The Plant Cell, Vol. 6, 995–1006, July 1994 © 1994 American Society of Plant Physiologists

The Geminivirus BR1 Movement Protein Binds Single-Stranded DNA and Localizes to the Cell Nucleus

Erica Pascal,[a] Anton A. Sanderfoot,[a] Brian M. Ward,[a] Richard Medville,[b] Robert Turgeon,[c] and Sondra G. Lazarowitz [a,1]

[a] Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
[b] Electron Microscopy Services, Ithaca, New York 14853
[c] Section of Plant Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Plant viruses encode movement proteins that are essential for infection of the host but are not required for viral replication or encapsidation. Squash leaf curl virus (SqLCV), a bipartite geminivirus with a single-stranded DNA genome, encodes two movement proteins, BR1 and BL1, that have been implicated in separate functions in viral movement. To further elucidate these functions, we have investigated the nucleic acid binding properties and cellular localization of BR1 and BL1. In this study, we showed that BR1 binds strongly to single-stranded nucleic acids, with a higher affinity for single-stranded DNA than RNA, and is localized to the nucleus of SqLCV-infected plant cells. In contrast, BL1 binds only weakly to single-stranded nucleic acids and not at all to double-stranded DNA. The nuclear localization of BR1 and the previously demonstrated plasma membrane localization of BL1 were also observed when these proteins were expressed from baculovirus vectors in *Spodoptera frugiperda* insect cells. The biochemical properties and cellular locations of BR1 and BL1 suggest a model for SqLCV movement whereby BR1 is involved in the shuttling of the genome in and/or out of the nucleus and BL1 acts at the plasma membrane/cell wall to facilitate viral movement across cell boundaries.

INTRODUCTION

Because plant cells are surrounded by cell walls, plant viruses must be more aggressive than animal viruses in their spread from cell to cell. To accomplish this, plant viruses encode movement proteins (MPs) that are essential for infection of the host plant, but not required for viral replication or encapsidation (Atabekov and Dorokhov, 1984; Hull, 1991). Genetic evidence implicates viral MPs in both local cell-to-cell movement and long-distance transport through the vascular system and argues for viral movement being actively facilitated by these proteins (Hull, 1991; Leisner and Turgeon, 1993). However, the mechanism of action of viral MPs is yet to be defined. The MPs of RNA viruses, in particular the 30-kD MP of tobacco mosaic virus (TMV), remain the best characterized, and studies of TMV (Citovsky et al., 1990; Ding et al., 1992) have suggested a model for MP function that has generally been supported by subsequent studies of other plant viruses which, like TMV, replicate in the cytoplasm (Linstead et al., 1988; Citovsky et al., 1991; Osman et al., 1992; Fujiwara et al., 1993). According to this model, the TMV MP interacts with the genome and targets it to the plasmodesmata in the walls between adjacent cells, where it acts to facilitate passage of the genome through these cell wall "pores" to neighboring uninfected cells (Wolf et al., 1989; Citovsky et al., 1992a; Ding et al., 1992). This could explain local spread of infection by direct cell-to-cell movement. However, the TMV studies have also indicated that the MP is not sufficient for movement into the vascular system for long-distance viral spread, suggesting that additional viral functions are required (Dawson et al., 1988; Saito et al., 1990; Ding et al., 1992).

Unlike RNA viruses such as TMV that replicate in the cytoplasm, the bipartite geminivirus squash leaf curl virus (SqLCV) is a DNA virus that replicates in the nucleus (Goodman, 1981). Therefore, for viral spread, transport in and out of the nuclear compartment is required in addition to movement across cell boundaries. Furthermore, unlike most RNA viruses that have one MP, SqLCV encodes two ~30 kD MPs, BR1 and BL1 (Lazarowitz, 1992). This has led us to hypothesize that if MPs must bind the viral genome to facilitate movement, then for SqLCV at least one MP should localize to the nucleus and function to shuttle the viral genome into and out of the nucleus as well as to the periphery of the cell. This potential nuclear localization presents a particular advantage for identifying viral MP–genomic complexes and determining the requirements for such complexes in facilitating viral movement.

Current studies on bipartite geminiviruses do not resolve whether BL1 and BR1 function in concert or separately to facilitate local and long-distance viral movement. Recent studies do suggest that the two SqLCV MPs carry out different functions in viral movement. Biochemical fractionation of

[1] To whom correspondence should be addressed.

infected plant cell extracts localizes BL1 to the plasma membrane and cell wall fractions and BR1 to a separate vesicle-containing membrane fraction. In addition, expression of these MPs in transgenic tobacco has demonstrated that, in contrast to the normal appearance and growth properties of BR1-expressing transgenic plants, expression of BL1 alone is sufficient to produce all the symptoms of SqLCV infection (Pascal et al., 1993). Taken together, this evidence suggests that the processes involved in viral movement may be partitioned between the two MPs. We have suggested that BL1 may be involved in direct cell-to-cell movement, and BR1 may function in long-distance movement (Ingham and Lazarowitz, 1993; Pascal et al., 1993), although other investigators have reached the opposite conclusion (von Arnim et al., 1993). Another potential model is that BR1 is necessary for the transport of the genome into and out of the nucleus, whereas BL1 directs movement across cell boundaries; a recent study by Noueiry et al. (1994) for bean dwarf mosaic virus (BDMV) purports to support this latter model.

To further elucidate the potential functions of BR1 and BL1, we have begun to investigate their biochemical properties. Because unlike many plant viruses, SqLCV and other bipartite geminiviruses do not require encapsidation for infection (Gardiner et al., 1988; A.A. Sanderfoot and S.G. Lazarowitz, unpublished results), we propose that either or both of the MPs interact directly with the DNA genome. To address these issues, we have employed several experimental systems to investigate the nucleic acid binding properties and potential nuclear localization of BR1 and BL1. Our results reported here demonstrate that BR1 is a single-stranded DNA binding protein. In addition, we used immunogold cytochemistry to more precisely localize BR1 to the nucleus of infected plant cells. We further establish that this nuclear localization of BR1, as well as the plasma membrane localization of BL1, are also observed when these MPs are expressed from baculovirus vectors in insect cells, making this expression system a powerful one for the investigation of MP localization and interactions. These findings support our earlier results, suggesting a partitioning of movement functions between the two MPs, and lead us to conclude that BR1 is responsible for nuclear transport of the viral genome, whereas BL1 functions in viral movement between cells.

RESULTS

Nucleic Acid Binding Properties of BR1 and BL1

To investigate the nucleic acid binding properties of the SqLCV MPs, ^{35}S-methionine–labeled BR1 and BL1 proteins were synthesized in an in vitro transcription/translation system and tested for their ability to bind DNA coupled to cellulose. Each protein was incubated with double-stranded DNA (dsDNA)–cellulose, single-stranded DNA (ssDNA)–cellulose, or control uncoupled cellulose resin. Following an initial binding period, the resins were extensively washed with the binding buffer containing 50 mM KCl, and protein was eluted stepwise with binding buffer containing increasing KCl concentrations from 100 mM to 1 M. Fractions collected at each salt step were separated by SDS-PAGE, and proteins were detected by autoradiography. As shown in Figure 1, BR1 bound to both ssDNA– and dsDNA–cellulose resins, but was not bound to

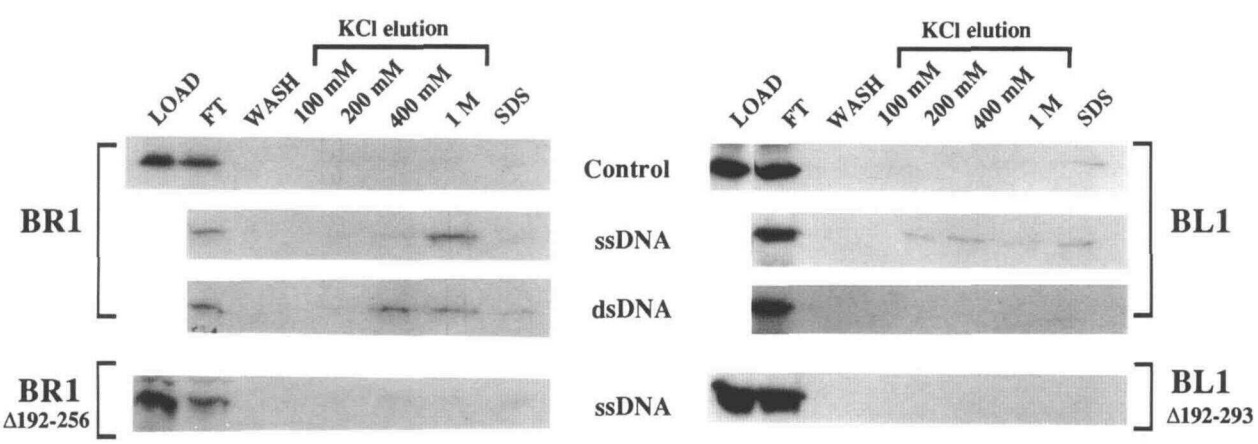

Figure 1. Binding of BR1 and BL1 to DNA-Cellulose.

