoplasm by 5 d posttransfection (Fig. 1D; Table I). All of these mutants are null mutants that have no infectivity in all hosts tested (class III, Ingham et al., 1995). This suggests that nuclear localization of BR1 is indeed required for its proper function in vivo.

Mutations in the C Terminus of BR1 Affect Interactions with BL1

Previous studies show that when BL1 and BR1 are co-expressed in Sf9 cells or Xanthi protoplasts, BL1 and BR1 appear to directly interact (Sanderfoot and Lazarowitz, 1995). In all cells co-transfected with these two MPs, BL1 remains at the cell periphery but redirects BR1 from the nucleus to the cell periphery. Furthermore, specific mutations in BL1 both affect this interaction and coordinately affect viral infectivity in planta, thus identifying a central domain in BL1 essential for this interaction. This ability of BL1 to redirect BR1 from the nucleus is independent of the correct subcellular targeting of BL1 to the cell periphery (Sanderfoot and Lazarowitz, 1995). Thus, to identify mutations in BR1 that would also affect this interaction, we co-expressed our BR1 Ala-scanning mutants and wild-type BL1 in our transient expression assays.

Those mutations that targeted potential NLSs in the N terminus of BR1 had no effect on the ability of BR1 to interact with BL1. BR1$^{K25A/R26A}$, BR1$^{R36A/R37A/R38A}$, and BR1$^{K97A/R98A/R100A}$, all of which localized to the nucleus, were each redirected to the cell periphery when co-expressed with BL1 in either Sf9 cells or Xanthi protoplasts (Fig. 1E; Table I). Mutations in the middle of BR1, BR1$^{F148A/D149A}$ and BR1$^{D170A/R171A}$, were also correctly targeted to the nucleus and found to be relocalized to the cell periphery when co-expressed with BL1 (Table I).

$BR1^{R89A/R91A}$, even though it mislocalized to the cytoplasm, was also redirected to the cell periphery when co-expressed with BL1 (Fig. 1F; Table I), thus suggesting that correct nuclear targeting of BR1 is independent of and not required for BR1 to interact with BL1. $BR1^{K25A/R26A}$, $BR1^{R36A/R37A/R38A}$, and $BR1^{K97A/R98A/R100A}$ were all also relocalized, as is wild-type BR1, from the nucleus to the cytoplasm by $BL1^{W208A/K211A}$ (Table I), a BL1 mutant that mislocalizes to the cytoplasm (Sanderfoot and Lazarowitz, 1995). These findings thus extended our previous studies by demonstrating that correct subcellular targeting of BL1 is independent of and not required for its ability to interact with BR1.

In contrast to these findings for N-terminal BR1 mutants, C-terminal mutations $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, $BR1^{N219A}$, $BR1^{N224A}$, $BR1^{K227A/N228A}$, and $BR1^{\Delta 195-256}$ all did affect the ability of BR1 to interact with BL1 (Fig. 1, G–J; Table I). $BR1^{R215A/D216A}$ was the only C-terminal mutant that was not impaired in its ability to interact with BL1. When co-expressed with BL1 in Xanthi protoplasts, $BR1^{R215A/D216A}$ relocalized to the cell periphery at all times posttransfection, whether it had been in the perinuclear region or nucleus when expressed alone at that particular time posttransfection (Fig. 1G; Table I). Thus, it appeared that localization to the perinuclear region and delay in final subcellular targeting per se were not indicative of global misfolding of BR1. The C-terminal mutants $BR1^{N224A}$ and $BR1^{\Delta 195-256}$ in Sf9 cells and mutant $BR1^{K227A/N228A}$ in Xanthi protoplasts, however, when co-expressed with either wild-type BL1 or mutant $BL1^{W208A/K211A}$ were not redirected to the cell periphery or cytoplasm at any time posttransfection, each remaining in the perinuclear region or distributed throughout the cytoplasm, depending on the day posttransfection (Table I). These BR1 mutants are all null mutants having no infectivity in any host tested (Ingham et al., 1995).

