Sonchus Yellow Net Rhabdovirus Nuclear Viroplasms Contain Polymerase-Associated Proteins

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We have initiated a study of the cytopathology of nucleorhabdoviruses by analyzing the subcellular localization of sonchus yellow net virus (SYNV) genomic and antigenomic RNAs and the encoded polymerase proteins. In situ hybridizations demonstrated that the minus-strand genomic RNA sequences are restricted to the nuclei of infected cells, while the complementary plus-strand antigenomic RNA sequences are present in both the nuclei and the cytoplasm. Immunofluorescence and immunogold labeling experiments also revealed that the nucleocapsid (N) protein and phosphoprotein (M2) are primarily localized to discrete regions within the nuclei and in virus particles that accumulate in perinuclear spaces. The N protein antisera specifically labeled the nuclear viroplasms, whereas the M2 antisera was more generally distributed throughout the nuclei. Antibody detection also indicated that the polymerase (L) protein is present in small amounts in the viroplasms. When the N and M2 proteins were expressed individually from the heterologous potato virus X (PVX) vector, both proteins preferentially accumulated in the nuclei. In addition, viroplasm-like inclusions formed in the nuclei of cells infected with the PVX vector containing the N gene. Fusions of the carboxy terminus of β-glucuronidase to N and M2 resulted in staining of the nuclei in staining of the nuclei of infected cells following expression from the PVX vector. Deletion analyses suggested that multiple regions of the N protein contain signals that are important for nuclear localization.

Sonchus yellow net virus (SYNV) belongs to the Rhabdoviridae family of nonsegmented, negative-strand RNA viruses. This family is one of the most widely distributed virus families in nature, and it contains members that infect animals, plants, and insects (47). With this diversity, it is not surprising that individual members of the family differ with respect to requirements for infection and the sites of viral replication and morphogenesis. All of the well-characterized animal rhabdoviruses replicate in the cytoplasm, whereas the plant rhabdoviruses are divided into two groups depending on whether their morphogenesis occurs in association with cytoplasmic or nuclear membranes (16). The cytorhabdoviruses, which replicate in the cytoplasm and bud in association with the endoplasmic reticulum, include lettuce necrotic yellow virus, strawberry crinkle virus, and barley yellow striate mosaic virus, among others. The nucleorhabdoviruses, of which SYNV, sowthistle yellow vein virus, potato yellow dwarf virus, and maize mosaic virus are members, appear to replicate in the nucleus and bud from the inner nuclear envelope into perinuclear spaces. Furthermore, over 50 plant rhabdoviruses have yet to be assigned to specific groups because of insufficient information about their replication and morphogenesis (50).

SYNV is the most extensively characterized plant rhabdovirus, and its genome has been completely sequenced (5, 6, 10, 12, 14, 42, 51, 52). The 13,720-nucleotide (nt) SYNV RNA encodes six proteins in a negative-sense orientation (see Fig. 1), and all of these are found in association with virus particles (42). A ribonucleoprotein core that can be released from virions (17), and from the nuclei of infected plants (44), consists of the nucleocapsid (N) protein, the phosphoprotein (M2), and the polymerase (L) protein complexed with SYNV RNA. The glycoprotein (G) is thought to traverse the viral lipid envelope and associate with the matrix (M1) protein, which probably also has a role in coiling of the ribonucleoprotein core (16). A sixth protein, sc4, has no known function but can be released from virions by treatment with mild nonionic detergents (42). Two putative regulatory regions, located at the 3′ and 5′ ends of the SYNV genome, flank the six minus-sense genes. The 144-nt leader sequence is located at the 3′ end of the genome (44, 51), and the 160-nt untranslated trailer sequence is present at the 5′ end of the genomic RNA. The transcribed plus-sense leader RNA is encapsidated by the N protein, and the extent of encapsidation is thought to regulate transcription versus replication (49). Sixteen of the eighteen 5′-terminal trailer nucleotides are complementary to those at the 3′ terminus of the genome and potentially could form a circular structure by base pairing (6).

