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Characterization of the Nucleic Acid Binding Properties of Tomato Spotted Wilt Virus Nucleocapsid Protein

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Tomato spotted wilt tospovirus (TSWV) is the type member of the plant-infecting viruses of the genus *Tospovirus* in the family Bunyaviridae. The three TSWV RNAs are encapsidated with nucleocapsid (N) protein to form ribonucleoprotein (RNP) which serves as the template for viral transcription and replication. Regions of the open reading frame coding for the N protein on the small (S) RNA were subcloned into pET protein expression vectors and expressed in *Escherichia coli* BL21 (DE3) cells. Full-length N, N amino and carboxy halves, and two N carboxy-terminal regions were expressed and isolated by metal chelate affinity chromatography. The N protein, both of its halves and the extreme carboxy-terminal region, bound cooperatively and irrespective of sequence to radiolabeled single-stranded RNA produced by runoff transcription of clones of either TSWV S RNA or cowpea chlorotic mottle virus RNA3. N protein did not bind to radiolabeled double-stranded TSWV RNA. The density of the synthetic RNase-sensitive N protein–RNA complexes was 1.32 g/ml, similar to the density of authentic Bunyaviridae RNPs. These studies are the first to indicate differences in the nucleic acid binding abilities of *Tospovirus* and *Hantavirus* nucleocapsid proteins, the only characterized nucleocapsid proteins of the family Bunyaviridae.

INTRODUCTION

Tomato spotted wilt tospovirus (TSWV) is the type member of the plant-infecting viruses in the genus *Tospovirus* in the family Bunyaviridae (Murphy *et al.,* 1995). TSWV is transmitted by thrips (*Thysanoptera:* Thripidae) to over 650 different plant species in temperate and tropical regions (Goldbach and Peters, 1994). Virions consist of a host-derived lipid membrane envelope, containing two viral glycoproteins (G1 and G2), which surrounds ribonucleoprotein (RNP) [small (S), medium (M), and large (L) RNAs individually encapsidated with many copies of nucleocapsid (N) protein]. In addition, virions contain small amounts of the putative RNA-dependent RNA polymerase (L) (German *et al.,* 1992). Both the S and M RNAs have an ambisense coding strategy, with the S RNA encoding N protein (28.8 kDa) and a nonstructural NS_s protein (52.4 kDa) (de Haan *et al.,* 1990), and the M RNA encoding both a nonstructural NS_m protein (33.6 kDa) (Kormelink *et al.,* 1992) and a polyprotein which is cleaved to yield two glycoproteins, G1 and G2 (95 and 58 kDa). The L RNA is negative sense, encoding L protein (3315 kDa) (de Haan *et al.,* 1991). The genomic RNAs have terminal complementary sequences that may be involved in maintaining the panhandle or "pseudo-circular" structure of TSWV RNP (Peters *et al.,* 1991).

Consistent with other segmented negative strand viruses (Tordo *et al.,* 1992; Krug *et al.,* 1989), the Bunyaviridae RNP serves as template for both viral transcription and replication. It is this RNP that putatively interacts with other viral proteins during viral transcription, replication, and, in the case of TSWV, movement from cell to cell in plants (Kormelink *et al.,* 1994). Additionally, during maturation, interactions between the N protein of the RNP complex and viral glycoproteins are thought necessary to initiate the budding of virions through host membranes (Matsuoka *et al.,* 1991). Interestingly, apart from the role N plays in the formation of the RNP complex and its associated interactions, the La Crosse virus (Bunyaviridae, *Bunyavirus*) N protein has also been found to regulate its own synthesis through the encapsidation of the N mRNA (Hacker *et al.,* 1989). Thus, the manner in which N protein binds RNA is significant for the role it plays in RNP formation as well as other, possibly regulatory, roles it has in the viral life cycle.

