

BHK Cell Proteins That Bind to the 3' Stem-Loop Structure of the West Nile Virus Genome RNA

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The first 83 3' nucleotides of the genome RNA of the flavivirus West Nile encephalitis virus (WNV) form a stable stem-loop (SL) structure which is followed in the genome by a smaller SL. These 3' structures are highly conserved among divergent flaviviruses, suggesting that they may function as *cis*-acting signals for RNA replication and as such might specifically bind to cellular or viral proteins. Cellular proteins from uninfected and WNV-infected BHK-21 S100 cytoplasmic extracts formed three distinct complexes with the WNV plus-strand 3' SL [(+)3'SL] RNA in a gel mobility shift assay. Subsequent competitor gel shift analyses showed that two of these RNA-protein complexes, complexes 1 and 2, contained cell proteins that specifically bound to the WNV (+)3'SL RNA. UV-induced cross-linking and Northwestern blotting analyses detected WNV (+)3'SL RNA-binding proteins of 56, 84, and 105 kDa. When the S100 cytoplasmic extracts were partially purified by ion-exchange chromatography, a complex that comigrated with complex 1 was detected in fraction 19, while a complex that comigrated with complex 2 was detected in fraction 17. UV-induced cross-linking experiments indicated that an 84-kDa cell protein in fraction 17 and a 105-kDa protein in fraction 19 bound specifically to the WNV (+)3'SL RNA. In addition to binding to the (+)3'SL RNA, the 105-kDa protein bound to the SL structure located at the 3' end of the WNV minus-strand RNA. Initial mapping studies indicated that the 84- and 105-kDa proteins bind to different regions of the (+)3'SL RNA. The 3'-terminal SL RNA of another flavivirus, dengue virus type 3, specifically competed with the WNV (+)3'SL RNA in gel shift assays, suggesting that the host proteins identified in this study are flavivirus specific.

The flavivirus West Nile encephalitis virus (WNV) has a single-stranded, positive-polarity RNA genome that is approximately 11 kb in length. The genome RNA is the only viral mRNA and encodes a single open reading frame of about 10 kb (40). Ten mature viral proteins are processed by proteolytic cleavage from the large polyprotein precursor translated from this open reading frame (11). The functions of the seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5, are not currently well understood (40). The genome coding region is flanked by 5' and 3' noncoding regions (NCRs) that in the WNV genome are 96 and 580 nucleotides (nt) long, respectively. Computer folding predictions and RNase cleavage studies have indicated that the 3'-terminal nucleotides of the flavivirus genome form a stable stem-loop (SL) structure (10). The shape of this 3' SL structure is highly conserved among divergent flaviviruses, even though the sequences located in the stem are not well conserved (10, 20, 31, 40, 47). However, short conserved sequences located in the loop regions of the flaviviruses' 3' structures are conserved (10). The 5' NCRs of the genomes of all flaviviruses sequenced to date have also been predicted to form a conserved SL structure (9). The predicted 5' SL structure is much less stable than the 3' SL structure. The 3' NCR of the minus strand, which is complementary to the 5' end of the genome, contains a corresponding SL structure (45a).

Flavivirus plus- and minus-strand RNAs are synthesized disproportionately; approximately 10 to 100 times more plus-strand RNA than minus-strand RNA is produced during the flavivirus replication cycle (46). Both sequence and structural elements present within the flavivirus 3'-terminal SL structures may function as *cis*-acting signals for the initiation of viral

RNA transcription and may play regulatory roles during viral RNA synthesis. The interaction of cellular proteins with the flavivirus 3' termini may also be involved in regulating the rate of viral RNA transcription initiation.

Data indicating that host factors are components of RNA virus replicase complexes have been reported for phage Q β , influenza virus, Sindbis virus, and cucumber mosaic virus (4, 22, 26, 35). Purified poliovirus RNA-dependent RNA polymerase is incapable of specifically initiating viral RNA synthesis *in vitro* without the addition of host proteins (2, 17, 32). The possibility that at least one host factor is involved in flavivirus RNA replication was suggested by previous studies with congenic inbred flavivirus-resistant and -susceptible mouse strains. These mice differed in the efficiency with which they replicated flavivirus RNA (7, 16). A single, dominant gene confers the flavivirus-resistant phenotype (42). This locus, designated Flv', has recently been mapped to the murine chromosome 5 (44).