Several studies have provided evidence that the nucleus is the site of SYNV replication. Nuclear inclusions that can be visualized by light microscopy are a prominent feature of leaf cells infected with SYNV (7, 19). Electron microscopy studies have also shown that many enveloped bacilliform particles are present in the perinuclear spaces surrounding the nuclei, and viral cores at various stages of morphogenesis can be seen...
budding through the inner nuclear envelope (7, 15, 19, 43). Incubation with tunicamycin, a glycosylation inhibitor, interrupts morphogenesis and leads to accumulation of a striking array of nucleocapsid cores at the periphery of the nuclei and at the outer edges of greatly enlarged viroplasm (43). An active polymerase complex contained within the nucleoprotein core has also been isolated from purified tobacco nuclei (20, 44, 45). This complex is involved in transcription because it is capable of synthesizing the polyadenylated leader RNA and each of the six mRNAs in a polar fashion to yield decreasing amounts as follows: N > M2 > 4c > M1 > G > L (44, 45). A temporal switch from transcription to replication is thought to occur at the intermediate stages of the infection process because SYNV genomic RNAs accumulate later than the mRNAs in infected protoplasts (22). This strategy of transcription and replication is similar to that of vesicular stomatitis virus (VSV) and other monopartite negative-strand viruses (2).

In SYNV-infected tissue, a dynamic series of events occurs that suggest intimate interactions of host and viral components. The nuclei swell, distinct nuclear inclusions appear, and large numbers of virus particles accumulate in the perinuclear spaces (7, 16). Observations of SYNV in protoplasts indicate that following entry into the cell, one of the early events involves fusion of the viral envelope with the endoplasmic reticulum and subsequent release of the nucleocapsid core into the cytoplasm (43). These cores are then thought to enter the nucleus and establish a discrete viroplasm that functions in transcription and replication. To mediate these integral events in the viral life cycle, the host macromolecular transport machinery must carry out several essential steps requiring nucleocytoplasmic transport of viral components. For example, unspliced SYNV mRNAs are thought to be transcribed in the nucleus (44, 45) and exported to the cytoplasm, where they are partitioned into free and membrane-associated polysomes during translation (30, 31). After protein synthesis, some of the virus-encoded proteins are post-translationally modified in the nucleus and participate in formation of the viroplasm complex. However, the interactions culminating in formation of the viral viroplasm are not yet understood. To begin to investigate these processes, we examined the subcellular localization of SYNV RNAs and the polymerase-associated proteins in infected cells. Additional experiments were performed to determine the fate of exogenously expressed N and M2 proteins and to identify regions of the N protein that are required for nuclear localization.

MATERIALS AND METHODS

General. SYNV (ATCC PV-263) was propagated by serial passages in Nicotiana benthamiana under ambient greenhouse conditions (18). SYNV and potato virus X (PVX) systemically infected tissue was harvested 11 to 14 days postinoculation (dpi) for various experiments.

Construction and expression of PVX expression vectors. A wild-type version of the N gene (p3ZN2, nt 203 to 1627 in the 3'-to-5' orientation of the SYNV genome; GenBank accession no. L32603) was constructed from plasmids p3ZN1 (44) and pAS41 (52). The XbaI/PstI fragment of p3ZN1 which contains a premature termination codon in the coding sequence was removed and replaced with the wild-type sequence by inserting the analogous XbaI/PstI fragment from pAS41 to generate plasmid pNW1. The N gene of pNW1 was then digested with BamHI and EcoRI, blunted with Klenow enzyme, and cloned into the EcoRV site of the PVX vector pPC25 (4) to construct the vector PVX-N. The M2 gene (nt 1750 to 2637 of the SYNV genome) was cloned into the EcoRV site of pPC25 by digestion of the DNA clone p3F11 (44) with EcoRI and PstI and subsequently blunt-ended with Klenow enzyme to produce PVX-M2. The β-glucuronidase (GUS) gene was excised from plasmid pRTL2-GUS/NaIDbm (36) by digestion with PstI, Blul, and subsequently blunt-ended with Klenow enzyme to produce PVX-M2-GUS. This was accomplished by digestion of plasmid pRTL2-GUS/NaIDbm with BglI, followed by a partial fill-in (A, G, and T) reaction using Klenow enzyme prior to digestion with BamHI. The N gene was digested with NheI, partially filled in (A, C, and T) with Klenow enzyme, and subsequently digested with BamHI. The resulting plasmid, pRTL2-GUS/NIaDBam, was digested with EcoRI and BamHI, blunt-ended with Klenow enzyme, and cloned into the EcoRV site of pPC2S to produce PVX-GUS/NLS. The putative nuclease (N) amino acid sequence (14) was amplified by PCR using the 5' CTGAAGCTTCTACATTCCTCTCT 3' and 5' TTCTAACATGGAAGCTAGTATTTTTCTG3' encoding the 5' and 3' NLS sequences with BglII and SalI sites located at the 5' and 3' termini, respectively, were annealed prior to ligation into pRTL2-GUS/NaIDbm that had been digested with BglII and SalI to generate plasmid pRTL2-GUS:NLS. PVX-GUS:NLS was then made by digesting with PstI and PstII in the linker (pRTL2-GUS/NIaDBam) and subsequently digested with EcoRI and BamHI, and the resulting fragment was blunt-ended with Klenow enzyme and cloned into the EcoRV site of pPC2S. To facilitate cloning of GUS:N gene mutations, the Apal-to-SacI fragment of PVX-GUS:N, which contains nt 4954 to 6545 of the PVX genome and the GUS fusion, was cloned into pGemZel (+) (Promega, Madison, Wis.) and religated. An in-frame fusion of GUS and M2 (GUS:M2 fusion) was generated by ligation of an EcoRI/PstI fragment from plasmid pRTL2-GUS/NaIDbm containing the tobacco etch virus untranslated leader followed by the GUS gene, into the EcoRI and NcoI sites of the plasmid pZ2S (44, 45). Prior to ligation, the BglII site at the 3' end of the GUS gene and the NcoI site at the 5' end of the M2 gene were made compatible by filling in with Klenow enzyme. The resulting plasmid, pZ2GT1, was digested with EcoRI and HindIII, and the fragment containing the tobacco etch virus untranslated leader followed by the GUS:M2 fusion was blunt-ended with Klenow enzyme and cloned into the EcoRV site of pPC2S to generate PVX-GUS:M2.