^{35}S-methionine–labeled BR1, BR1$^{\Delta 192-256}$, BL1, and BL1$^{\Delta 192-293}$ were incubated with uncoupled cellulose resin (control), or ssDNA, or dsDNA coupled to cellulose resin. Protein was eluted from each resin by successive washes in buffer containing increasing concentrations of KCl as indicated above each lane. Residual protein was eluted by boiling in SDS sample buffer (SDS lanes). Equivalent amounts of each fraction, including the input and final wash (WASH) fractions, were separated by SDS-PAGE. Gels were impregnated for fluorography, dried, and exposed to x-ray film. LOAD denotes total input protein; FT denotes flow-through protein not bound to DNA-cellulose.

the control uncoupled resin. BR1 appeared to have a higher affinity for ssDNA–cellulose than for dsDNA–cellulose, eluting from the ssDNA resin at 1 M KCl, whereas it began to elute from the dsDNA resin at 400 mM KCl. As BR1 is a very basic protein (pI of 10.8), it was possible that these interactions with DNA were solely due to its charge. To eliminate this possibility, we tested the binding properties of a truncation of BR1, $BR1^{\Delta 192-256}$, which lacks the C-terminal 64 amino acids of the 256–amino acid BR1 protein but deletes only five of the 40 basic residues. $BR1^{\Delta 192-256}$ did not bind to either ssDNA– or dsDNA–cellulose resin (Figure 1 and data not shown), thus indicating that specificity in the interaction of BR1 with DNA is not simply due to the overall basic charge of the protein.

In contrast to the apparently tight binding of BR1, BL1 bound only weakly to ssDNA–cellulose, eluting at lower salt concentrations than did BR1, and did not bind at all to the dsDNA–cellulose (Figure 1). Although a small amount of BL1 also bound the control resin, it did not elute in a salt-dependent manner. Unlike BR1, which bound to the DNA–cellulose resin at room temperature (25°C), BL1 required incubation at 4°C for binding. This may have been due to either an inherent instability in BL1 protein structure or an unstable protein–DNA complex at higher temperatures. To confirm the specificity of this protein–DNA interaction, we tested a deletion of the C-terminal 101 amino acids of the 293–amino acid BL1, $BL1^{\Delta 192-293}$, which has approximately the same charge density as full-length BL1. This deletion mutant was unable to bind to the ssDNA–cellulose resin (Figure 1). As an additional control for both BR1 and BL1, we have performed the same DNA–cellulose binding assays using full-length native BR1 and BL1 (Pascal et al., 1993) produced in recombinant baculovirus–infected *Spodoptera frugiperda* (Sf9) cells and obtained the identical results (data not shown).

We used a protein blot binding assay (Sukegawa and Blobel, 1993) to further define the nucleic acid binding specificity of BR1 and BL1. Proteins extracted from *Escherichia coli* expressing either BR1 or BL1 were separated by SDS-PAGE and transferred to nitrocellulose. Following a renaturation step, these membranes were incubated with radiolabeled RNA, ssDNA, or dsDNA probes derived from SqLCV genomic sequences. The membranes were then washed with buffers containing 250, 400, or 600 mM KCl and exposed to film. As shown in Figure 2, BR1 bound to all three nucleic acid probes at 250 mM salt. However, at higher salt concentrations, BR1 no longer bound the dsDNA probe but did remain bound to both the ssDNA and RNA probes (Figure 2, compare lane 13 to lanes 8 and 18). Whereas little apparent decrease in the interactions of BR1 with RNA and ssDNA was detected at 400 mM KCl, at 600 mM salt the binding of BR1 to these probes was much reduced (Figure 2, compare lanes 8 and 9 to lanes 18 and 19). Under these same salt conditions, BL1 did not bind to any of the probes (Figure 2, lanes 6, 11, and 16). As a control for nonspecific binding of the probes due to large amounts and/or the basic nature of protein on the blots, we included BSA and lysozyme on each blot. Neither of these proteins bound to any of the nucleic acid probes tested under our assay

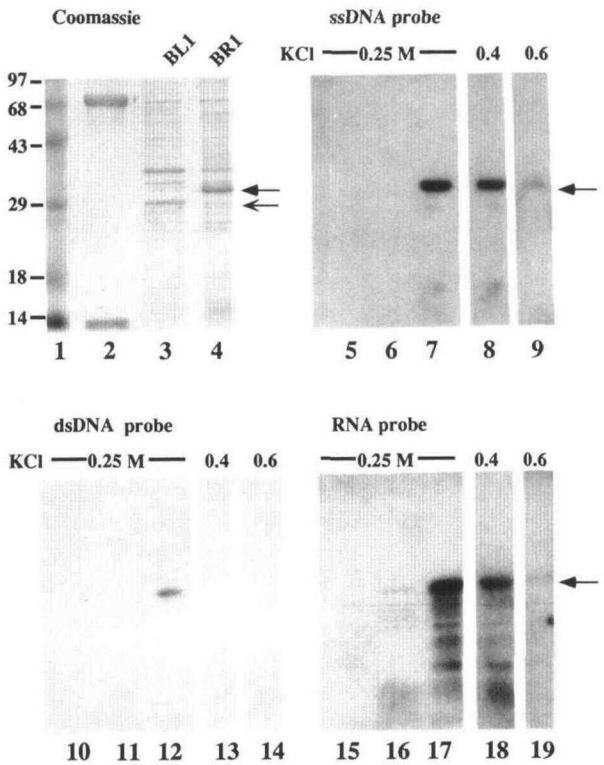

Figure 2. Protein Blot Binding Assay of BR1 Nucleic Acid Binding Specificity.

Whole cell extracts of E. coli expressing BL1 (lanes 3, 6, 11, and 16) or BR1 (lanes 4, 7 to 9, 12 to 14, and 17 to 19) were separated by SDS-PAGE and visualized by Coomassie blue staining (lanes 1 to 4) or transferred to nitrocellulose. Nitrocellulose-bound proteins were incubated in renaturing buffer and probed with ^{32}P-labeled ssDNA (lanes 5 to 9), dsDNA (lanes 10 to 14), or RNA (lanes 15 to 19). Following washes in buffer containing concentrations of KCl as indicated above each panel (0.25, 0.4, or 0.6 M), those proteins binding nucleic acids were *visualized by autoradiography. Lane 1 contains* prestained protein molecular mass markers with sizes indicated in kilodaltons at the left. Lanes 2, 5, 10, and 15 contain BSA and lysozyme (1 μg each) as controls for nonspecific binding. Filled-in upper arrows indicate the position of *E. coli*–expressed BR1; the lower thin arrow marks the position of BL1.

conditions (Figure 2, lanes 2, 5, 10, and 15). The results of this protein blot binding assay are consistent with our DNA–cellulose binding assays, demonstrating that BR1 interacts with both double- and single-stranded nucleic acids, having a higher affinity for the latter.