None of the C-terminal mutations affected NLSs within BR1 (see below); however, each did exhibit a delay of 4 d during which they were localized in the perinuclear region prior to their final targeting to the cell nucleus and/or cytoplasm of Sf9 cells and/or Xanthi protoplasts. The C-terminal mutants $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, and $BR1^{N219A}$ were found to be only partially defective in their ability to interact with BL1 in Xanthi protoplasts. Neither $BR1^{N201A/K202A/R203A}$ nor $BR1^{N219A}$ was redirected to the periphery of Sf9 cells when co-expressed with BL1: $BR1^{N201A/K202A/R203A}$ remained in the perinuclear region or nucleus depending on the day posttransfection, and $BR1^{N219A}$ remained distributed throughout the cytoplasm (Table I). However, when co-expressed with BL1 in Xanthi protoplasts at 24 to 48 h posttransfection, $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, and $BR1^{N219A}$ were all in part relocalized to the cell periphery, with approximately equal staining being detected for each at the cell periphery and in the perinuclear region (Fig. 1H; Table I). At 3 d and later times posttransfection, these three BR1 mutants were no longer relocalized to the periphery of Xanthi protoplasts by BL1; each remained in the perinuclear region prior to 5 d posttransfection when $BR1^{N201A/K202A/R203A}$ and $BR1^{D212A}$ were found localized to the nuclei (Fig. 1I; Table I), and $BR1^{N219A}$ was found equally distributed between nuclei and cytoplasm (Fig. 1J). $BR1^{N201A/K202A/R203A}$ and $BR1^{N219A}$ are both class I mutants, the defective phenotypes of which are masked by the presence of viral CP (see above; Ingham et al., 1995).

Although prolonged residence in the perinuclear region prior to final subcellular targeting did not appear to indicate global misfolding of BR1 mutants, our findings suggested that mutations within the C terminus did affect the correct timing of the subcellular targeting of BR1. This complicated the interpretation of mutations $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, and $BR1^{N219A}$ as targeting a domain in BR1 required for proper interaction with BL1. Hence, to directly examine whether the C terminus of BR1 did contain a domain that specified interactions with BL1, we made translational fusions of the N-terminal 113 amino acids or the C-terminal 146 amino acids of BR1 to the C terminus of GUS, thereby creating GUS-$BR1^{1-113}$ and GUS-$BR1^{110-256}$, respectively. These were each tested for their ability to redirect GUS from the cytoplasm to the cell periphery when co-expressed with BL1, as judged by indirect immunofluorescent staining with anti-GUS antibodies and confocal microscopy. When individually expressed in Xanthi protoplasts, the GUS-$BR1^{1-113}$ fusion was localized to the nuclei, whereas the GUS-$BR1^{110-256}$ fusion was detected uniformly throughout the cytoplasm at all times posttransfection (data not shown). When co-expressed with BL1, the GUS-$BR1^{1-113}$ fusion protein remained in the nuclei of the transfected Xanthi protoplasts (Fig. 1K); however, the GUS-$BR1^{110-256}$ fusion was now relocalized from the cytoplasm to the cell periphery (Fig. 1L) in a manner identical to that observed for wild-type BR1 (Sanderfoot and Lazarowitz, 1995; and above). These findings suggested that the C terminus of BR1 did contain a domain that is necessary and sufficient for its ability to specifically interact with BL1. It further demonstrated that all of the NLSs in BR1 were contained within the N-terminal region of the protein.

The N Terminus of BR1 Contains Two NLSs

Our immunofluorescent studies of the localization of the fusion proteins GUS-$BR1^{1-113}$ and GUS-$BR1^{110-256}$ argued that the NLSs in BR1 were located within the N-terminal 113 amino acids of the protein. In addition, the presence of the bipartite consensus NLS at residues 25 to 39 and the SV40-type consensus NLS at residues 87 to 95, and the finding that mutants $BR1^{K25A/R26A}$ and $BR1^{R89A/R91A}$, in which these potential NLSs were mutated, were defective in their nuclear targeting (see above), with the latter mutant mislocalizing to the cytoplasm and having a null phenotype in infectivity, further suggested that these are potential NLSs. Therefore, to further investigate this point and clearly identify possible NLSs in BR1, we constructed a series of GUS-BR1 fusions designed to test the ability of different regions and specific small peptide segments of BR1 to redirect GUS from the cytoplasm to the nuclei of Xanthi protoplasts when fused to the C terminus of GUS in the expression vector pRTL2-GUS (Restrepo et al., 1990).