All the PVX expression vectors were linearized with SpeI prior to in vitro transcription reactions. Capped transcripts were synthesized in vitro by using bacteriophage T7 polymerase and used to inoculate N. benthamiana plants as described elsewhere (41).

In situ hybridization. The SYNV plasmids pJ3N1 (nt 380 to 1633), pJ3T1 (nt 2011 to 2857), and pJ3L1 (nt 12788 to 13630) were used as templates to generate probes for recognition of the N, M2, and L regions, respectively, of the genomic and antigenic RNAs (44). A probe designed to recognize the genomic or antigenic leader RNA was constructed by PCR amplification of pJ3W2 (44) with the following primers: 5'-GGGAGTCAGACAGAAACCTCAGAAAAT AC3' and 5'-GGGAGTCAGACAGAAACCTCAGAAAAT AC3'. The PCR fragment was digested with KpnI and SacI sites (indicated in bold in the primer sequences) and cloned into the KpnI and SacI sites of pSP65+ II (Strategene, La Jolla, Calif.) as described previously (28). Digoxygenin-labeled probes were synthesized by using either bacteriophage T3 or T7 polymerase. Probes recognizing regions of the negative-strand (genomic) leader, N, M2, and L genes were generated with T7 polymerase, using the following templates linearized with the designated restriction enzyme: pJ3L1 digested with HindIII, pJ3T1 digested with BamHI, pJ3T1/SacI, and pJ3L1/SacI. The p35N1-HindIII, pJ3T1-XbaI, pJ3L1-KpnI, and p33J1-KpnI linearized plasmids provided templates for T3 transcription probe of recognizing the leader, N, M2, and L regions. A negative-strand (antigenic) RNAs, RNA1, RNA2, RNA3, respectively. Uncapped, linearized probes were generated as described previously (41) except that the reactions included a modified digoxigenin nucleoside triphosphate labeling mix (250 μM digoxigenin-11-UTP [Boehringer Mannheim, Indianapolis, Ind.]; 0.5 μM ATP, GTP, and CTP; 0.25 mM UTP). Following transcription, DNase (5 U) was added and reaction mixtures were incubated for 15 min at 37°C before ethanol precipitation of the probes. The N, M2, and L probes were hydrolyzed for 45 min before use in an equal volume of 2× carbonate buffer (80 mM NaHCO3, 120 mM Na2CO3 [pH 10.2], neutralized with 0.5% [vol/vol] acetic acid, precipitated, and resuspended in 50% formamide. Probe concentrations were estimated by analyzing aliquots on 1% agarose gels.

SYNV-infected and uninfected N. benthamiana leaf tissue was vacuum infiltrated with FAA (3.7% formalin, 5% acetic acid, 50% ethanol) and fixed overnight at room temperature. Samples were embedded in paraffin, sectioned, prehybridized, and hybridized with individual probes (Fig. 1) as previously described (21). Posthybridization washes, digoxigenin detection, and slide mounting were performed as described elsewhere (28). The slides were viewed with a Zeiss Axioshot microscope using differential interference contrast.