We further investigated the specificity of BR1 binding using a gel shift assay. Nuclear extracts containing BR1 expressed in recombinant baculovirus–infected Sf9 cells produced a characteristic band shift when incubated with a ssDNA probe, as shown in Figure 3. That BR1 was the binding protein was inferred from our finding that this band shift was not observed with nuclear extracts from uninfected or BL1 recombinant

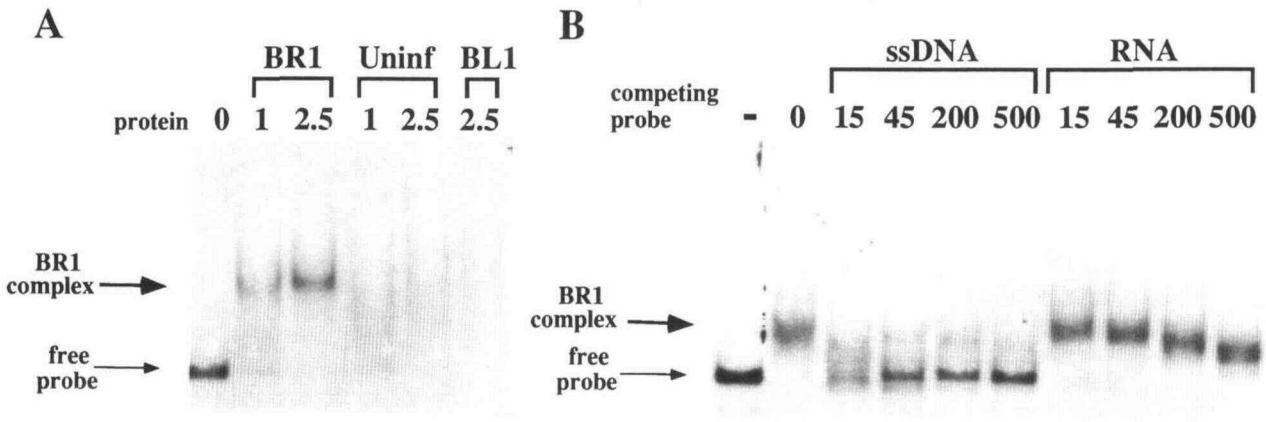

Figure 3. Gel Shift Assays of BR1 Binding to ssDNA.

(A) Nuclear extracts from baculovirus-infected Sf9 cells expressing BR1 or BL1, or from uninfected Sf9 cells (Uninf) were incubated with the ssDNA probe (~0.25 ng per lane) containing SqLCV B_E nucleotides 2554 to 270. Amounts of protein in micrograms are indicated above each lane. Positions of the free (unbound) probe and the BR1–probe complex are marked. See text for details.

(B) Nuclear extracts containing BR1 were incubated with labeled ssDNA probe (~0.25 ng per lane SqLCV B_E nucleotides 270 to 631), followed by addition of competing unlabeled calf thymus ssDNA or RNA as indicated. Shown above each lane is the amount of competing nucleic acid in nanograms. The (–) denotes no protein extract added; 0 denotes no addition of competing nucleic acid.

virus–infected Sf9 cells, both of which produced a diffuse shift, suggesting unstable interactions of an endogenous insect cell protein(s) with the DNA probe (Figure 3A). More direct evidence for the identity of the band shift was obtained by the addition of antibodies to the binding reactions. Whereas preimmune sera had no effect, anti-BR1 antibodies inhibited the BR1 gel shift, thus demonstrating that BR1 was responsible for the binding of the DNA probe (data not shown).

Using this gel shift assay, we were able to demonstrate a difference in the affinity of BR1 for ssDNA and RNA by a competition assay (Figure 3B). The addition of unlabeled ssDNA efficiently competed the formation of BR1–ssDNA complexes, with 15 ng disrupting a significant fraction of the BR1-labeled ssDNA complexes and 45 ng eliminating most of these complexes. In contrast, much higher amounts of RNA were required to disrupt the BR1–ssDNA complexes, with no competition for BR1 being detected with 15 ng of RNA, and even 500 ng of RNA not fully disrupting the complexes to yield significant amounts of the free labeled ssDNA probe. These differences in the competitive ability of ssDNA and RNA were observed both when the competitor nucleic acids were preincubated with BR1-containing extracts before adding the labeled ssDNA, or when BR1 and labeled ssDNA were preincubated and subsequently challenged by the addition of the unlabeled competing nucleic acids. These results demonstrate that BR1 has a higher affinity for ssDNA than it does for RNA.

To investigate the potential sequence specificity of the BR1–ssDNA interaction, we tested a number of different SqLCV genomic fragments in both the protein blot and gel shift DNA binding assays. In particular, sequences from the entire SqLCV B_E component were tested as overlapping restriction fragments, including coding and noncoding regions labeled to detect binding to either viral DNA strand. However, we did not detect any differences in affinity of BR1 for these different viral ssDNA probes. We further investigated the potential sequence specificity of binding using BR1 purified to near homogeneity from insect cells by cation exchange chromatography. Although we again did not detect sequence-specific binding with this purified BR1, as shown in Figure 4 (lanes 4 and 6), we did observe the formation of multiple complexes

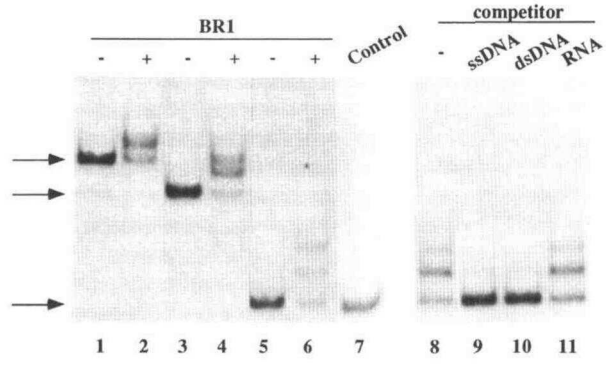

Figure 4. Gel Shift Assays of Purified BR1.

ssDNA probes containing SqLCV B_E sequences (nucleotides 270 to 631, lanes 1 and 2; nucleotides 2554 to 270, lanes 3 and 4; or nucleotides 50 to 270, lanes 5 through 11) were incubated with purified baculovirus-expressed BR1 (lanes 2, 4, 6, and 8 through 11) or the equivalent fraction from control uninfected insect cells (lane 7). Arrows mark the positions of migration of the three ssDNA probes without the addition of protein. In each lane, 0.2 to 0.25 ng of probe was used. In lanes 9 through 11, 5 ng of the competitor nucleic acid (indicated above each lane) was added to the BR1–ssDNA binding reaction.

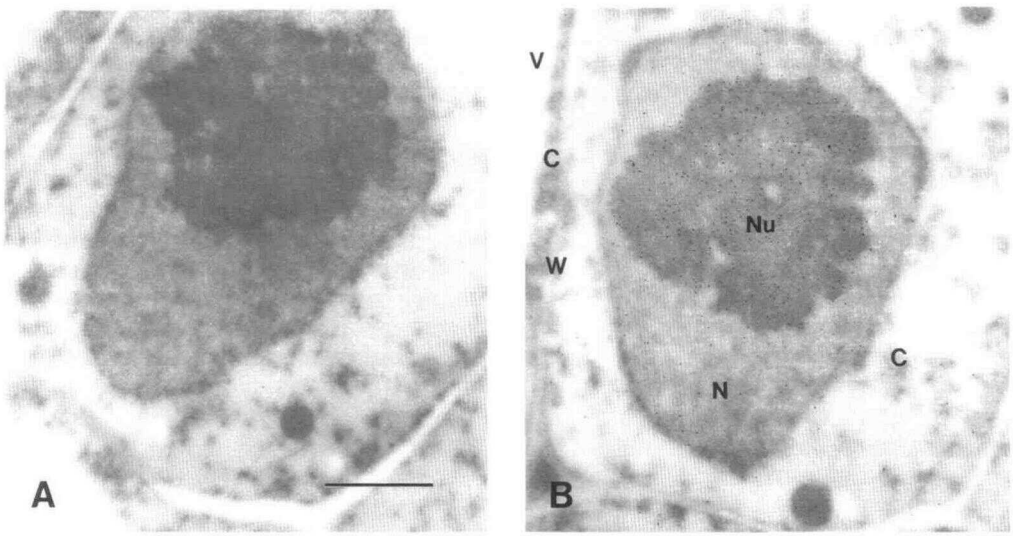

Figure 5. Immunolocalization of BR1 to a Phloem Parenchyma Cell Nucleus.