Evidence for specific binding of cell proteins to cell or viral RNAs has been obtained from a number of systems (30). While in some cases proteins recognize RNA in a sequence-specific manner (13, 30), in other cases the specificity of protein binding depends on the presence of RNA secondary or tertiary structures (15, 18, 23, 33, 34, 41). Recent studies have demonstrated that host cell proteins can bind specifically to regions within the 3' and 5' NCRs of RNA viruses. Three cytoplasmic proteins have been shown to bind to SL structures located in the 3' region of rubella virus genome RNA (33, 34). One of these proteins and two additional cell proteins bind specifically to the 3' end of the rubella virus minus-strand RNA (33). Similarly, specific binding of cell proteins to the 3' end of the Sindbis virus minus-strand RNA (36, 37), to the 3' end of the poliovirus genome RNA (23), and to the 3' end of the mouse hepatitis virus minus-strand RNA (19) has been reported. Interestingly, for both rubella virus and mouse hepatitis virus, different cellular proteins have been found to bind to

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the 3' end of the minus-strand RNA and to the complementary 5' end of the plus-strand RNA (19, 38). It has been proposed that the interaction of cellular and viral proteins with the 5' end of some plus-strand RNA genomes brings about the release of the 3' ends of nascent minus strands (1, 39). Cellular proteins which bind specifically to the 3' end of the measles virus genome RNA have also recently been reported (28). These data suggest that host proteins are intimately involved in transcription initiation events occurring from both plus- and minus-strand RNA templates.

In the present study, we have identified two BHK cell proteins which interact specifically with the plus-strand 3' SL [(+)3'SL] structure of the WNV genome. These results support the hypothesis that cellular proteins are involved in the initiation of flavivirus RNA transcription.

MATERIALS AND METHODS

Cells and virus. BHK-21/W12 cells (referred to hereafter as BHK cells) were used as the source of uninfected and infected cytoplasmic extracts (48). Confluent monolayers of BHK cells in T-150 flasks were infected with WNV strain E101 at a multiplicity of infection of 1 as described previously (10). Cell extracts were prepared 24 h after infection as described below.

Preparation of cDNA clones. The scheme used for construction of pWNV(+)'3'SL, the plasmid used for in vitro synthesis of WNV (+)'3'SL RNA, is shown in Fig. 1. A clone [pWNV(+)'3'NCR] that contains the entire WNV genome 3' NCR and approximately 150 nt of the NS5 gene of WNV was constructed by reverse transcription of viral RNA (24) followed by PCR amplification, as described in the legend to Fig. 1. The PCR product was cloned directly into plasmid pCR1000 (Invitrogen) by the TA cloning method, and the resulting pWNV(+)'3'NCR clone was sequenced by the dideoxy method (43). pWNV(+)'3'SL was subcloned from pWNV(+)'3'NCR by PCR amplification using a forward primer (5'-ACGTGAATTCGAATGCA*AGTATCCTGTGTTCTCGCAC-3') complementary to the 3' terminus of the genome RNA that contained a tail with two restriction sites. The *EcoRI* site (underlined) was added for subsequent use in unidirectional cloning, and the adjacent *BsmI* site (boldface) was added to generate an RNA product after in vitro transcription with an exact 3' end (the asterisk represents the *BsmI* cut site). The reverse primer (5'-TTAGGGCCCATATGATACCTGGGA-3') was located 109 nt from the 3' end of the genome and contained an *ApaI* site (underlined). The resulting PCR product was digested with *ApaI* and *EcoRI* and ligated into a similarly digested pCR1000 vector DNA. This vector was chosen because the *ApaI* site is positioned adjacent to the T7 promoter, thus minimizing the number of nucleotides added to the 5' end of the RNA transcription product.

Additional plasmids were constructed to transcribe truncated WNV and dengue virus type 3 (Den3) (+)'3'SL RNAs. These constructs, designated pWNV(+)'3'SL-trunc1, pDen3(+)'3'SL-trunc1, and pDen3(+)'3'SL-trunc2, were each prepared by directly annealing complementary synthetic oligonucleotides. These oligonucleotides were designed so that once annealed they produced sticky ends for unidirectional cloning into pCR1000 DNA that had been digested with *ApaI* and *SacI*. The sequences and secondary structures of these RNA transcripts are shown below (see Fig. 7C).