Electron microscopy. Virus-infected and uninfected N. benthamiana leaf tissue was processed by using a modified 800-W safety-exhausted microscope designed for tissue series and Ted Pella Inc., Redding, Calif. as described previously (9). Samples were fixed twice in 2% paraformaldehyde–0.25% glutaraldehyde in phosphate-buffered saline (PBS; 10 mM phosphate buffer [pH 7.4], 150 mM NaCl) by treatment for 40 s in the microscope. The tissue samples were then rinsed in PBS and treated using the methylamine and acetone series. Tissue was infiltrated in the microscope for 15 min in 1:1 ace-
Gene expression was analyzed using RT-PCR in infected and uninfected N. benthamiana leaves as described previously (22). Localizations of GUS:N and GUS:M2 fusions were determined in PVX-infected protoplasts isolated from 11- to 14-dpi systemically infected leaves. The protoplasts were incubated in GUS substrate buffer (50 mM Na2PO4 [pH 7.0], 10 mM EDTA [pH 8.0], 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 3 mM MgO, and 0.25 mg of 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid per ml) for 1 h at 37°C before visualization under bright-field illumination with a Zeiss Axiophot microscope (40). Computer images were acquired with an Optromics 450 Color charge-coupled device camera and captured at high resolution by using a Scion CG-7 RGB framegrabber board. Figures were assembled on a Power Macintosh computer using Photoshop and Canvas software.

Immunogold labeling with the SYNV antiserum revealed immunogold particles are restricted to the nuclei and nuclear membranes, the presence of large numbers of gold particles located over nuclear inclusion bodies were detected in control experiments relative to the plane of sectioning. Neither virus particles nor gold-conjugated (10-nm gold particles) goat anti-rabbit immunoglobulin G at a 1:20 dilution in blocking buffer. The grids were then rinsed in PBS, fixed in 0.5% glutaraldehyde in PBS, rinsed in water, and stained with 2% aqueous uranyl acetate and Reynolds’s lead citrate (37). Sections were viewed at 80 kV in a JEO100 CX transmission electron microscope.

RESULTS

Genomic and antigenic RNAs are abundant in nuclei of SYNV-infected cells. Electron microscopy has previously indicated the presence of viroplasms in the nuclei of SYNV-infected cells and accumulation of virosomes in the perinuclear spaces (16), but the subcellular localization of SYNV RNAs has not been visualized directly in infected cells. To obtain this information, in situ hybridization experiments were performed with single-stranded ribonucleases designed to recognize specific regions of the genomic or antigenic RNAs (Fig. 1). The specificity of the probes used in these experiments was verified by Northern blot analysis of poly(A)+ RNA extracted from SYNV-infected and uninfected leaves (not shown). Probes designed to recognize the plus-strand leader RNA, and the N and M2 mRNAs hybridized to the appropriately sized poly(A)+ RNAs (38). Corresponding complementary probes hybridized to RNA blots containing the minus-sense genomic sequences (44). In sections of fixed tissue, probes specific for the leader and M2 regions of the genomic RNA hybridized within nuclei throughout infected mesophyll tissue but failed to hybridize to uninfected tissue (Fig. 1). Similar results were obtained with the N and L probes (data not shown). These results thus suggest that the overwhelming proportion of the genomic RNA is confined to the nucleus, a finding that is consistent with previous experiments suggesting that SYNV replication occurs in the nucleus (16, 45). The antigenic RNA also appeared to be concentrated predominantly in the nuclei, as expected from its putative role as the template for genomic RNA synthesis. However, in contrast to the strict nuclear hybridization of the genome specific probes, the antigenomic leader, N, M2, and L RNA probes produced a diffuse cytoplasmic labeling pattern (Fig. 1 and data not shown). These observations provide additional verification of previous hybridization results indicating cytoplasmic partitioning of polyadenylated SYNV mRNAs (30, 31). In addition, our results show unexpectedly that a portion of the polyadenylated leader RNA is present in the cytoplasm (Fig. 1), where it possibly influences host metabolism and/or has a role in regulating viral replication.

The N, M2, and L proteins are components of the nuclear inclusion bodies. No direct experiments have previously been conducted to determine which SYNV proteins are associated with the nuclear viroplasms observed in SYNV-infected cells (16) or to identify the subcellular location of the N, M2, and L polymerase-associated proteins (45). Therefore, we conducted immunofluorescence experiments to determine the subcellular localization of the SYNV proteins in combination with DAPI staining to identify nuclei. As shown in Fig. 2, SYNV-specific antibodies raised against disrupted virus particles resulted in intense fluorescence around the nuclear envelope, with fainter fluorescence within the nuclei of protoplasts isolated from infected N. benthamiana. The nuclear envelope is the site of SYNV morphogenesis (16, 43), and hence the bright signal around the periphery of the nuclei correlates with the presence of large numbers of virus particles that accumulate in the perinuclear spaces and react with antibodies that recognize the G protein on the surface of the virosomes. In contrast, antibodies raised against the N and M2 proteins were not as readily detected in virus particles because N and M2 within the virosomes are less accessible to the antibodies. Therefore, the N and M2 antibodies exhibited less intense fluorescence at peripheral regions and more intense reactions at discrete sites within the nuclei of SYNV-infected protoplasts (Fig. 2). Moreover, areas showing the most intense signal following labeling by the N and M2 antibodies resided within regions of the nuclei which had minimal DAPI staining, suggesting that chromatin is excluded from sites containing the N and M2 proteins. Staining was not observed in protoplasts isolated from uninfected tissue with any of the antibodies described above (Fig. 2 and data not shown).