The specificity and conditions under which TSWV N protein binds RNA have not been previously described. *Hantavirus* nucleocapsid proteins share no significant homology to other Bunyaviridae nucleocapsid proteins. Hantaviruses are the only members for which nucleocapsid protein binding has been studied (Gott *et al.,* 1991). To better understand RNP complex formation and the possible regulatory roles N may have *in vivo,* we

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have recently carried out experiments to characterize isolated TSWV N protein binding to TSWV RNA *in vitro.*

RESULTS

Expression of the TSWV N protein and binding regions

Western blot analysis demonstrated that bacterially expressed TSWV N protein produced a single band with an estimated molecular weight of 29 kDa, similar to that of authentic TSWV N protein (28.8 kDa). The NN, NC, N3, and N4 regions (Fig. 1) were also analyzed and each showed a predominant band with the estimated molecular weights of 14.4, 14.4, 7.2, and 7.2 kDa, respectively (data not shown). The full-length and partial-length N proteins reacted specifically with antisera raised against the TSWV N protein.

Gel shift assay

To determine if purified full- and partial-length N proteins could bind nucleic acid, binding assays were performed with TSWV and/or nonviral single-stranded RNA substrate (Figs. 2A and 2C). In gel shift assays the decreased mobility of the nucleic acid substrate is indicative of the increased mass of the protein–nucleic acid

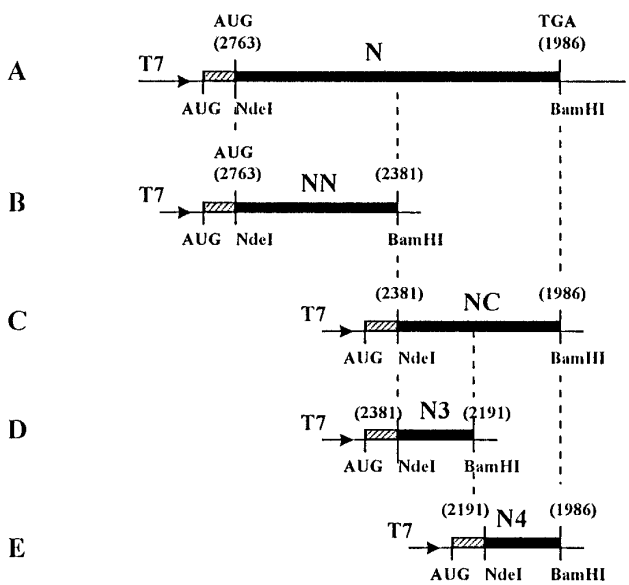

FIG. 1 TSWV nucleocapsid protein bacterial expression cassettes. For all plasmids, T7 denotes the T7 RNA polymerase promoter. (A) Clone pNHis, the TSWV N open reading frame (—) (ORF) (bases 2763–1986) in the pET-14b bacterial expression vector (Novagen) *Nde*I/*Bam*HI sites, allowing for the addition of six histidine residues (denoted by rectangle with diagonal lines) to the amino terminus of the expressed N protein. (B) pNNHis, the TSWV amino half of the nucleocapsid protein (bases 2763–2381 in the TSWV N gene). (C) pNCHis, the TSWV carboxy half of the nucleocapsid protein (bases 2381–1986 in the TSWV N gene). (D) pN3His (bases 2381–2191 in the TSWV N gene). (E) pN4His (bases 2191–1986 in the TSWV N gene) in the pET-15b expression vector (Novagen).

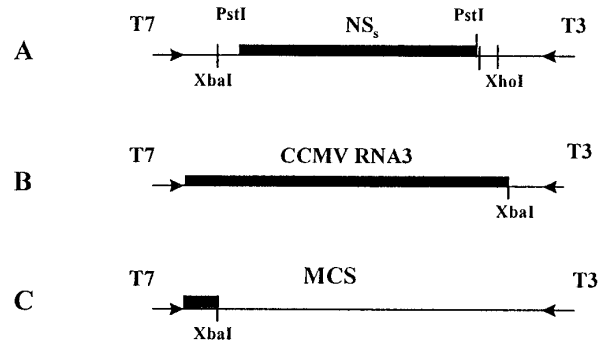

FIG. 2. RNA transcription cassettes used to generate runoff transcripts for gel shift assays. For all plasmids, T7 denotes the T7 RNA polymerase promoter, T3 denotes the T3 RNA polymerase promoter, and (—) denotes the RNA transcript used as substrate in binding assays. (A) Clone pNS$_s$, the TSWV NS$_s$ open reading frame (—) cloned into the *Pst*I site in pBluescript(KS$^+$). (B) pCCMV RNA3, the RNA3 segment (—) from cowpea chlorotic mottle virus. (C) pBluescript(KS$^+$) multiple cloning site (MCS).