Immunogold labeling of unstained thin sections of a major vein from an SqLCV systemically infected leaf is shown at 10 days postinfection.
(A) Section incubated with preimmune sera and protein A coupled to 10-nm gold particles.
(B) Serial section of the same nucleus as shown in **(A)** incubated with anti-BR1 antibodies and protein A coupled to 10-nm gold particles. The diffuse appearance of cytoplasm may be due to fixation conditions and/or cytopathology associated with viral infection. N, nucleus; NU, nucleolus; C, cytoplasm; W, cell wall; V, vacuole. Magnification in both panels is ×13,954. Bar = 1 μm.

with certain specific ssDNA probes that contained the virion strand of the viral common region, the ~200-nucleotide intergenic region that is identical in sequence in the viral A and B components and contains the viral replication origin (Lazarowitz, 1992; Lazarowitz et al., 1992). These multiple complexes were not disrupted when competed with RNA (Figure 4). They were competed by ssDNA and dsDNA (Figure 4), although titrations showed that approximately five- to 10-fold higher amounts of dsDNA than ssDNA were required to fully compete with BR1–ssDNA complex formation in this assay (data not shown). Whether these higher molecular weight complexes represent protein interactions at multiple sites on the specific ssDNAs or BR1 protein–protein interactions is not clear at present.

Subcellular Localization of BR1

Given the ssDNA genome of SqLCV, we have hypothesized that at least one viral MP accesses the nucleus, the site of viral DNA replication. Subcellular fractionation of plant cells has localized BL1 to the cell wall and plasma membrane and BR1 to a distinct membrane-containing fraction (Pascal et al., 1993). These data, together with the nucleic acid binding properties of BR1, suggested that it might be the MP that functions in nuclear transport. To further investigate the potential role of BR1 in nuclear transport of the viral genome, we more precisely localized BR1 in infected plant cells and recombinant baculovirus–infected Sf9 cells through immunolocalization studies.

Immunogold labeling of thin sections from SqLCV systemically infected pumpkin leaves clearly localized BR1 to the nuclei of specific cells within the vascular system, as shown in Figure 5. Anti-BR1 antisera specifically labeled nuclei of phloem parenchyma cells. From 40 to 200 gold particles were clearly visible over the nuclei in different thin sections (Figure 5B). Labeling was more concentrated over the nucleoli, but also evident in the nucleoplasm of these cells. A small number of gold particles were also seen in the cytoplasm. Labeling of serial sections of the same cells with preimmune antibodies showed no gold particles, either in the nucleus or other parts of the cell (Figure 5A), nor was labeling observed when leaf sections from uninfected plants were incubated with anti-BR1 antibodies (data not shown). Vascular bundles of the primary veins (branches from the midvein) were examined. We also looked at secondary veins and extrafascicular phloem in primary veins. Phloem parenchyma in all of these was labeled for BR1. The labeling of phloem parenchyma cells by BR1 was not a rare event. The majority of parenchyma cells in phloem were clearly and specifically labeled over their nuclei. No labeling of mesophyll cells was observed. This phloem localization of BR1 was not unexpected based on the reported phloem restriction of geminivirus infections (Goodman, 1981).

Given the conservation in evolution of nuclear localization signals (Raikhel, 1992), we investigated whether BR1 also localized to the nuclei in Sf9 cells infected with a BR1-expressing

recombinant baculovirus. In particular, this system would provide a powerful approach for analyzing the cellular localization and potential protein interactions of BR1 mutant proteins known to alter viral infectivity or host specificity. Furthermore, our previous studies suggest that BR1 and BL1 are correctly modified in this system (Pascal et al., 1993). BR1 expressed in recombinant baculovirus–infected Sf9 cells fractionated with the nuclear pellet and could be partially released in high salt buffers (data not shown). To more precisely localize BR1, we used confocal microscopy and indirect immunofluorescent staining of fixed Sf9 cells with rabbit polyclonal anti-BR1 antibodies followed by rhodamine-conjugated anti–rabbit secondary antibodies. As shown in Figure 6D, BR1 localized to the nuclei of BR1-expressing Sf9 cells. This nuclear localization of BR1 was confirmed by demonstrating its precise colocalization with the DNA stain chromomycin A (data not shown). No staining of BR1-expressing Sf9 cells was detected using preimmune sera (Figure 6C), nor were uninfected Sf9 cells stained with anti-BR1 antibodies and the rhodamine-conjugated secondary antibodies (data not shown).

Using anti-BL1 antibodies, we found BL1 to specifically localize to the plasma membrane of BL1-expressing Sf9 cells (Figure 6B). No staining was detected with preimmune antibodies (Figure 6A) or when uninfected Sf9 cells were incubated with anti-BL1 antibodies and the secondary rhodamine conjugate (data not shown). This plasma membrane localization of BL1 is consistent with biochemical fractionation data for BL1 transgenic and SqLCV-infected plant tissue showing partitioning of BL1 with the plasma membrane in a PEG–dextran phase separation (Pascal et al., 1993). Whereas many membrane proteins appear to follow a default pathway to the plasma membrane when expressed in Sf9 cells (Bustos et al., 1988), these results do indicate that BL1 is processed as a membrane protein and has the properties of an integral or peripheral membrane protein. Thus, it appears that for both BL1 and BR1, the Sf9 cells are a useful expression system for protein localization assays, as well as a source of protein for biochemical assays.

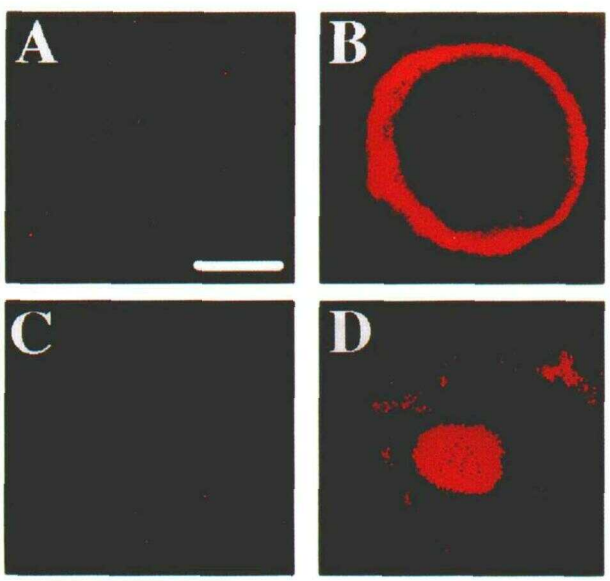

Figure 6. Indirect Immunofluorescent Staining of BR1 and BL1 in Baculovirus-Infected Sf9 Cells.

(A) and **(B)** Sf9 cells infected with recombinant baculovirus expressing BL1.

(C) and **(D)** Sf9 cells infected with recombinant baculovirus expressing BR1.

Fixed cells were incubated with **(A)** and **(C)** preimmune sera, **(B)** anti-BL1 immune sera, or **(D)** anti-BR1 immune sera, followed by rhodamine-conjugated goat anti–rabbit secondary antibody IgG, and visualized using a dual-beam Bio-Rad laser confocal microscope. Bar = 10 μm.

Post-Translational Modifications of BL1 and BR1

The strong correlation between the cellular localizations of the MPs in the insect cells and in SqLCV-infected plant tissue suggested that many of the features that dictate the properties of BR1 and BL1 are conserved in eukaryotic cells. Furthermore, the migration properties in SDS-PAGE of BR1 and BL1 expressed in Sf9 cells and plant cells suggest that the same modifications occur in each (Pascal et al., 1993). We therefore investigated potential post-translational modifications of BR1 and BL1 using the Sf9 expression system. Both SqLCV MPs have consensus glycosylation and phosphorylation sites in their conceptual amino acid sequences, modifications of potential relevance for membrane proteins and/or nucleic acid binding proteins. In particular, the heterogeneous migration of BL1 in SDS-PAGE at higher than expected molecular weights suggests that at least this protein is post-translationally modified (Pascal et al., 1993; von Arnim et al., 1993). To address these possibilities, we labeled Sf9 cells expressing BL1 or BR1 with either ^{32}P-orthophosphate, ^{3}H-fucose, or ^{3}H-glucosamine.