Electron microscopy in combination with immunogold labeling was used to investigate the subcellular localization of the SYNV proteins at higher resolution. Our observations revealed large numbers of bacilliform particles present in the perinuclear spaces and occasional particles that appeared to be budding from the inner nuclear envelope (Fig. 3A, inset). Viroplasms of different shapes were also present in the swollen nuclei of infected tissue that were reminiscent of the granular structures previously observed in SYNV-infected cowpea protoplasts (43). The nuclei of SYNV-infected tissue were enlarged considerably and, from observations of a large number of sections, were estimated to be two- to threefold greater in volume than those of uninfected tissue. The sizes of the viroplasm varied considerably, and their shapes ranged from oval to spherical, possibly as a reflection of varied orientations relative to the plane of sectioning. Neither virus particles nor nuclear inclusion bodies were detected in control experiments with uninfected tissue (data not shown).

Immunogold labeling with the SYNV antiserum revealed the presence of large numbers of gold particles located over the virosomes and in the viroplasms (Fig. 3B). The remaining gold particles were restricted to the nuclei and nuclear membranes, but no appreciable label was found in the cytoplasm (data not shown). To localize each of the polymerase-associated proteins (N, M2, and L) in the infected cells, labeling experiments using...
specific antibodies were performed. Within the nucleus, the N protein antibodies decorated discrete regions associated with the viroplasms (Fig. 3C). In contrast, the M2 nuclear staining was more diffuse, and rather than being restricted to the viroplasms, some gold particles were distributed throughout the nuclei (Fig. 3D). Much less intense labeling was observed with the L antibody, but small numbers of gold particles were also present in the viroplasms, suggesting that compared to the N and M2 proteins, the viroplasms contain smaller amounts of the L protein (data not shown). These observations are consistent with previous studies with purified virions, where the antibody reactions approximate the relative abundance of the N, M2, and L proteins, and with the polymerase complex extracted from the nuclei of infected cells, where a relatively low abundance of L protein was also present (45). In several experiments, antibodies raised against the M1 protein (14) and the G protein (10) labeled the virus particles intensely but failed to label the viroplasms (data not shown). These results thus suggest that the N, M2, and L proteins are the major virus-specific components of the viroplasm, and they support our previous results (44, 45) implicating the N, M2, and L proteins as the major virus-specific components of the polymerase that can be recovered from infected nuclei.

The N and M2 proteins expressed from a PVX vector localize to the nuclei. To determine whether the N and M2 proteins can localize to the nucleus independently of SYNV infection, these proteins were expressed separately from the PVX vector pPC2S (4, 40), and systemically infected leaf tissue was collected at 11 to 14 dpi for electron microscopy. Plants infected with PVX-N and PVX-M2 developed a wild-type PVX symptom phenotype, although the appearance of symptoms was delayed slightly relative to those of wild-type infections. However, the cytopathologies of the PVX-N- and PVX-M2-infected cells differed substantially from each other (Fig. 4) and

FIG. 1. In situ hybridization of SYNV-infected and uninfected N. benthamiana leaf tissue, using probes recognizing specific regions of the genomic and antigenomic RNAs. Tissue collected at 11 to 14 dpi was fixed, sectioned, and hybridized with digoxigenin-labeled RNA probes that specifically recognized the leader or M2 region of the genomic or antigenomic SYNV RNA. The leaf sections were viewed by differential interference contrast microscopy and photographed. The bars below the depiction of the SYNV genome represent the locations of the probes used to recognize genomic or antigenomic RNAs. The size bar above the L gene represents 1 kb. E, P, and S indicate the epidermal cells, the palisade parenchyma, and the spongy mesophyll cells, respectively. Bar, 15 μm.
from that of the PVX-infected cells (data not shown). PVX-infected cells contained large paracrystalline inclusion bodies in the cytoplasm, but the nuclei were similar in size to those of uninfected cells and contained no obvious inclusions. The cytoplasmic paracrystalline inclusion bodies consisted of rod-shaped PVX particles whose morphology was typical of the banded inclusions observed frequently in potexvirus infections (34). In sharp contrast, the PVX-N-infected cells had greatly enlarged nuclei containing pronounced viroplasm-like inclusions (Fig. 4A). The N antibody label in the nuclei was restricted primarily to the viroplasms (Fig. 4A, inset), although small amounts of label were present in the cytoplasm associated with the PVX banded inclusions.