complex formed in a binding event. A variety of binding conditions were compared, including those used for Hantavirus nucleocapsid proteins (Gott *et al.,* 1991) and influenza virus NS1 protein (Hatada *et al.,* 1992). Significant binding was seen only when a modified version of the binding buffer of Gott *et al.* (1991) was used. Gel shift assays demonstrated that when N bound to large single-stranded RNA substrates, the complexes formed remained in the well of the gel, with no intermediate shifts noted (Fig. 3A, lane N). In order to examine if the carboxy terminus of the nucleocapsid protein could bind to RNA, as was observed for Hantavirus (Gott *et al.,* 1991), regions of the N protein (Figs. 1B–1E) were also expressed and used in gel shift assays (Fig. 3A, lanes NN, NC, N3, N4). Of the proteins tested, only NN, NC, and N4 protein cooperatively bound RNA substrates. The observation that the RNA substrate remained in either a "free" or a "bound" state indicated cooperative binding. No change in RNA mobility was noted with reactions in which the nucleocapsid proteins had been heat treated or the reaction was amended with detergent or proteinase K (Fig. 3B). These results indicate that the shift in RNA mobility is due to an interaction with protein. To ensure that the histidine tag of the N protein was not responsible for binding, a control using a similarly histidine-tagged calmodulin kinase, a generous gift from E. Hrabak, was used in gel shift assays (data not shown). The addition of bovine serum albumin (BSA) was used to assess whether nonspecific protein interactions could be the cause of substrate shifting (data not shown). Since no shift was apparent with either of these substitutions, binding does not appear to be due to the presence of the histidine tag or from nonspecific protein–nucleic acid interactions.

To confirm that the gel shift was not due to an aggregation of N protein, an additional, shorter, RNA template

(Fig. 2C) consisting of a runoff-linearized vector transcript (~95 bases) was also used in gel shift assays (Fig. 4). As N protein concentrations are increased in the standard binding reaction a ladder-like profile of protein–RNA complexes is observed, indicating the formation of discrete complexes prior to the putative encapsidation of the RNA substrate.

Binding of N protein to single-stranded RNA was not sequence-specific since complex formation was seen regardless of whether the RNA competitor was TSWV or CCMV RNA3 (Figs. 5A and 5B). Yeast tRNA and single-strand DNA were also able to compete effectively (Fig. 5A) when added simultaneously with the radiolabeled transcript. However, if competitors were added after complexes were allowed to form, then no competition was seen (Fig. 5B).

Due to the observation of a stable pseudo-circular panhandle structure formed by TSWV nucleocapsids (Peters *et al.,* 1991) purified N protein was also examined for its ability to bind double-stranded RNA. As no gel shift was observed (data not shown), N protein did not bind to double-stranded RNA.