Based on immunoblots and autoradiography of whole cell extracts, no labeling of either BL1 or BR1 was detected in cells labeled with the two sugars, nor did tunicamycin alter the mobility of either protein in SDS-PAGE (data not shown). However, both BL1 and BR1 were found to be phosphorylated, as shown in Figure 7. In total cell extracts separated by SDS-PAGE, a phosphorylated protein at ~30 kD was detected in both BL1- and BR1-expressing Sf9 cells that was not present in the control uninfected cells (Figure 7A). Each of these ^{32}P-labeled bands precisely comigrated with BL1 and BR1, respectively, on protein immunoblots probed with anti-BL1 or anti-BR1 antisera (data not shown). To confirm the identity of these phosphoproteins as BL1 and BR1, each was immunoprecipitated from solubilized Sf9 cell extracts derived from a crude nuclear fraction that also contained large membrane fragments and insoluble proteins (the solubility properties of BL1 during preparation of extracts accounted for its presence in this fraction). In extracts from BL1-expressing cells, the anti-BL1 antibodies specifically immunoprecipitated the ~30 kD phosphoprotein from the high salt crude nuclear extract (Figure 7B,

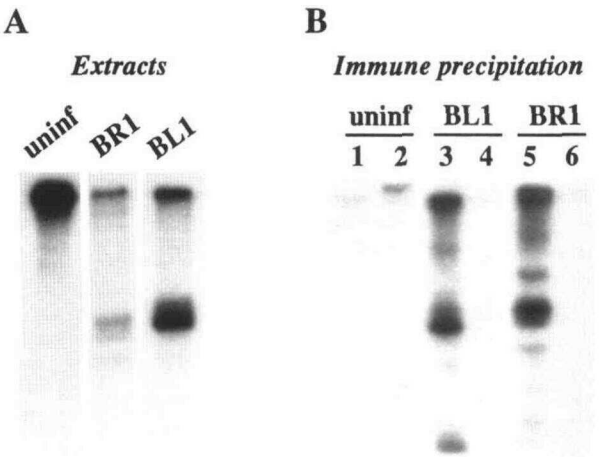

Figure 7. Phosphorylation of BR1 and BL1 in Sf9 Cells.

(A) ^{32}P-labeled whole cell extracts from uninfected (uninf) or baculovirus-infected Sf9 cells expressing BR1 or BL1 were analyzed by SDS-PAGE and visualized by autoradiography. Equivalent amounts of extract were loaded in each lane.

(B) Immunoprecipitation of ^{32}P-labeled Sf9 extracts. Extracts were incubated with preimmune sera (lanes 4 and 6), anti-BL1 sera (lanes 1 and 3), or anti-BR1 sera (lanes 2 and 5) followed by protein A–Sepharose. Proteins were separated by SDS-PAGE and visualized by autoradiography.

lane 3). No ^{32}P-labeled protein was immunoprecipitated from these extracts by preimmune sera (Figure 7B, lane 4) or by anti-BR1 antisera (data not shown). Similarly, in extracts from BR1-expressing cells, anti-BR1 antibodies, but not the preimmune sera or anti-BL1 antisera, specifically immunoprecipitated the ~30 kD phosphoprotein from the high salt nuclear extract (Figure 7B, lanes 5 and 6, respectively; and data not shown). Neither specific immune sera precipitated any proteins from the mock-infected Sf9 cell extract (Figure 7B, lanes 1 and 2). Thus, both BL1 and BR1 appear to be phosphoproteins.

DISCUSSION

Although the mechanisms by which MPs facilitate viral movement through the host plant have not yet been defined, it is intriguing that some viruses such as the bipartite geminivirus SqLCV require two MPs, whereas other plant viruses such as TMV and cauliflower mosaic virus have only a single MP. This poses the question of whether MP functions have been divided between two proteins in bipartite geminiviruses or whether additional functions are needed for geminivirus movement and fulfilled by a second MP. Our studies reported here suggest that the partitioning of MP functions to two separate proteins reflects a requirement for two types of movement for a DNA virus such as SqLCV, namely transport of the DNA genome into and out of the nucleus for replication and the movement of the genome across cell boundaries for the local and systemic spread of the virus through the plant.

Our results indicated that BR1 is likely to be the MP involved in nuclear transport of the viral genome. In the context of viral movement, we suggest that BR1 may be a nuclear shuttling protein, several of which have been identified in eukaryotic cells (reviewed in Laskey and Dingwall, 1993). BR1 exhibits strong affinity for single-stranded nucleic acids, binding more avidly to ssDNA than to RNA. Notably, under conditions where BR1 forms gel-shifted complexes with ssDNA, it does not complex with dsDNA. BR1 also specifically localizes to the nucleus of phloem parenchyma cells in infected plants, as well as the nuclei of Sf9 cells in our baculovirus expression system. Whereas some small molecules and proteins can diffuse into the nucleus, nuclear uptake of karyophilic proteins is often an active process specified by short nuclear localization signals (NLSs) within the protein (Forbes, 1992). BR1, in addition to being a very basic protein, contains two potential NLSs centered around amino acids 37 and 95, the former fitting the consensus of a bipartite NLS. The ability of these sequences to specify nuclear localization is currently being assessed in our baculovirus expression system. Recent findings indicate that mutations in either potential NLS severely impair or eliminate viral infectivity (D.J. Ingham and S.G. Lazarowitz, unpublished results). The potential nuclear localization and basic domains of BR1 may allow this protein to bind the SqLCV ssDNA genome and import it into the nucleus for replication. This scenario is similar to that of Agrobacterium, where the VirD and VirE proteins, both containing NLSs, are thought to direct the transport of the infectious single-stranded T-DNA to the nucleus of the infected plant cell (Citovsky et al., 1992b).

Of particular interest for viral movement is the potential function of BR1 in export of the viral ssDNA genome from the nucleus to the cell periphery. Many animal DNA viruses are released upon cell lysis and thus do not require an active mechanism to export progeny genomes from the cell (Batterson et al., 1983; Greber et al., 1993). More relevant here are influenza viruses, retroviruses, and hepadnaviruses that do require export of the viral genome from the nucleus. Studies of the influenza viruses suggest that the viral matrix protein M1 interacts with replicated viral ribonucleoprotein particles (RNPs) in the nucleus to facilitate their transport to the cytoplasm for assembly into progeny virions (Martin and Helenius, 1991). Recent studies lead to the conclusion that nuclear export involves specific domains for nuclear retention rather than specific export signals (Schmidt-Zachmann et al., 1993). Thus, as suggested for M1 protein–RNP interactions (Martin and Helenius, 1991), BR1 may interact with replicated SqLCV ssDNA to mask retention sites in replication complexes and permit export of the viral genome. With the capsid protein being completely dispensable for SqLCV infection of cucurbits (A.A. Sanderfoot and S.G. Lazarowitz, unpublished results), a BR1–viral ssDNA complex may be the form that moves systemically in the plant and initiates infections at distant sites in the phloem.

In contrast to BR1, BL1 exhibits only very weak affinity for ssDNA and no apparent ability to bind dsDNA, implying that BL1 alone cannot interact directly with the viral genome to facilitate viral movement. Thus, whereas some properties of BL1, namely its cell wall and plasma membrane localization, basic charge, and phosphorylation, resemble those of the single MP encoded by TMV (Deom et al., 1990; Atkins et al., 1991; Moore et al., 1992; Watanabe et al., 1992) and red clover necrotic mosaic virus (RCNMV) (Osman and Buck, 1991), BL1 is quite distinct in its inability to bind nucleic acids. Recently, based on microinjection of individual mesophyll cells, Noueiry et al. (1994) have concluded that an *E. coli*–synthesized BL1 fusion protein can increase the size exclusion limits of plasmodesmata and facilitate the transport of dsDNA. Based on our biochemical studies, and contrary to the suggestion of Noueiry et al. (1994), we suggest that BL1 cannot do this by directly binding to nucleic acids, but requires additional interactions with other SqLCV proteins or endogenous plant proteins to participate in the cell-to-cell transport of the viral genome. The studies of Noueiry et al. (1994) do not exclude a passive role for BL1 in facilitating dsDNA movement under their experimental conditions, and these researchers did not test for movement of a BR1–ssDNA complex in the presence of BL1. Further studies are needed to define both the nature of the relevant protein and nucleic acid interactions and the form in which viral DNA is transported.