A clone containing the WNV plus-strand 5' NCR [(+)'5'NCR] and most of the capsid gene [pWNV(+)'5'NCR-Capsid] was constructed by reverse transcription PCR using a forward primer (5'-CTAGTTCCTTCTTGAATC-3') located 356 nt downstream of the 5' end of the genome and a reverse primer (5'-AGTAGTTCGCTGTGTGAGC-3') that was identical to the 5'-terminal 20 nt of the WNV genome RNA. The resulting PCR product was cloned directly into pCR1000 by the TA cloning method. A clone containing the WNV (+)'5'NCR-Capsid insert in the correct orientation was chosen after dideoxy sequencing.

Preparation of RNA transcripts. To synthesize RNA in vitro, purified plasmid DNA was first linearized; plasmid pWNV(+)'3'SL was linearized with *BsmI*, and plasmids pWNV(+)'3'SL-trunc1, pDen3(+)'3'SL-trunc1, and pDen3(+)'3'SL-trunc2 were linearized with *SacI*. A ³²P (specific activity; ~50,000 cpm/ng)-labeled WNV (+)'3'SL RNA probe was produced by in vitro transcription with T7 RNA polymerase (100 U; Ambion) in a 20- μ l reaction volume containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol (DTT), 0.5 mM ribonucleotides (A, C, and U), 12 μ M GTP, 50 μ Ci of [α -³²P]GTP (3,000 Ci/mmol; Amersham), 1 μ g of linearized DNA template, and RNasin (20 U). The photoactive nucleotide analog 5-azidouridine triphosphate (5N₃-UTP; Research Products International Corp.) was substituted for UTP during the synthesis of ³²P-WNV (+)'3'SL RNA in some experiments. The reaction mixture was incubated for 2 h at 37°C. RNase-free DNase I (20 U; Boehringer Mannheim Biochemicals) was added, and the incubation was continued for 10 min at 37°C. After ethanol precipitation, the ³²P-WNV (+)'3'SL RNA was resuspended in 10 μ l of loading buffer (7 M urea and 0.025% bromophenol blue) and gel purified on a 6% polyacrylamide-urea sequencing gel (14). The ³²P-labeled RNA was eluted from the gel slice with 0.5 ml of elution buffer