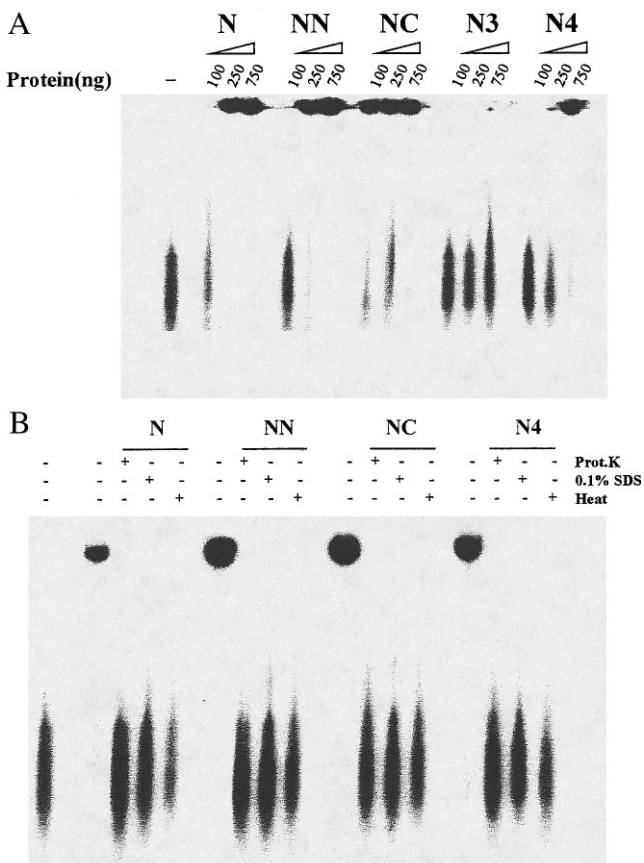

FIG. 3. Gel shift assay with full or partial TSWV N proteins. Autoradiographs of binding reactions after electrophoresis for 15 h at 8 V/cm on a 1% TBE agarose gel. (A) Binding reactions (as described under Materials and Methods) with pNS_s runoff transcripts (5 ng) and nucleocapsid proteins (N, NN, NC, N3, N4) in the indicated concentrations. (B) Binding reactions with pNS_s runoff transcripts (5 ng) and nucleocapsid protein (750 ng) were either amended with proteinase K (1 μg), 0.1% SDS or the nucleocapsid protein was heated to 100°C for 10 min prior to addition to binding reactions.

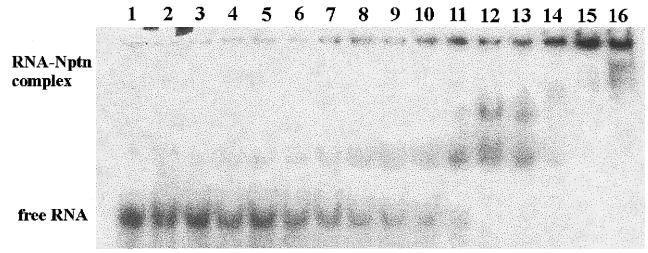

FIG. 4. Gel shift assay with TSWV N protein. Autoradiograph of binding reactions after electrophoresis for 4 h at 8 V/cm on a 6% polyacrylamide gel. Binding reactions (as described under Materials and Methods) containing 100 pmol radiolabeled pBluescript(KS^+) runoff transcripts and nucleocapsid protein in concentrations ranging from 0 μM (lane 1) to 10 μM (lane 16).

Effect of ionic concentration on binding

To determine if the binding of N protein to single-stranded RNA was a strong and stable interaction, binding reactions were performed under a range of ionic conditions. Gel shift assay results show that the binding of N protein to single-stranded RNA is partially disrupted at 0.3 M NaCl, and the binding is eliminated at 0.6 M NaCl (Fig. 6).

Density determination of TSWV N:RNA complexes

Experiments to compare the properties of synthetic (*in vitro*) complexes to the known properties of authentic Bunyaviridae RNP complexes were undertaken. Since the N protein not only binds but encapsidates genomic RNAs *in vivo,* the density of the *in vitro* N protein–RNA complexes was determined by isopycnic centrifugation through CsCl gradients. Complexes were shown to migrate to a density of 1.32 g/ml (data not shown). In addition, the synthetic complexes, like authentic Bunyaviridae RNPs, were not RNase resistant, as shown by treatment at 30°C for 30 min with DNase-free RNase (Boehringer-Mannheim) prior to gel shift analysis (data not shown).

DISCUSSION

We have shown that the TSWV N protein has both an amino and a carboxy region capable of binding RNA. To our knowledge, this is the first demonstration of multiple RNA binding domains in a segmented negative strand virus nucleocapsid protein. Similar research on the Hantavirus N protein (Gott *et al.,* 1991) suggested one domain of RNA binding, but focused on only the first and last 100 amino acids of the protein. The observation that the carboxy terminus retained the ability to bind RNA does not eliminate the possibility that an RNA binding