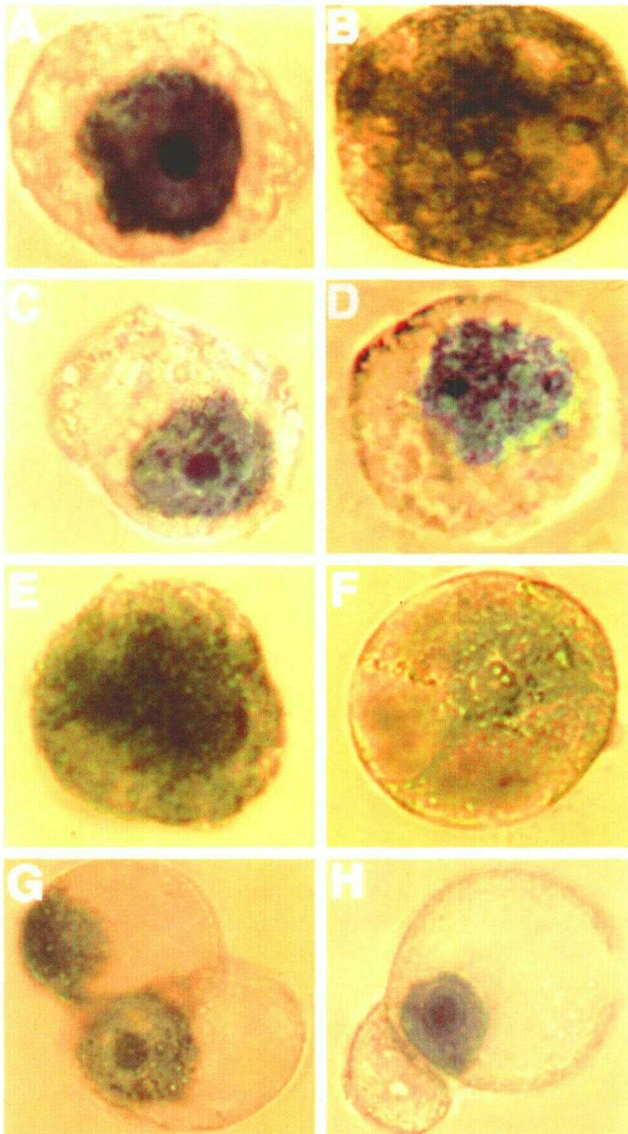

Figure 2. Localization in transfected Xanthi protoplasts of GUS activity by GUS-BR1 translational fusions for histochemical staining with X-gluc at 24 h posttransfection. A, GUS-BR1$^{1-113}$; B, GUS-BR1$^{110-256}$; C, GUS-BR1$^{1-65}$; D, GUS-BR1$^{57-113}$; E, GUS-BR1$^{1-65:K25A/R26A/R36A/R37A/R38A}$; F, GUS-BR1$^{57-113:R89A/R91A}$; G, GUS-BR1$^{21-42}$; H, GUS-BR1$^{81-96}$. Cells are shown at same magnification as in Figure 1.

Each fusion protein was localized within protoplasts at 24 h posttransfection based on histochemical staining for GUS activity (Jefferson et al., 1987). When GUS itself was transiently expressed in Xanthi protoplasts, staining with X-gluc showed that all GUS activity was uniformly distributed throughout the cytoplasm (data not shown), as previously reported by others (Jefferson et al., 1987; Restrepo et al., 1990; Howard et al., 1992; Citovsky et al., 1992). In contrast, GUS-BR1$^{1-113}$ activity was specifically targeted to the nuclei of the transfected protoplasts (Fig. 2A); however, GUS-BR1$^{110-256}$ activity was found distributed throughout the cytoplasm in a manner identical to GUS alone (Fig. 2B), thus confirming the results of our GUS immunofluorescent staining assays.