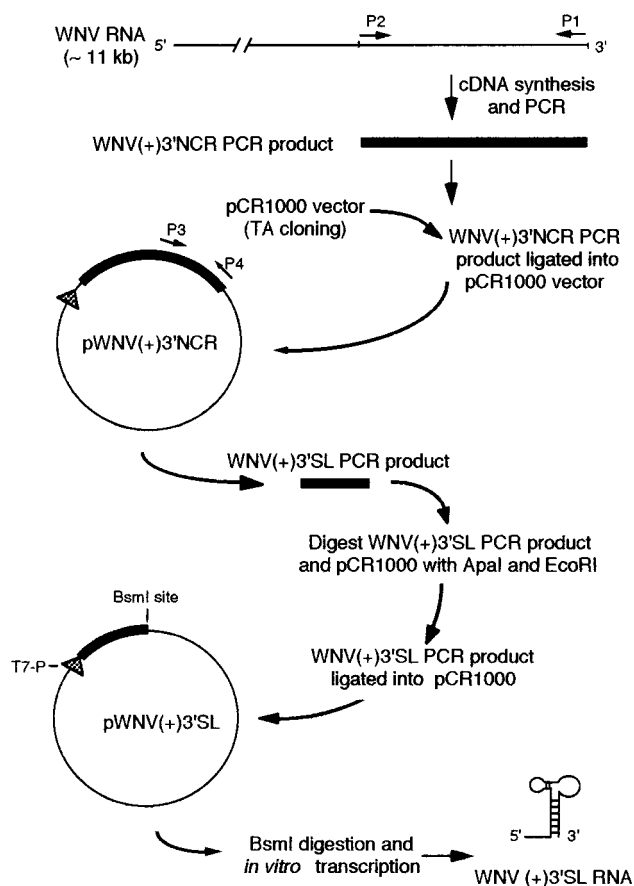


FIG. 1. Construction of plasmid pWNV (+)'3'SL. The 3'-terminal nucleotides of the WNV genome were cloned into pCR1000 by reverse transcription PCR to generate pWNV(+)'3'NCR. The primer, P1, used for first-strand cDNA synthesis was complementary to the genome 3'-terminal nucleotides. P2, located 747 nt upstream from the 3' end, and P1 were then used for PCR amplification of the cDNA. Plasmid pWNV(+)'3'NCR was subcloned from pWNV(+)'3'NCR following PCR amplification with primers P3 and P4. P3 was located 109 nt from the 3' end of the genome and had an *ApaI* site at its 5' end. P4 was identical to P1 except that it had an *EcoRI* site at its 5' end. The resulting PCR fragment [WNV (+)'3'SL DNA] and the vector (pCR1000) were each digested with *ApaI* and *EcoRI* and then ligated together. The resulting plasmid, pWNV(+)'3'SL, was digested with *BsmI* and used as a template for in vitro transcription of WNV (+)'3'SL RNA.

(0.5 M ammonium acetate, 0.1% sodium dodecyl sulfate [SDS], and 1 mM EDTA), precipitated with ethanol, and stored at -70°C . Prior to use, an RNA aliquot was pelleted, washed with 70% ethanol, dried, and resuspended in buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM KCl, 2 mM MgCl₂, 5% glycerol, and 5 mM DTT.

Plasmid pWNV(+)'5'NCR-Capsid was used to synthesize the minus-strand 3' SL RNA [WNV (-)'3'SL RNA]. A PCR fragment was amplified from pWNV(+)'5'NCR-Capsid by using a forward primer (5'-CCAAGCTCAATACGACTCACTATAGGGCTGGTTCTTACATC-3') located 112 nt from the 5' end of the WNV genome RNA. This primer contained complementary viral sequence (boldface) at its 3' end and the T7 promoter sequence (underlined) at its 5' end. The reverse primer was identical to the first 20 5' nt of the WNV genome (described above). The resulting PCR product was used to synthesize WNV (-)'3'SL RNA by in vitro transcription reaction as described below.

Nonspecific competitors tRNA (Life Technologies) and poly(I)-poly(C) (Boehringer Mannheim Biochemicals) were resuspended in RNase-free H₂O at a concentration of 1.0 μ g/ μ l. D10 RNA was in vitro transcribed from a 90-bp PCR product that contained the T7 promoter followed by the 67 bp that form U1 stem II and the D10 epitope as described elsewhere (5). The DNA fragment used to produce D10 RNA was a gift from J. Keene (Duke University, Durham, N.C.). An SL structure located near the 3' end of the rubella virus genome RNA was used as another competitor in this study (34). Gel-purified rubella virus (+)'3'SL RNA was a gift from H. Nakhasi (Food and Drug Administration, Bethesda, Md.).

Large-scale transcription reaction mixtures (0.5 ml) were used to synthesize the following unlabeled competitor RNAs: WNV (+)3'SL, WNV (-)3'SL, WNV (+)3'SL-trunc1, Den3 (+)3'SL-trunc1, Den3 (+)3'SL-trunc2, and D10. The reaction conditions were carried out as described above except that no ^{32}P -labeled ribonucleoside triphosphate was added and the final concentration of each unlabeled ribonucleoside triphosphate was 0.5 mM. Each competitor RNA was ethanol precipitated, pelleted, resuspended in 100 μl of 20 mM Tris-HCl (pH 7.5), and then purified by anion-exchange chromatography on a MonoQ HR 5/5 column (Pharmacia) over a 40-ml gradient from 0 to 100% with 20 mM Tris-HCl (pH 7.5)-1 M NaCl. Peak fractions were pooled and ethanol precipitated. The RNA was then pelleted, resuspended, and quantitated spectrophotometrically.