An unresolved issue for both MPs is the specificity of interactions between the proteins and the viral genome. We find that BR1 has a higher affinity for ssDNA than for RNA or dsDNA. We did not detect clear sequence-specific binding of BR1 to ssDNA using SqLCV fragments from the viral B_E component, although purified BR1 formed multiple complexes in gel shift assays with ssDNA probes that included the viral common region. Thus, like several other ssDNA binding proteins, such as the phage T4 gene 32 protein (Jensen et al., 1976) and a chicken hepatocyte estradiol-responsive protein (Feavers et al., 1989), BR1 can interact with both ssDNA and RNA, although it binds more tightly to the former. The TMV and RCNMV MPs interact in a sequence-nonspecific manner with both RNA and ssDNA (Citovsky et al., 1990, 1992a; Osman et al., 1992), and no difference in affinity for these has been shown in vitro. However, in plant injection studies the RCNMV MP has been reported to facilitate the movement of only RNA and not ssDNA (Fujiwara et al., 1993). Nucleic acid and sequence specificity may be conferred through MP interactions with other viral or host proteins.

Our evidence suggests that the two MPs BL1 and BR1 act coordinately to facilitate viral movement. Preliminary results demonstrate that BR1 relocalizes from the nucleus to the plasma membrane in Sf9 cells coexpressing BL1 (A.A. Sanderfoot and S.G. Lazarowitz, unpublished results). This indicates that BR1 has the properties expected for an MP that shuttles the viral genomes from the nucleus to the cell periphery, and suggests that BR1 either directly interacts with BL1 or has an intrinsic affinity for membranes when released from the nucleus. Our immunogold localization of BR1 in plant cells did not clearly demonstrate a cytoplasmic or membrane localization of BR1 in infected plant cells, although low numbers of particles were seen in the cytoplasm and near the plasma membranes of phloem parenchyma cells. Transient relocalization or a small subpopulation of BR1 localized outside the nucleus may have been missed by this technique. Most shuttling proteins at equilibrium are predominantly nuclear, having small cytoplasmic pools that are not detected by conventional fractionation or immunological techniques (Borer et al., 1989; Laskey and Dingwall, 1993; Schmidt-Zachmann et al., 1993). This is clearly consistent with our results for BR1 in both infected plant and Sf9 insect cells. Resolution of this problem awaits more sensitive detection assays and direct assays for viral movement designed to trap the MPs in the act of transporting the viral genome. Noueiry et al. (1994) microinjected single mesophyll cells with *E. coli* fusion proteins to study the function of BR1 of BDMV and have postulated a similar model for BR1 function. However, of concern is their inability to detect localization of BR1 to the nuclei of mesophyll cells. This is in striking contrast to our nuclear localization of BR1 in both infected plant and insect cells and suggests problems with the microinjection approach as a result of the methods used to obtain soluble protein for microinjection and/or the amounts of protein and site of injection. Given our current results, we favor a model in which BR1 shuttles the viral ssDNA into and out of the nucleus, targeting replicated genomes to the cell plasma membrane and wall where BL1 acts to facilitate genome movement to neighboring cells, perhaps by acting at the plasmodesmata as reported for the TMV MP (Deom et al., 1990; Ding et al., 1992) and inferred for RCNMV (Fujiwara et al., 1993) and BDMV (Noueiry et al., 1994) MPs. Our current immunolocalization studies of BL1 should provide the biological context for further defining its function.

The localization of SqLCV BR1 exclusively to phloem cells parallels the tissue restriction reported for the majority of geminiviruses and argues for the utility of SqLCV movement in probing the requirements for transport in vascular tissue. What has become increasingly clear, as typified by studies of sucrose transport, is that the details of intercellular movement differ between different cell types and different plant species (Leisner and Turgeon, 1993). SqLCV infects a variety of plants, primarily cucurbits but also *Nicotiana benthamiana* (tobacco) and bean (Lazarowitz and Lazdins, 1991), and it is of interest whether the differences in vascular structure among these plants (Leisner and Turgeon, 1993) affect the cell type–specific localization of BR1 and BL1 or the requirements for viral transport. Indeed, mutations in both BR1 and BL1 can distinguish between viral hosts, with cucurbits being more permissive for movement than *N. benthamiana* (Ingham and Lazarowitz, 1993; D.J. Ingham, E. Pascal, and S.G. Lazarowitz, unpublished results). Investigations of the role of SqLCV MPs in intercellular and systemic movement in these different hosts should provide information on the physical and signal transduction pathways involved in these important processes.

METHODS

Expression Vectors

Bacterial pET3 clones expressing BR1 and BL1, and pGBL1, a pGEM7Zf(−) clone expressing BL1, have been described previously (Pascal et al., 1993). To construct pGBR1 expressing BR1, a BglII fragment from pSQBR1E (Pascal et al., 1993) containing nucleotides 632 to 1669 of the squash leaf curl virus (SqLCV) B_E component was cloned into the BamHI site of pGEM7Zf(−) (Promega). Both BL1 and BR1 were transcribed from the SP6 promoter.

The baculovirus expression vectors for BL1 and BR1 have been described previously (Pascal et al., 1993). Recombinant *Autographica californica* nuclear polyhedrosis virus (AcMNPV) expressing each SqLCV protein was generated by cotransfection of *Spodoptera frugiperda* (Sf9) lepidopteran cells with the appropriate pVL1393 recombinant clone and Baculogold baculovirus DNA (PharMingen, San Diego, CA) and amplified and plaque purified as described by Summers and Smith (1987). Expression of each protein was determined both by Coomassie Brilliant Blue R 250 staining of cell extracts analyzed on polyacrylamide gels and protein immunoblots using appropriate antisera as previously described (Pascal et al., 1993).

Antisera

The generation of polyclonal rabbit antibodies against BR1 and BL1 expressed in *Escherichia coli* has been described previously (Pascal et al., 1993).

Protein Expression and Cell Extracts

^{35}S-methionine–labeled BR1 and BL1 were produced using a reticulocyte in vitro transcription/translation system (Promega) according to manufacturer's instructions. Proteins were stored in the presence of 10% (v/v) glycerol at −20°C. In vitro–synthesized BL1$^{\Delta 192-293}$ was produced from pGBL1 truncated by digestion at the XbaI site within the BL1 coding region. BR1$^{\Delta 192-256}$ was made from pGBR1 digested at the NcoI site within the BR1 coding region.

To obtain *E. coli*–expressed BR1 and BL1 for protein blot binding assays, BL21 (DE3) pLysS cultures carrying the appropriate pET3 clones were grown at 37°C and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (Bethesda Research Laboratories) for 3 to 5 hr. Cell pellets were washed and resuspended in 1 mL of HEMG (Pascal et al., 1993) containing 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF) and, following sonication on ice for 50 sec, were centrifuged at 10,000*g* for 5 min at 4°C. The pellet was resuspended in 1 mL of SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.003% bromophenol blue), and proteins were resolved on SDS-containing 12.5% polyacrylamide discontinuous buffer gels (SDS-PAGE) (Laemmli, 1970).

To prepare extracts containing baculovirus-expressed BR1 or BL1, Sf9 cells were infected with recombinant AcMNPV stocks as described by Summers and Smith (1987) and harvested at 36 to 72 hr postinfection. Cells (~6×10^6) were washed in PBS, resuspended in 3 mL of buffer C (10 mM Tris-HCl, pH 6.8, 10 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM PMSF), and lysed by addition of Nonidet P-40 to 0.05% (v/v) for 2 min at 25°C. The soluble "cytoplasmic extract" and crude nuclear pellet were separated by centrifugation at 900*g* for 5 min at 4°C. The nuclear pellet was extracted by incubation in 6 mL of buffer N (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM PMSF) containing 1% (v/v) Nonidet P-40 at 4°C for 20 min, following which it was centrifuged at 900*g* for 5 min at 4°C. The supernatant from this spin ("nuclear extract") was used for gel shift assays. Protein concentrations were determined by the method of Bradford (1976). For the experiment shown in Figure 4, BR1 or control nuclear extract was applied to a HiTrap-SP cation exchange column (Pharmacia) in buffer N containing 1% Nonidet P-40. Fractions were eluted with buffer N containing 0.1% Nonidet P-40 and 1 mM DTT and steps of increasing NaCl concentrations at 250 mM, 600 mM, and 1 M salt. BR1 eluted almost exclusively in the 1 M NaCl fraction. The 1 M NaCl fractions obtained from both the BR1 and control extracts were dialyzed overnight at 4°C against buffer N containing 0.1% Nonidet P-40, 1 mM DTT, and 10% glycerol before being used in binding assays.