To determine whether the regions that contained the potential bipartite NLS and SV40-type NLS consensus sequences could separately function for nuclear targeting, we further subdivided the N-terminal 113 amino acid segment of BR1 to create the translational fusions GUS-BR1$^{1-65}$ and GUS-BR1$^{57-113}$. The activity of each of these two GUS fusions was specifically targeted to the nuclei of transfected Xanthi protoplasts (Fig. 2, C and D), thus indicating that there are at least two distinct NLSs within this N-terminal region of BR1. The two basic regions of the potential bipartite NLS within segment BR1$^{1-65}$ are altered by Ala substitutions in mutants BR1$^{K25A/R26A}$ and BR1$^{R36A/R37A/R38A}$, and the SV40-type NLS within segment BR1$^{57-110}$ is mutated in the same manner in BR1$^{R89A/R91A}$ (see above). As reported by others who have mutated only one of the basic regions in a bipartite NLS (Howard et al., 1992; Citovsky et al., 1992), when the N-terminal 65 amino acids from either BR1$^{K25A/R26A}$ or BR1$^{R36A/R37A/R38A}$ were fused to GUS, the activity of resulting fusion proteins GUS-BR1$^{1-65:K25A/R26A}$ and GUS-BR1$^{1-65:R36A/R37A/R38A}$ was still targeted to the nuclei of transfected protoplasts (Fig. 3). However, when these two mutations were combined to alter both basic regions of the potential bipartite NLS in the fusion GUS-BR1$^{1-65:K25A/R26A/R36A/R37A/R38A}$, this peptide was no

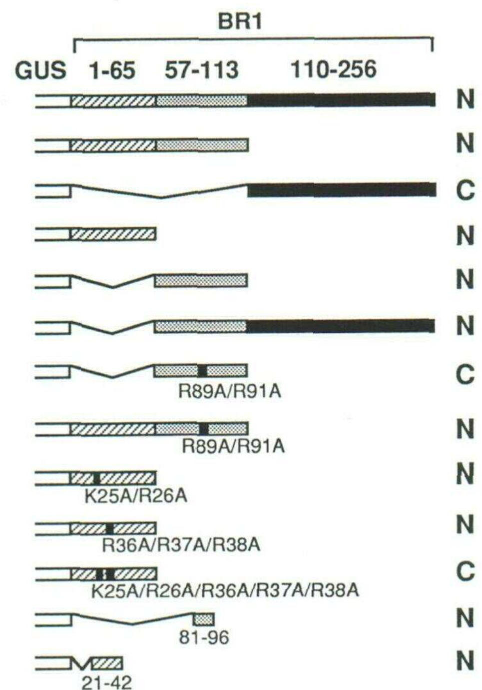

Figure 3. Subcellular localization of GUS activity for GUS-BR1 translational fusions in Xanthi protoplasts, based on histochemical staining with X-gluc. Diagrammed is each fusion protein assayed and the location of GUS activity as being nuclear (N) or cytoplasmic (C). Shown at the top are the segments of BR1 included in each fusion construct, indicated by residue numbers (amino acids 1–65, 57–113, or 110–256). Black boxes indicate location of point mutants as written beneath each construct. Numbers below the two bottom constructs indicate BR1 amino acids contained within each peptide fused to GUS. ▨, Amino acids 1 to 65 of BR1; ▤, amino acids 57 to 113 of BR1; ■, amino acids 110 to 256 of BR1.

longer able to target GUS to the nucleus and all GUS activity remained in the cytoplasm (Figs. 2E and 3). In a similar manner, segment $BR1^{57-110}$ from mutant $BR1^{R89A/R91A}$, containing Ala substitutions for two basic residues within the SV40-type consensus NLS, was no longer capable of targeting GUS activity to protoplast nuclei: $GUS\text{-}BR1^{57-113:R89A/R91A}$ activity was found located throughout the cytoplasm (Figs. 2F and 3). Based on these mutational studies, it appeared that there was both a bipartite NLS (defined by mutants $BR1^{K25A/R26A}$ and $BR1^{R36A/R37A/R38A}$) and an SV40-type NLS (defined by mutant $BR1^{R89A/R91A}$) within BR1. The cytoplasmic location of $GUS\text{-}BR1^{1-65:K25A/R26A/R36A/R37A/R38A}$ and $GUS\text{-}BR1^{57-113:R89A/R91A}$ further suggested that these were the only NLSs within BR1.