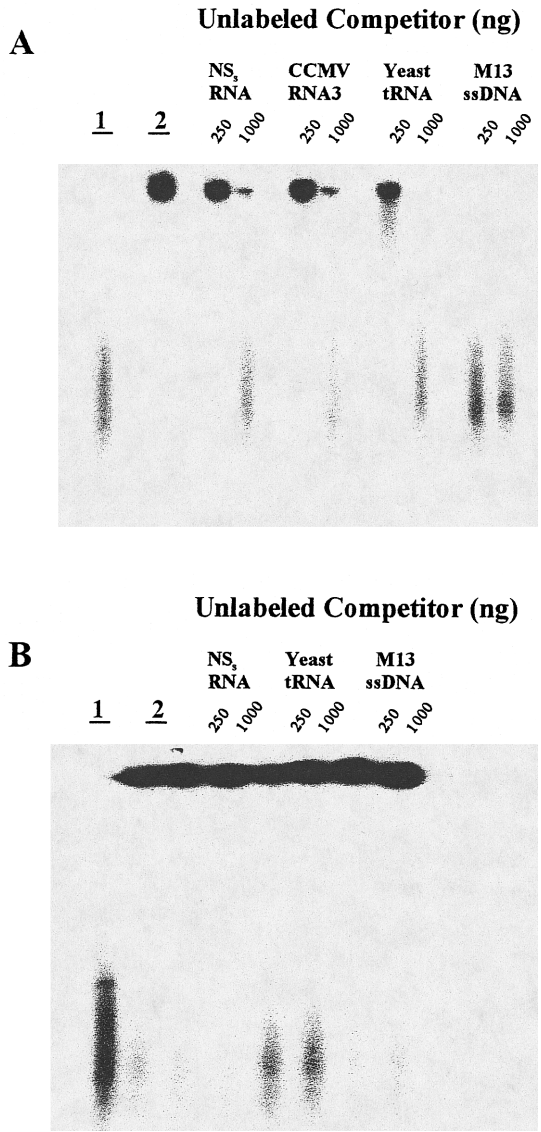

FIG. 5. Autoradiograph of encapsidation competition binding reactions after electrophoresis for 15 h at 8 V/cm on a 1% TBE agarose gel. In both figures are (lane 1) NS_S runoff transcripts (5 ng) and (lane 2) NS_S runoff transcripts (5 ng) and TSWV N protein (750 ng). Binding reactions containing NS_S runoff transcripts (5 ng) and TSWV N protein (750 ng) were amended with unlabeled competitor RNA (250 ng, 1000 ng), (A) prior to incubation at 30°C and (B) after incubation at 30°C. Homologous (NSs runoff transcripts) and heterologous (cowpea chlorotic mottle virus, RNA3) RNA, yeast tRNA, and ss M13 DNA were used as unlabeled competitors.

domain is present in a larger region of the amino terminus of Hantavirus N. Sequence analysis of the TSWV N open reading frame did not indicate any conserved RNA binding motifs or regions of NN and N4 that were similar and may play a role in RNA binding. The presence of multiple binding domains may allow for a stronger and more sensitive RNA binding capability or be necessary for the various roles N protein is thought to play in viral genome encapsidation, replication, and transcription.

Similar to *Escherichia coli*-expressed Hantavirus N protein (Gott *et al.,* 1991), TSWV N protein is not sequence-specific in its binding to single-strand RNA *in vitro,* a feature common to other negative strand RNA nucleocapsid proteins (Buchholz *et al.,* 1993; Kingsbury *et al.,* 1987; Masters and Bannerjee, 1988). These data, coupled with the apparent exclusive encapsidation of viral RNA *in vivo,* lead to the speculation that the binding specificity occurs only *in vivo.* This may be conferred by the presence of other viral and/or host components or by specific protein or ion concentrations. Alternatively, electron microscopy of infected thrip cells indicates that TSWV proteins are compartmentalized (Ullman *et al.,* 1995) and N protein is found only in cytoplasmic dense masses (presumed areas of nucleocapsid accumulation) and viroplasms. Such compartmentalization of the required RNA and protein components may obviate the need for sequence specificity in binding.