The nuclei of PVX-M2-infected cells were also larger than those of PVX infected cells (data not shown), but unlike the PVX-N infection, the PVX-M2 infection did not induce viroplasm-like inclusions (Fig. 4B). Instead, the immunogold labeling of PVX-expressed M2 was typical of that observed in SYNV-infected cells, since label was distributed more uniformly throughout the nucleus (Fig. 4B, inset). However, large numbers of amorphous electron-dense aggregates were present in the nuclei of PVX-M2-infected cells. These aggregates did not appear to contain M2 protein because gold labeling was not concentrated within the aggregates (Fig. 4B, inset). Although the vast majority of the M2 label was associated with the nuclei, some signal was also present in the cytoplasm in association with the paracrystalline PVX inclusions (Fig. 4B). These minor amounts of cytoplasmic M2 may result from a combination of high levels of expression of M2 from the PVX vector coupled with inefficient transport of M2 to the nucleus. Antibodies raised against PVX virions labeled the cytoplasmic PVX inclusions very heavily, whereas the nuclei were devoid of label (data not shown). These results demonstrate that the N and M2 proteins are able to accumulate in the plant cell nucleus independently of the presence of other SYNV proteins.

**GUS:N and GUS:M2 protein fusions are transported to the nuclei.** Examination of the N protein sequence revealed the presence of a putative nucleoplasmin-like bipartite NLS from amino acids 446 to 461 located near the carboxy terminus but that the M2 protein did not have regions with obvious homology to previously described NLSs (11, 33). To investigate nuclear import of the N and M2 proteins further, GUS:N and GUS:M2 fusions were expressed from PVX vectors, and the protoplasts isolated from systemically infected leaves were analyzed for GUS expression (Fig. 5). Protoplasts recovered from PVX-infected leaves expressing GUS alone had uniform staining throughout the cytoplasm (Fig. 5C). In contrast, protoplasts infected with PVX-GUS:N exhibited intense nuclear staining (Fig. 5A). The PVX-GUS:M2 derivatives also elicited GUS activity in the nuclei of infected protoplasts, but some cytoplasmic staining was also observed (Fig. 5B). These results indicate that the 122-kDa GUS:N and 106-kDa GUS:M2 fusion proteins can localize to the nucleus independently, although the GUS:M2 localization was less pronounced than that of the GUS:N. The PVX-GUS:N fusion results also reinforced the pronounced nuclear localization obtained in the immunogold labeling experiments. This strict nuclear localization permitted us to examine the putative nucleoplasmin-like NLS and to conduct mutagenesis experiments to define the distribution of the NLSs residing within the N protein.

To identify the karyophilic signals in the N protein and to delineate their functional activity in PVX-GUS:N, three mutations were constructed in the N gene sequence. These mutations resulted in (i) removal of about 50% of the carboxy-terminal portion of the N protein (GUS:Nstu), (ii) introduction of a stop codon prior to the putative bipartite NLS sequence from the N protein (GUS:Nstop), and (iii) fusion of the putative NLS to the carboxy terminus of GUS (GUS:NLS). Each of these alterations compromised GUS nuclear staining to some extent. The GUS:Nstu fusion, containing amino acids 1 to 210, reduced nuclear transit considerably because GUS staining was detected in the cytoplasm of the infected protoplasts, although some nuclear staining was also...
FIG. 3. Cytopathology of SYNV-infected *N. benthamiana* leaf tissue. Tissue isolated 14 dpi was embedded in LR white by using a microwave. (A) Enlarged nucleus of an infected cell that contains virus particles in the perinuclear spaces. The arrow indicates a region containing three incompletely enveloped virions, which are magnified in the inset. Bar, 750 nm. Panels B to D show reactions with rabbit antisera raised against disrupted virus particles (B), the N protein (C), and the M2 protein (D). Following incubation, sections were washed in PBS and subsequently incubated with gold-conjugated anti-rabbit immunoglobulin G. The sections were stained with 2% aqueous uranyl acetate and Reynolds’s lead citrate and viewed in a JEOL 100 CX transmission electron microscope operating at 80 kV. Bars, 150 nm. V, virus particle; VP, viroplasm.
FIG. 4. Subcellular distribution of the N and M2 proteins expressed separately from PVX in infected N. benthamiana. Tissue was prepared 11 dpi, and immunogold labeling was performed as described in the legend to Fig. 3. (A) PVX-N-infected tissue incubated with antiserum raised against the N protein; (B) PVX-M2-infected tissue treated with antiserum specific for the M2 protein. The insets represent higher magnifications of each of the cells to aid in visualization of the gold particles. E, electron-dense aggregates; I, viroplasm-like inclusion; P, PVX particles; VC, vacuole; arrows indicate nuclear envelope. (A) Bar, 750 nm; (B) bar, 500 nm.
evident (Fig. 5D). The GUS:Nstop derivative, with a stop codon engineered at amino acid 435, just prior to the putative NLS, retained appreciable nuclear staining, but substantial diffuse staining was also observed in the cytoplasm (Fig. 5E). However, the GUS:NLS construct (NLS amino acids 446 to 461) was severely compromised in localization of the fusion protein to the nucleus, but some nuclear staining was still present (Fig. 5F). Although there are some complications in the interpretation of these experiments due to possible aberrant protein folding and instability of PVX constructs, the results of the carboxy-terminal and NLS deletions suggest that efficient nuclear import of GUS:N is complex. Although deletion of the putative NLS had a substantial impact on localization, this motif alone appears not to be sufficient to facilitate efficient import of the GUS fusion protein, and so it is evident that several other unidentified regions of the N protein have synergistic effects on the nuclear transit process.