To more precisely define the NLSs within BR1, oligonucleotides encoding small peptides that encompassed the potential bipartite NLS (residues 21–42, RTGVKRSYGAARGDDRRRPNVV, consensus underlined) or SV40-type consensus (residues 81–96, SYVKTVPNRTRTYIKL) were translationally fused to the C terminus of GUS in pRTL2-GUS and each tested for its ability to redirect GUS to the nuclei of Xanthi protoplasts. As shown in Figure 2, G and H, both $GUS\text{-}BR1^{21-42}$ and $GUS\text{-}BR1^{81-96}$ targeted GUS activity to the nuclei of Xanthi protoplasts. Thus, BR1 contained two NLSs, a bipartite NLS located within the 22-residue peptide defined by $BR1^{21-42}$ and an SV40-type NLS located within the 16-residue peptide defined by $BR1^{81-96}$. We have termed the bipartite NLS (residues 21–42) "NLS-A" and the SV40-type NLS (residues 81–96) "NLS-B."

The results of all of our GUS-BR1 fusion studies and our BR1 localization and interaction studies are summarized in Figures 3 and 4, respectively.

DISCUSSION

Movement of the viral genome, both intracellularly to reach the cell periphery and intercellularly to cross the plant cell wall, is essential for all plant viruses. The mechanism of intracellular transport is of particular importance in providing directionality to this movement. For nuclear replicating viruses such as the bipartite geminiviruses, this process requires that genome movement be coordinated between two intracellular compartments, namely, the nucleus and cytoplasm. Our previous and current studies suggest that bipartite geminiviruses such as SqLCV accomplish this through the cooperative interaction of BR1 and BL1: BR1 acts as a nuclear shuttle protein to bind newly replicated viral ssDNA genomes and move these into and out of the cell nucleus; BL1 provides directionality by specifically trapping BR1-ssDNA complexes in the cytoplasm and redirecting these to the cell periphery and across the cell plasma membrane and wall to adjacent uninfected cells (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995). In this study, we have tested Ala-scanning and deletion mutants of BR1 in our transient expression assays to further examine the function of BR1 as a nuclear shuttle protein, thereby specifically identifying regions of BR1 essential for its interaction with BL1 and correct subcellular

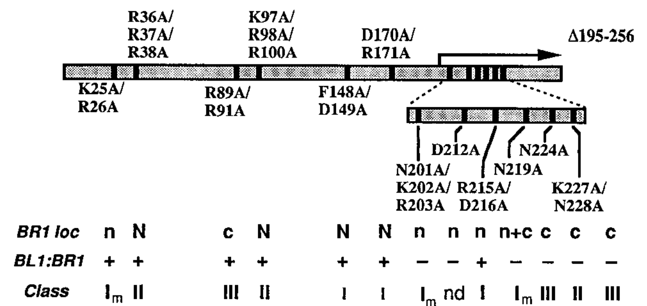

Figure 4. Summary of the subcellular location, BR1 interactions, and infectivity phenotype of BR1 mutants. Diagrammed is the *BR1* coding sequence. Point mutations are indicated by black boxes, and the C-terminal truncation Δ195–256 is indicated by an arrow. *BR1 loc,* Subcellular location of BR1 as nuclear (N), cytoplasmic (C), or delayed in perinuclear region prior to nuclear (n) or cytoplasmic (c) targeting. *BL1:BR1,* Ability of the mutated BR1 protein to be relocalized (+) or not relocalized (−) by BL1. *Class,* Infectivity defect of each mutant as characterized by Ingham et al. (1995). The C-terminal region of BR1 is drawn expanded for clarity. See text for details.

targeting to the nucleus. As reported here, and previously for BL1 (Sanderfoot and Lazarowitz, 1995), these two functions of BR1 could be independently mutated, thus allowing us to identify regions of BR1 essential for each separate function (Fig. 4). Also, as for BL1, we found excellent correlations between the defects in subcellular targeting and MP interaction for each BR1 mutant protein and its effect on SqLCV infectivity, thus further validating our transient expression assays, particularly in Xanthi protoplasts, as model systems for dissecting SqLCV MP function. Our results indicated that BR1 has a domain-type structure, with an N-terminal region that contains two NLSs and a C-terminal region that specifies interaction with BL1.