Since double-stranded regions of *Bunyavirus* genomic RNA, such as the panhandle structure, may be involved in the regulation of viral replication and/or transcription, it is interesting to note that the TSWV N protein binds to single-stranded RNA but not double-stranded RNA. The lack of binding to double-stranded RNA seen *in vitro* suggests that TSWV N protein may bind *in vivo* to the single-stranded regions of the panhandle. Similar to TSWV (de Haan *et al.,* 1989) the complementary terminal ends of the Hantavirus S RNA are not fully double stranded (Schmaljohn *et al.,* 1986). In previous work, the Hantavirus N protein bound cooperatively to a partially double-stranded substrate consisting of the exact 5′ and 3′ Hantavirus S RNA complementary termini, and the noncooperative binding of single-stranded RNA was inhibited by fully double-stranded competitor RNA (Gott *et*

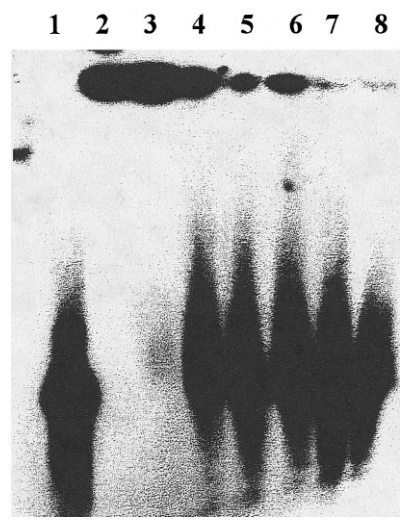

FIG. 6. Effect of salt concentration on nucleic acid binding by TSWV N protein. Autoradiograph of binding reactions conducted over a range of NaCl concentrations. Lane 1, single-stranded radiolabeled RNA; lanes 2–8, radiolabeled RNA and N protein (0.5 μg) binding reactions modified to have NaCl concentrations ranging from 100 mM (lane 2) to 700 mM (lane 8), in 100 mM increments.

al., 1991). This suggests differences between the two bacterially expressed nucleocapsid proteins with respect to RNA binding and may indicate different strategies for viral replication or regulation. Alternatively, since the S RNAs of Hanta- and Nairoviruses encode a nucleocapsid protein (49–50 kDa) larger than that of TSWV, and no nonstructural protein, differences in binding may be due to multiple functions of the larger N protein.

The complementary terminal ends of TSWV genomic RNAs are not fully double stranded (de Haan *et al.,* 1989). The speculation that N protein binds *in vivo* to the single-stranded regions of this panhandle does not contradict the lack of double-stranded RNA binding activity seen with N protein expressed in and isolated from *E. coli.*

Since single-stranded RNA–N protein complexes form over a large range of ionic concentrations, the binding appears to be strong and not solely due to electrostatic interactions. This stability is consistent with the observation that synthetic as well as authentic Bunyaviridae RNPs are capable of surviving CsCl density centrifugation. Our synthetic RNPs have a density of 1.32 g/ml, similar to that of authentic Bunyaviridae RNPs [1.31 g/ml (Kolakofsky *et al.,* 1991)], suggesting that *in vitro* N protein encapsidates the input single-stranded RNA. This observation indicates the high stability of N:RNA complexes once they are formed and is consistent with the stability we observed during the competition assays.

The manner by which the specificity of N protein binding is determined *in vivo* is not known. Although an initiation site of encapsidation has been speculated to exist (Raju and Kolakofsky, 1987), other evidence indicates that binding is somewhat nonspecific, possibly dependent on protein concentration (Hacker *et al.,* 1989). Similarities have been noted between electron microscopy images of single-strand binding (ssb) protein of *E. coli* and N protein–nucleic acid complexes of the Bunyaviridae (Hewlett and Chiu, 1991). In addition, hallmarks of ssb protein binding such as nonspecificity, cooperativity, and its ability to bind in the presence of high salt concentrations are also characteristics of N protein *in vitro* binding. This, and evidence of N protein compartmentalization, may indicate that the nonspecific binding activity of TSWV N protein *in vitro* accurately reflects the binding activity of N protein *in vivo.* Since expressed N protein encapsidates single-stranded RNA to form complexes similar to authentic Bunyaviridae RNPs, the binding assay described may serve as the foundation for more detailed studies of the Bunyaviridae RNP complex and the requirements for its formation.