**DISCUSSION**

Our studies support a model predicting that early in SYNV infection, virions or viral cores move into the nucleus to establish a viroplasm that functions to mediate transcription of mRNAs as well as the antigenomic and genomic replicative RNAs. According to this model, polyadenylated mRNA transcripts are exported to the cytoplasm, where the mRNAs are translated differentially on free or membrane-bound polysomes (30, 38). The present in situ experiments show that both the genomic and the antigenomic RNAs are associated with the nuclei, and they most likely are present as replicating complexes within the viroplasm. The results also complement previous studies showing that the mRNAs accumulate to high abundance in the cytoplasm (30, 31). Interestingly, sequences corresponding to the positive strand (antigenomic) leader RNA transcript appear in the cytoplasm as well as in the nucleus. Detection of a cytoplasmic pool of the polyadenylated SYNV leader RNA is of particular interest because the converse situation exists with the animal rhabdovirus VSV, in which the majority of the positive-sense leader RNA is transiently associated with the nuclei of infected cells very early in infection (24, 25). These transient associations may have important roles in regulating the rhabdovirus infection cycle because the extent of encapsidation of the leader RNA is thought to facilitate the switch between transcription and replication.
(1–3, 49). Evidence obtained with VSV suggests that during the initial stages of infection, the concentration of the newly synthesized N protein available to encapsidate the nascent leader RNAs is low and that mRNA transcription predominates. However, as the concentration of the N protein increases to levels sufficient to facilitate replication, transcribed leader sequences become increasingly encapsidated, and synthesis of full-length antigenic nucleocapsids commences (49). Although the transient nuclear association of VSV leader RNAs is still an enigma, it is intriguing that the highest proportion of nuclear association occurs during the primary transcription phase of replication and that a decrease in abundance of the leader RNA found in the nuclei coincides with the appearance of antigenic replicating molecules (24, 25). Thus, it is conceivable that during the early phases of infection, rhabdoviruses such as VSV that use a cytoplasmic mode of replication sequester transcribed leader RNAs in the nuclei to facilitate rapid amplification of replication-competent antigenic nucleocapsids. In the case of the nuclearrhabdovirus SYNV, transport of the leader RNAs to the cytoplasm could provide an analogous mechanism for reducing localized nuclear concentrations of transcribed leader RNA.

We have also demonstrated that the polymerase-associated proteins N, M2, and L accumulate in the nuclei of SYNV-infected cells, and the results of DAPI staining suggest that the viroplasms form distinct structures within the nuclei that exclude chromatin. Christie et al. (7) first observed that oval inclusions occur in the nuclei of SYNV-infected plants in association with bacilliform particles surrounding the nuclei, and this observation has been verified subsequently in both whole plant and protoplast infections (16, 19, 43). The present study provides the first direct evidence that the N, M2, and L proteins are components of the nuclear viroplasms whereas the M1 and G proteins are not. In addition, our immunogold labeling experiments suggest that considerably lower amounts of the L protein than the N and M2 proteins colocalize with the viroplasms, a finding that correlates with the relative levels of these proteins in the polymerase complex isolated from the nuclei of infected plants (45). Furthermore, these findings provide additional evidence that the polymerase complex recovered from nuclei is derived from viroplasms.