DNA Cellulose Binding Assays

Native double-stranded DNA (dsDNA) or denatured single-stranded DNA (ssDNA) coupled to cellulose (Pharmacia) or uncoupled CC31 cellulose (Whatman) (100-μL suspensions) was washed four times with buffer Z (50 mM Tris-HCl, pH 8.0, 12.5 mM $MgCl_2$, 1 mM EDTA, 0.1% Nonidet P-40, 20% glycerol, 1 mM PMSF, 1 mM DTT) containing 50 mM KCl (Z-50 buffer). ^{35}S-methionine–labeled in vitro transcribed and translated protein (10 μL) in 300 μL of Z-50 buffer was added to the pelleted resin and incubated 20 min at 25°C for BR1 and BR1$^{\Delta 192-256}$ or 2 hr at 4°C for BL1 and BL1$^{\Delta 192-293}$. Protein bound to resin was pelleted by centrifugation at 16,000*g* for 5 to 10 sec and washed five times with 300 μL of Z-50 buffer. Bound protein was eluted from the resin by successive washes with 300 μL of Z buffer containing 100 mM, 200 mM, 400 mM, and 1 M KCl. Washes and elutions were performed at the same temperature as the initial binding step. Following the 1 M KCl wash, residual bound protein was eluted by incubation in 300 μL of sample buffer at 100°C for 5 min. Fractions (20 μL) were analyzed by SDS-PAGE on 12.5% acrylamide gels, and gels were impregnated for fluorography (Laskey, 1980) before exposure to x-ray film to detect the ^{35}S-labeled proteins.

Protein Blot Binding Assays

These were essentially as described by Sukegawa and Blobel (1993), except that the reaction buffer contained 250 mM KCl, and after incubation with ^{32}P-labeled probes, blots were washed four times with reaction buffer containing 250, 400, or 600 mM KCl. DNA probes shown in Figure 2 were derived from pGBL1 (containing SqLCV B_E nucleotides 2575 to 1442) cut with HindIII and XhoI and labeled at their 5′ ends with γ-^{32}P-ATP and polynucleotide kinase (Bethesda Research Laboratories). Other DNA probes used for this assay included fragments of SqLCV B_E located between the following nucleotides: 2554 to 2229, 2554 to 270 (common region), 270 to 631, 814 to 1952, and 1952 to 814. Labeled DNA probes were isolated on 4% native acrylamide gels. For ssDNA probes, the labeled DNA was denatured by heating to 100°C for 10 min followed by rapid cooling. RNA probes were transcribed from pGBL1 in the T7 orientation (digested with XhoI) in the presence of α-^{32}P-CTP using the same procedures as for RNA transcription in the in vitro transcription/translation system.

Gel Shift Assays

Nuclear extracts (0.6 μg of protein in 15 μL) from uninfected Sf9 cells or Sf9 cells expressing BR1 or BL1 were incubated with ssDNA probes (~0.25 ng) in 10 μL of buffer Z containing 425 mM KCl for 20 min at 25°C (final incubation volume 25 μL containing 250 mM salt). Protein–DNA complexes were loaded onto 4% native acrylamide gels in 0.5 × TBE (1 × TBE is 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and resolved at 10 V/cm for 2 to 3 hr. Gels were dried and exposed to film overnight. The DNA probe shown in Figure 3A contained SqLCV B_E nucleotides 2554 to 270 (the common region) labeled at the 3′ end at nucleotide 270 (BssHII site) using α-^{32}P-dCTP, the remaining three unlabeled nucleotides, and the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Other probes also used in this assay encompassed the following SqLCV B_E nucleotides: 2554 to 2229, 270 to 631, 2554 to 270, and 50 to 270. Fragments were separately labeled at either 3′ end to obtain strand-specific probes, and probes of both strand specificities were tested. For competition assays, BR1 and labeled ssDNA probe (B_E nucleotides 270 to 631) were mixed as given above. Following 5 min of incubation, unlabeled competitor nucleic acids were added, and the reactions were incubated for an additional 15 min and then loaded onto native acrylamide gels as described above. Calf thymus DNA (Sigma) was sheared by sonication to ~1 kb in length and denatured by heating to 100°C for 10 min, followed by rapid cooling on ice. Calf thymus RNA was obtained from Sigma. For gel shift assays of purified BR1, ~2.5 ng of protein was incubated with ~0.25 ng of DNA probes (molar ratio protein: DNA of ~30:1) as described above.

Immunolocalization Assays

For electron microscopy and immunogold localization of BR1, tissue sections (1 mm) from SqLCV-infected pumpkin leaves (10 days postinfection) were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in 50 mM Pipes, pH 7.2, 2 mM $CaCl_2$ for 2 hr at 25°C, followed by three 30-min washes in 50 mM Pipes, pH 7.2, 2 mM $CaCl_2$ at 4°C. All subsequent steps were performed at 4°C. Sections were dehydrated in a series of 30-min ethanol washes (30, 50, 70, and 95%), followed by four washes in 100% ethanol. Tissue was then successively incubated in solutions of ethanol and London Resin (LR) white (Electron Microscopy Sciences) at ratios of 3:1 for 1 hr, 1:1 for 1 hr, and 1:3 for 3 hr; followed by incubations for 12 and 24 hr each in 100% LR white. Tissue was placed in fresh LR white and embedded in gelatin capsules (Electron Microscopy Sciences) at 52°C for 24 hr. Thin sections (~0.1 μm) were cut on an LKB Ultramicrotome (Pharmacia) using a diamond knife. Immunogold labeling using preimmune or rabbit polyclonal anti-BR1 antisera (Pascal et al., 1993) and protein A coupled to 10-nm gold particles (Electron Microscopy Sciences) was done essentially as described by Hall and Hawes (1991), except that lead citrate was omitted from the staining procedure.

BR1 or BL1 was detected by indirect immunofluorescent staining of Sf9 cells using our specific rabbit polyclonal antisera and rhodamine-conjugated goat anti-rabbit IgG (Jackson Laboratories, Bar Harbor, ME). Cells (1.5 × 10^5) were seeded in 10-mm chamber slides (Nunc, Naperville, IL) and inoculated with ~40 plaque forming units per cell of the appropriate recombinant AcMNPV stock. At ~72 hr postinfection, cells were washed three times in PBS and fixed by immersion in 95% ethanol at −20°C for 5 min. Fixed cells were stained essentially as described by Lazarowitz (1982). Samples were mounted in PBS containing 50% glycerol and visualized using a Bio-Rad 600 Krypton/Argon Dual Laser confocal system attached to an Optiphot microscope (Nikon) at a final magnification of ×1000. For colocalizations, nuclei were stained by incubation in 56 μM chromomycin A (Sigma) for 5 min, and the rhodamine and fluorescein images were superimposed (Leemann and Ruch, 1982).

Phosphate Labeling and Immunoprecipitation of BR1 and BL1

Sf9 cells at ~80% confluency were infected with recombinant AcMNPV expressing either BR1 or BL1 as described previously (Summers and Smith, 1987) and labeled for 4 hr at ~44 hr postinfection with 100 μCi/mL ^{32}P-orthophosphate (DuPont-New England Nuclear) in TMN-FH media (Summers and Smith, 1987) lacking phosphate. Labeled BR1- or BL1-expressing cells (~6 × 10^6) were harvested by pelleting at 500*g* for 5 min at 4°C and washed three times with PBS at 4°C. One-tenth of each sample was set aside ("whole cell extract"), and the remaining cells were fractionated into cytoplasmic and nuclear extracts as described above, except that the crude nuclear pellet was extracted in N buffer containing 500 mM rather than 137 mM NaCl. To analyze total ^{32}P-labeled proteins, pelleted intact cells were resuspended in SDS sample buffer and resolved by SDS-PAGE on 12.5% acrylamide gels.