MATERIALS AND METHODS

Cloning and expression of the TSWV N protein regions

TSWV N protein was isolated by metal chelate affinity chromatography from the expression of pNHis (Fig. 1A) in *E. coli* BL21 (DE3) cells by methods previously described (Ullman *et al.,* 1995). The amino and carboxy halves (NN and NC, respectively) and the N3 and N4 regions were amplified from pNHis by PCR (Figs. 1B–1E). For all regions, a primer containing an *Nde*I site followed by 15 bases complementary to the viral sense of the TSWV N open reading frame and a primer containing a *Bam*HI site followed by 15 bases in the viral sense were used for amplification. PCR products were digested with *Nde*I and *Bam*HI, purified, ligated into an *Nde*I–*Bam*HI pET-15b vector (Figs. 1B–1E), and expressed as above for pNHis. The pET-14b and pET-15b expression vectors (Novagen) code for the addition of six histidine residues to the amino terminus of the expressed protein. Purified N, NN, and NC protein was concentrated in a centriprep concentrator (Amicon), with a molecular weight cutoff of 10 kDa. The TSWV N protein and partial N regions were analyzed by SDS–polyacrylamide gel electrophoresis followed by either Coomassie staining or Western blot analysis (Bollag and Edelstein, 1991) with polyclonal anti-N and anti-TSWV antisera.

Substrate production

Radiolabeled single-stranded RNA transcripts were produced by runoff transcription with T7 RNA polymerase of *Xho*I-linearized TSWV pNS$_s$, *Xba*I-linearized pCCMV RNA3, or pBluescript (KS$^+$) (Stratagene, La Jolla, CA) (Figs. 2A–2C) (Sambrook *et al.,* 1989). To create a double-stranded RNA substrate, ^{32}P-radiolabeled single-stranded RNA transcripts of the TSWV NS$_s$ gene were made by T7 or T3 RNA polymerase runoff transcription of *Xba*I- or *Xho*I-digested pNS$_s$ (Fig. 2A), respectively. These transcripts were then combined and denatured at 95°C for 5 min, placed at 60°C, and allowed to anneal as the temperature was slowly decreased to 37°C over 2 h. The reactions were adjusted to 0.4 M NaCl prior to the addition of 0.75 μg RNase A and 15 units RNase TSWV and incubated at 37°C for 1 h. After phenol–chloroform extraction and ethanol precipitation (Sambrook *et al.,* 1989), the double-stranded RNA was resuspended and used in binding reactions with N protein.

Gel shift analysis

Purified N proteins and ^{32}P-radiolabeled single-stranded RNA transcripts were combined with a modified version of the binding buffer of Gott *et al.* (1991), (10 mM NaCl, 2 mM KCl, 0.5 mM $MgCl_2$, 0.05 mM EDTA, 0.2 mM dithiothreitol (DTT) 10 units of rRNasin (Promega), for a total reaction volume of 15 μl, and incubated at 30°C for 30 min. Products of binding reactions were then separated by electrophoresis on either a 6% polyacrylamide gel or a 1% agarose gel and visualized by autoradiography.

Competition assays were performed by amending the above binding reactions with either 250 or 1000 ng of

homologous RNA (NSs RNA), heterologous RNA (cowpea chlorotic mottle virus, RNA3), yeast tRNA, and M13 ssDNA, either at the beginning of the incubation at 30°C for 30 min or after incubation at 30°C for 30 min and continuing at 30°C for another 30 min.

Effect of ionic concentration on binding

Binding reactions were modified to have NaCl concentrations ranging from 0.1 to 1 M (in 0.1 M increments) and then placed at 30°C for 30 min. Products of binding reactions were then separated by electrophoresis on a 1% agarose gel and visualized by autoradiography.

Density determination

Binding reactions were loaded onto a 1.31 g/ml CsCl solution and centrifuged at 52.6 K rpm in a Vti65.2 rotor (Beckman) at 10°C for 44 h. Fractions from these gradients were then taken to determine both their density and radioactivity.

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