Our serological analyses also show that the M2 protein in SYNV infections is present throughout the nucleus. The observation that appreciable amounts of nucleus-associated M2 reside outside the viroplasms may be linked to our previous finding that M2-L protein complexes can be commounprecipitated from extracts of SYNV-infected protoplasts (45). These experiments show clearly that the M2-L complex consists of nonstoichiometric amounts of the two proteins, with a substantial excess of M2 predominating (45). We postulate that the non-viroplasm-associated M2 protein observed in the immunogold labeling experiments is a component of an M2-L complex that provides a precursor to the viral polymerase. The N protein is also localized to the nuclei in SYNV infections but, in contrast to the M2 protein, is concentrated in the viroplasms. This result suggests that in addition to nuclear targeting functions, the N protein has signals that mediate its localization to particular regions within nuclei.

During the course of SYNV infection, the N, M2, and L proteins must be transported from their cytoplasmic sites of synthesis to the nucleus, where they are required for transcription, replication, and formation of virus particles. The exogenous expression of the N protein from the PVX vector reveals that the N protein alone is able to localize to the nucleus and concentrate at localized sites in amounts sufficient to form pseudoviroplasms similar to those found in SYNV-infected cells. This finding suggests that the karyophilic N protein signals facilitate specific transport associations with host components that target macromolecules to particular compartments within the nuclei. In addition, it appears that the N protein alone is sufficient for induction of nuclear swelling and formation of inclusions similar to those found in SYNV-infected cells. The M2 protein expressed from PVX also moves to the nucleus and results in enlargement of the nuclei. However, M2 is distributed throughout the nuclei, and it fails to elicit viroplasm-like inclusions, although unusual electron-dense aggregates of unknown composition are present. Thus, the SYNV-encoded N and M2 proteins differ in the ability to accumulate at specific sites in the nuclei and to alter the nuclear morphology.

The carboxy terminus of the N protein contains a putative NLS composed of a bipartite basic sequence that is characteristic of a major class of nuclear import signals (11, 33). However, although fusion of the putative NLS to the reporter gene directs some GUS nuclear import, additional, undefined signals within multiple regions of the N protein are required for efficient localization. This heterogeneous distribution of multiple localization signals has some features in common with the karyophilic signals present in the proteins of other viruses that replicate in the nuclei of their hosts. For example, the influenza virus N protein has an NLS in the central region that does not resemble any known NLS (8). However, a second recently identified nonconventional NLS (TKRFxxxM) present in the amino terminus of the influenza virus N protein (48) has some similarity to a sequence (TSDKxxxM) present in the amino-terminal region (amino acids 69 to 76) of the SYNV N protein. This signal may be involved in nuclear localization because the amino-terminal portion of the SYNV N protein, which contains this signal, directs some nuclear import of the fusion protein. Interestingly, when the two identified NLSs in the influenza virus N protein are deleted, nuclear localization is compromised but some nuclear localization still is evident, suggesting that multiple NLSs are also present in the influenza virus protein (32). Multiple NLSs also appear to be present in the N protein of the negative-strand nonsegmented Borna disease virus (BDV). Of some significance is the presence of two forms of the N protein that are essentially identical except for the presence of an amino-terminal 13-amino-acid extension containing an NLS. However, the BDV N protein derivative lacking the amino-terminal extension sequence is still able to localize to the nucleus, albeit less efficiently (35). Therefore, it appears that multiple NLSs are present in nucleocapsid proteins of several negative-strand viruses that replicate in the nucleus. Discrete signals required for nuclear localization of the SYNV M2 protein are not evident, and other than a centrally located sequence (AKKKSKA) reminiscent of a simian virus 40-like NLS (PKKKRKV), no obvious bipartite or M9 signals are present (11, 33).

The stringent localization of the N protein in the viroplasms during infection, coupled with the more uniform nuclear distribution of M2, raises the possibility that these two components interact in complexes. Moreover, other viral (for instance, M2-L complexes) and/or host nuclear import proteins probably facilitate targeting of the polymerase complex proteins into specific sites within the nucleus as the viroplasms form early in infection. Some evidence also exists for the presence of subnuclear localization signals in other viral and nonviral proteins. Such signals are present in a DNA methyltransferase (27) and in the splicing factor Tra (13) proteins. In DNA methyltransferase, the NLS is distinct from the subnuclear localization signals, whereas in Tra the two signals overlap. Neither of these signals is present in the N protein, so the
determinants that facilitate its localization to specific sites in the nucleus are presently obscure. Other experiments showing that M2 is a phosphoprotein (46) also raise the possibility that the phosphorylation state of the protein can affect subcellular localization in an arginine/serine-rich splicing motif. Proc. Natl. Acad. Sci. USA 92:11524–11528.


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