This domain structure of BR1 was most clearly demonstrated by translationally fusing the N- and C-terminal regions of BR1 to the C terminus of GUS. The nuclear targeting of both GUS protein and activity for our fusion construct $GUS\text{-}BR1^{1-113}$ and the cytoplasmic location of both protein and GUS activity for $GUS\text{-}BR1^{110-256}$ clearly demonstrated that all NLSs were within the N-terminal 113 amino acids of BR1 (see Figs. 2 and 3). Mutants $BR1^{K25A/R26A/R36A/R37A/R38A}$ and $BR1^{R89A/R91A}$ further demonstrated that nuclear targeting of BR1 was essential for its function and, combined with our GUS fusion studies, clearly identified two NLSs within the N-terminal 96 residues of BR1: the bipartite NLS-A between residues 21 and 42 and the SV40-type NLS-B between residues 81 and 96. In addition, although BL1 was unable to redirect $GUS\text{-}BR1^{1-113}$ to the cell periphery, it did specifically relocalize $GUS\text{-}BR1^{110-256}$. These findings and the fact that $BR1^{D170A/R171A}$ and all other N-terminal BR1 mutants were relocalized by BL1 lead us to conclude that the domain(s) essential for BR1 to interact with BL1 is located within the C-terminal 86 residues of BR1.

Ala substitutions within this C-terminal region implicated a domain encompassing amino acids 201 to 219 as specifying this interaction. When co-expressed with BL1,

mutants $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, and $BR1^{N219A}$ were each partially relocalized from the perinuclear region to the periphery of Xanthi protoplasts up to 72 h posttransfection. At 3 to 4 d posttransfection these mutated proteins were found completely in the perinuclear region, and at 5 d and later $BR1^{N201A/K202A/R203A}$ and $BR1^{D212A}$ became concentrated in cell nuclei and $BR1^{N219A}$ was distributed between nuclei and cytoplasm (Fig. 1; Table I). This partial defect in the interation of these mutants with BL1 was well correlated with their partial defects in infectivity, since the defective phenotypes of *BR1*$^{N201A/K202A/R203A}$ and *BR1*^{N219A} are both masked by CP. This interactive domain in BR1 may extend more C-terminally than residue 219. More mutations are needed to examine this point because the cytoplasmic localization of mutants $BR1^{N224A}$, $BR1^{K227A/N228A}$, and $BR1^{\Delta 195-256}$, their inability to interact with BL1, and their null phenotypes in infectivity all suggested that these mutant proteins might be globally misfolded.

The one striking difference found between Sf9 cells and Xanthi protoplasts was in the dynamics of BR1-BL1 interaction and perhaps in BR1 shuttling (Sanderfoot and Lazarowitz, 1995; and above). In all cases infectivity results correlated well with assays in Xanthi protoplasts, even when these were at variance with findings in Sf9 cells. Although wild-type BL1 redirected BR1 to the cell periphery at all times examined posttransfection (up to 9 d) in Sf9 cells, in Xanthi protoplasts BR1 was redirected from the nucleus to the periphery up to 5 d posttransfection, after which BR1 was found in the perinuclear region, whereas BL1 remained at the periphery (A.A. Sanderfoot and S.G. Lazarowitz, unpublished results). This is of particular interest, since, according to our model, regulating the timing of BR1-BL1 interactions would be important: BL1 not only has to bind BR1-ssDNA complexes and direct these to the cell periphery, but must also release these complexes so they can enter adjacent cells. Further illustrating these differences between Sf9 cells and Xanthi protoplasts, all mutations that affected the ability of BR1 and BL1 to mutually interact were partially defective, both in their infectivity phenotypes and interactions in Xanthi protoplasts; however, all behaved as null mutations in their interaction in Sf9 cells. This was true for $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, and $BR1^{N219A}$, which were each partially relocalized to the periphery of Xanthi protoplasts for up to 48 h posttransfection, after which they no longer interacted with wild-type BL1, and correlated strikingly with our finding that the partially defective class II mutants $BL1^{K140A/K142A}$ and $BL1^{K147A/H148A}$ can only redirect wild-type BR1 to the periphery of Xanthi protoplasts prior to 48 h posttransfection (Sanderfoot and Lazarowitz, 1995) (see Table I).