Immune precipitations were done essentially as described previously (Lazarowitz, 1982). In brief, 100,000 cpm of each extract (50 μL) was incubated with 10 μL of appropriate antisera (preimmune, anti-BR1, or anti-BL1) for 30 min at 25°C, followed by the addition of 50 μL of a 50% suspension of protein A–Sepharose (Pharmacia) and further incubation for 20 min. Precipitates were pelleted at 10,000*g* for 1 min at 4°C, washed three times, and analyzed by SDS-PAGE as previously described (Lazarowitz, 1982).

ACKNOWLEDGMENTS

We thank Eric Spana and Chris Doe for help with the confocal microscopy and Jack Gladin and Richard Becker for expert photographic assistance. We also thank Mike Sepanski for initially exposing us to the wonders of electron microscopy. E.P. is Department of Energy-Energy Biosciences Research Fellow of the Life Sciences Research Foundation. This work was supported by National Institutes of Health Grant No. PHS AI27449 to S.G.L. and National Science Foundation Grant No. DCB-9104159 to R.T.

Received April 4, 1994; accepted May 13, 1994.

REFERENCES

Atabekov, J.G., and Dorokhov, Y.L. (1984). Plant virus-specific transport function and resistance of plants to viruses. Adv. Virus Res. **29,** 313–363.

Atkins, D., Roberts, K., Hull, R., Prehaud, C., and Bishop, D.H.L. (1991). Expression of the tobacco mosaic virus movement protein using a baculovirus expression vector. J. Gen. Virol. **72,** 2831–2835.

Batterson, W., Furlong, D., and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. **45,** 397–407.

Borer, R.A., Lehner, C.F., Eppenberger, H.M., and Nigg, E.A. (1989). Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell **56,** 379–390.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248.

Bustos, M.M., Luckow, V.A., Griffing, L.R., Summers, M.D., and Hall, T.C. (1988). Expression, glycosylation and secretion of phaseolin in a baculovirus system. Plant Mol. Biol. **10,** 475–488.

Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. (1990). The P30 movement protein of tobacco mosaic virus is a single-stranded nucleic acid binding protein. Cell **60,** 637–647.

Citovsky, V., Knorr, D., and Zambryski, P. (1991). Gene I, a potential cell-to-cell movement locus of cauliflower mosaic virus, encodes an RNA-binding protein. Proc. Natl. Acad. Sci. USA **88,** 2476–2480.

Citovsky, V., Wong, M.L., Shaw, A.L., Prasad, B.V.V., and Zambryski, P. (1992a). Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. Plant Cell **4,** 397–411.

Citovsky, V., Zupan, J., Warnick, D., and Zambryski, P. (1992b). Nuclear localization of the VirE2 protein in plant cells. Science **256,** 1802–1805.

Dawson, W.O., Bubrick, P., and Grantham, G.L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement and symptomatology. Phytopathology **78,** 783–789.

Deom, C.M., Schubert, K.R., Wolf, S., Holt, C.A., Lucas, W.J., and Beachy, R.N. (1990). Molecular characterization and biological function of the movement protein of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA **87,** 3284–3288.

Ding, B., Haudenshield, J.S., Hull, R.J., Wolf, S., Beachy, R.N., and Lucas, W.J. (1992). Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. Plant Cell **4,** 915–928.

Feavers, I.M., McEwan, I.J., Liang, H., and Jost, J.-P. (1989). An estradiol dependent protein from chicken liver binds single-stranded DNA and RNA. J. Biol. Chem. **264,** 9114–9117.

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

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel, S.A., and Lucas, W.J. (1993). Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the red clover necrotic mosaic virus movement protein. Plant Cell **5,** 1783–1794.

Gardiner, W.E., Sunter, G., Brand, L., Elmer, J.S., Rogers, S.G., and Bisaro, D.M. (1988). Genetic analysis of tomato golden mosaic virus: The coat protein is not required for systemic spread or symptom development. EMBO J. **7,** 899–904.

Goodman, R.M. (1981). Geminiviruses. In Handbook of Plant Virus Infection and Comparative Diagnosis, E. Kurstak, ed (New York: Elsevier/North Holland Biomedical Press), pp. 879–910.

Greber, U.F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. Cell **75,** 477–486.

Hall, J.L., and Hawes, C. (1991). Electron Microscopy of Plant Cells. (San Diego: Academic Press Inc.).

Hull, R. (1991). The movement of viruses within plants. Semin. Virol. **2,** 89–95.

Ingham, D.J., and Lazarowitz, S.G. (1993). A single missense mutation in the BR1 movement protein alters the host range of the squash leaf curl geminivirus. Virology **196,** 694–702.

Jensen, D.E., Kelly, R.C., and von Hippel, P.H. (1976). DNA "melting" proteins II. Effects of bacteriophage T4 gene 32 protein binding on the conformation and stability of nucleic acid structures. J. Biol. Chem. **251,** 7215–7228.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Laskey, R.A. (1980). The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. **65,** 363–371.

Laskey, R.A., and Dingwall, C. (1993). Nuclear shuttling: The default pathway for nuclear proteins? Cell **74,** 585–586.

Lazarowitz, S.G. (1982). Simian virus 40 mutant with transposed T-antigen and VP1 genes. J. Virol. **41,** 1025–1037.

Lazarowitz, S.G. (1992). Geminiviruses: Genome structure and gene function. Crit. Rev. Plant Sci. **11,** 327–349.

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.

Lazarowitz, S.G., Wu, L.C., Rogers, S.G., and Elmer, J.S. (1992). Sequence-specific interaction with the viral AL1 protein identifies a geminivirus DNA replication origin. Plant Cell **4,** 799–809.

Leemann, U., and Ruch, F. (1982). Cytofluorometric determination of DNA base content in plant nuclei and chromosomes by the fluorochromes DAPi and chromomycin A3. Exp. Cell Res. **140,** 275–282.

Leisner, S.M., and Turgeon, R. (1993). Movement of virus and photoassimilate in the phloem: A comparative analysis. Bioessays **15,** 741–748.

Linstead, P.J., Hills, G.J., Plaskitt, K.A., Wilson, I.G., Harker, C.L., and Maule, A.J. (1988). The subcellular localization of the gene 1 product of cauliflower mosaic virus is consistent with a function associated with virus spread. J. Gen. Virol. **69,** 1809–1818.

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.

Moore, P.J., Fenczik, C.A., Deom, C.M., and Beachy, R.N. (1992). Developmental changes in plasmodesmata in transgenic tobacco expressing the movement protein of tobacco mosaic virus. Protoplasma **170,** 115–127.

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.

Osman, T.A.M., and Buck, K.W. (1991). Detection of the movement protein of red clover necrotic mosaic virus in a cell wall fraction from infected *Nicotiana clevelandii* plants. J. Gen. Virol. **72,** 2852–2856.

Osman, T.A.M., Hayes, R.J., and Buck, K.W. (1992). Cooperative binding of the red clover necrotic mosaic virus to single-stranded nucleic acids. J. Gen. Virol. **73,** 223–227.

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.

Raikhel, N. (1992). Nuclear targeting in plants. Plant Physiol. **100,** 1627–1632.

Saito, T., Yamanaka, K., and Okada, Y. (1990). Long-distance movement and viral assembly of tobacco virus mutants. Virology **176,** 329–336.

Schmidt-Zachmann, M.S., Dargemont, C., Kuhn, L.C., and Nigg, E.A. (1993). Nuclear export of proteins: The role of nuclear retention. Cell **74,** 493–504.

Sukegawa, J., and Blobel, G. (1993). A nuclear pore complex protein that contains zinc finger motifs, binds DNA and faces the nucleoplasm. Cell **72,** 29–38.

Summers, M.D., and Smith, G.E. (1987). A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. (College Station: Texas Agricultural Experiment Station).

von Arnim, A., Frischmuth, T., and Stanley, J. (1993). Detection and possible functions of African cassava mosaic virus DNA B gene products. Virology **192,** 264–272.

Watanabe, Y., Ogawa, T., and Okada, Y. (1992). In vivo phosphorylation of the 30-kDa protein of tobacco mosaic virus. FEBS Lett. **313,** 181–184.

Wolf, S., Deom, C.M., Beachy, R.N., and Lucas, W.J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science **246,** 377–379.