In addition to these findings for BR1-BL1 interactions in Xanthi protoplasts, we found that all mutations within the C-terminal 61 amino acids of BR1 (including *BR1*$^{N201A/K202A/R203A}$, *BR1*^{D212A}, and *BR1*^{N219A}) caused BR1 to reside in the perinuclear region for 4 d prior to final subcellular targeting to the nucleus and/or cytoplasm. This perinuclear localization does not appear to result from global protein misfolding per se, since $BR1^{N201A/K202A/R203A}$, $BR1^{D212A}$, $BR1^{R215A/D216A}$, and $BR1^{N219A}$ were each relocalized from the perinuclear region to the cell periphery by BL1 during the first 48 h posttransfection and did eventually correctly target to the nucleus in Xanthi protoplasts. Furthermore, $BR1^{R215A/D216A}$ was relocalized by BL1 at all times posttransfection, whether it resided in the nucleus or perinuclear region (Fig. 2G). Thus, taken together with the perinuclear location of wild-type BR1 following 5 d of interaction with BL1 in Xanthi protoplasts and the fact that wild-type BR1 following 2 d of interaction with defective mutants $BL1^{K140A/K142A}$ and $BL1^{K147A/H148A}$ is found in the nucleus (Sanderfoot and Lazarowitz, 1995), these results suggested that posttranslational modification of BR1, and possibly BL1, may be important in regulating the interaction and function of these two MPs.

What might such posttranslational modifications be? No obvious structural motifs are evident from inspection of the sequence in this C-terminal region of BR1. However, since both BR1 and BL1 are phosphoproteins (Pascal et al., 1994), we suggest that altered patterns of phosphorylation could be important in regulating BR1-BL1 interaction and may explain the behavior of C-terminal BR1 mutants in Xanthi protoplasts. The perinuclear localization we have observed for BR1 and its C-terminal mutants in Xanthi protoplasts has been reported for other nuclear localized cellular and virus-encoded proteins in response to changes in phosphorylation state, developmental stage, or the availability of appropriate ligands (Mukaigawa and Nayak, 1991; Faure and Posner, 1993; Whiteside and Goodbourn, 1993; Worrad and Caradonna, 1993; Studinger et al., 1995). The perinuclear localization we found bears a striking resemblance to that reported for nuclear proteins in the absence of Importin, a factor essential for their transport through nuclear pores (Gorlich et al., 1994), and for mutant forms of several virus-encoded phosphoproteins such as the influenza polymerase subunit PB2, the capsid protein VP1 of SV40, and UL3 of herpesvirus (Carswell and Alwine, 1986; Mukaigawa and Nayak, 1991; Worrad and Caradonna, 1993). Particularly relevant here may be changes in the nuclear and cytoplasmic localization of many transcription factors in response to alterations in their phosphorylation state (Whiteside and Goodbourn, 1993). A number of potential Ser/Thr and Tyr phosphorylation sites are clustered within the C terminus of BR1, and it has been suggested that function of the tobacco mosaic virus 30-kD MP may be regulated by changes in its state of phosphorylation (Citovsky et al., 1993). Thus, we suggest that the functions of BR1 and BL1, as well as their mutual interaction, may be posttranslationally regulated through phosphorylation or other possible modifications and that the perinuclear localization of BR1 C-terminal mutants may reflect alterations in their modified states. Additional mutational and biochemical studies should elucidate the importance of posttranslational phosphorylation and other potential modifications in regulating the interaction and function of BR1 and BL1.

It is interesting that those infectious mutants that have an early perinuclear "phase" prior to entering the nucleus in Xanthi protoplasts—$BR1^{K25A/R26A}$, $BR1^{N201A/K202A/R203A}$, and $BR1^{N219A}$—all have their infectivity defects masked by

CP (see above; Ingham et al., 1995). Co-expression of CP in our transient assays did not affect this perinuclear localization (data not shown), thus indicating that masking by CP does not involve direct interaction with BR1. We have suggested that CP masking may result from the induction of high levels of viral ssDNA synthesis found to occur in the presence of CP (Ingham et al., 1995), and our findings here for the subcellular localization of $BR1^{K25A/R26A}$, $BR1^{N201A/K202A/R203A}$, and $BR1^{N219A}$ are consistent with this model. Each of these "masked" mutant BR1 proteins is delayed in its nuclear accumulation, taking several days to reach wild-type BR1 levels, and none of these mutants are defective in DNA binding (D.J. Ingham and S.G. Lazarowitz, unpublished data). In addition, $BR1^{N219A}$ eventually partitions between the nucleus and cytoplasm (Fig. 1C). Hence, the high levels of CP-induced viral ssDNA, to which BR1 will bind, could compensate for the lower levels of BR1 found at early times in the nucleus for these mutants. In this manner CP would mask their defective infectivity phenotypes by providing higher concentrations of ssDNA substrate that would favor BR1 binding. Without CP, $BR1^{K25A/R26A}$ and $BR1^{N201A/K202A/R203A}$ are null in infectivity, and $BR1^{N219A}$ infectivity is delayed and severely depressed. This, together with their different patterns of targeting in Xanthi protoplasts, leads us to suggest that $BR1^{K25A/R26A}$ and $BR1^{N201A/K202A/R203A}$ are delayed in nuclear import, but $BR1^{N219A}$ may be defective in nuclear retention. Thus, $BR1^{N219A}$, unlike $BR1^{K25A/R26A}$ and $BR1^{N201A/K202A/R203A}$, may still rapidly enter the nucleus, and this kinetic difference could explain why its infectivity is reduced but not abolished in the absence of CP.

Our results have demonstrated that nuclear targeting of BR1, as well as its ability to interact with BL1, is essential for its function in SqLCV infection. In addition, the partitioning of $BR1^{N219A}$ between the cytoplasm and nuclei of Xanthi protoplasts, combined with the absence of NLSs in this C-terminal region of BR1 and our previous studies (Sanderfoot and Lazarowitz, 1995), argues that shuttling of BR1 may be essential for its proper function as well. Nuclear shuttle proteins are important components of intercompartmental communication in all eukaryotic cells and have historically been identified based on their nuclear redistribution in heterokaryons or microinjection studies (Bataille et al., 1990; Guichon-Mantel et al., 1994). The only other reported virus-encoded nuclear shuttle protein, the matrix protein M1 of influenza A virus, was deduced based on immunogold localization of M1 to the cytoplasm and nucleus at different times following infection of animal cells (Martin and Helenius, 1991b). We have inferred the function of BR1 as a nuclear shuttle protein based on the unique specificity of its interaction with and relocalization by BL1. It has been suggested that all karyophilic proteins may potentially function as shuttle proteins (Schmidt-Zachmann et al., 1993), and recent studies indicate that NLSs may be required for nuclear export as well as import (Guichon-Mantel et al., 1994). However, Leu-rich regions have also been reported to potentially function as nuclear export signals (Fischer et al., 1995; Wen et al., 1995), and indeed four such motifs are present in BR1 that conform to the proposed export signal consensus, one of which resides in the region of residue 219. Given our ability to use Xanthi protoplasts to experimentally examine the import of BR1, as well as its export in the presence of BL1, our further investigation of $BR1^{N219A}$ and additional *BR1* mutants should elucidate some of the fundamental properties of nuclear shuttle proteins and the mechanisms by which their action is regulated. In addition, given the dynamics of BR1-BL1 interaction and virus movement, we predict that within our mutant collections we should also identify BR1 and BL1 mutants that bind too avidly to the other MP and would therefore have a dominant interfering phenotype. Such mutants would be prime candidates for engineering plants resistant to infection by SqLCV and perhaps other bipartite geminiviruses as well.

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