Preparation of cell extracts. Confluent monolayers of BHK cells grown in T-150 flasks were washed three times with ice-cold phosphate-buffered saline (PBS), then harvested with a rubber policeman, and pelleted by centrifugation at $150 \times g$ for 3 min. Pelleted cells were resuspended in PBS, and aliquots containing approximately 10^7 cells in 0.5 ml of PBS were stored at -70°C in microfuge tubes. When needed, an aliquot was thawed on ice and briefly centrifuged to collect the cells. The protein concentration of the PBS supernatant was measured by using the bicinchoninic acid assay (Pierce) and found to contain $<1\%$ of the total protein in the cytoplasmic extract prepared as described below, indicating that very little cell lysis had occurred as a result of freezing and thawing. The cell pellet was resuspended in 200 μl of cytolysis buffer (10 mM HEPES [pH 7.9], 5 mM DTT, 20% glycerol, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of leupeptin per ml, and 1% Triton X-100 [Boehringer Mannheim]), vortexed for 30 s, and stored on ice for 15 min. The nuclei were removed by centrifugation at $2,000 \times g$ for 5 min at 4°C . The supernatant was clarified by centrifugation at $100,000 \times g$ for 15 min at 4°C . The resulting S100 supernatant was then transferred to a Centricon-30 microconcentrator and centrifuged as described by the manufacturer (Amicon). Concentration and buffer exchange were continued until the cell lysate volume was 50 to 100 μl in storage (S20) buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl_2 , 5 mM DTT, 0.1 mM PMSF, 10 μg of leupeptin per ml, and 50% glycerol. The cytoplasmic extract was stable for several weeks at -20°C under these conditions. The total protein concentration of the S100 extract was typically 2 to 10 $\mu\text{g}/\mu\text{l}$.

Partial purification of RNA-binding proteins. BHK cells from 25 T-150 flasks were washed three times with cold PBS, scraped, pooled, and resuspended into a volume equivalent to 5 packed cell volumes (about 5 ml) of cytolysis buffer as described above. The cell extract was centrifuged at $2,000 \times g$ for 10 min at 4°C to remove the nuclei, after which the supernatant was removed and centrifuged at $100,000 \times g$ for 2 h at 4°C . The resulting supernatant (S100) was diluted with 50 ml of cold buffer A (20 mM HEPES [pH 7.5], 10 mM NaCl, 5 mM DTT, 0.05% Triton X-100, and 10% glycerol) and loaded onto an anion-exchange column (MonoQ HR 5/5) that was pre-equilibrated with 10 column volumes of buffer A. Proteins were eluted with a 50-ml gradient of 0 to 100% buffer B (buffer A with 1 M NaCl) of increasing NaCl concentrations (10 mM to 1 M). Fractions (1.0 ml) were collected, and 2.0 μl of each fraction was immediately analyzed by the gel mobility shift assay as described below. Fractions having binding activity were concentrated approximately 20-fold into S20 buffer with Centricon-30 microconcentrators, and the total protein concentration was determined as described above.

Gel mobility shift assay. Approximately 1 μg of the S100 cytoplasmic extract or the partially purified protein fraction was incubated in GS buffer (14 mM HEPES [pH 7.5], 6 mM Tris-Cl [pH 7.5], 1 mM EDTA, 1 mM DTT, and 60 mM KCl) with ^{32}P -WNV (+)3'SL RNA ($\sim 20,000$ cpm per reaction mixture), poly(I)-poly(C) (100 ng), and RNasin (1 U) in a final volume of 10 μl for 30 min at room temperature. In competition assays, various amounts of a competitor RNA were preincubated with the protein extracts in the reaction mixture for 5 min prior to the addition of the probe. RNA-protein complexes were electrophoresed at 4°C on a nondenaturing 5% polyacrylamide gel (50:1, acrylamide-to-bisacrylamide ratio) containing 2.5% glycerol in $0.5 \times$ Tris-borate-EDTA. The gel was then dried, and the complexes were visualized by autoradiography. In some experiments, the RNA-protein complexes and unbound probe were quantitated by measurement on a Molecular Dynamics densitometer.

UV-induced cross-linking of RNA and protein. The RNA-protein binding reaction mixture was set up as described above except that it was diluted fivefold with GS buffer. After incubation for 10 min at room temperature, the binding reaction mixture was transferred to an ice bath and irradiated with a 254-nm UV lamp (Ultra-Violet Products, Inc.) held 3 cm from the reaction mixture for 1 to 30 min as described by Nakhasi et al. (34). After irradiation, RNase T₁ (2.5 U) was added, and the reaction mixture was incubated for 15 min at 37°C to digest unprotected RNA. The UV cross-linked products were boiled in Laemmli sample buffer for 3 min and analyzed on a discontinuous 10% polyacrylamide-SDS gel (25). The gel was fixed in 7% acetic acid and dried, and the complexes were visualized by autoradiography.

Protein standards (low range; Bio-Rad) were used to calculate the relative molecular masses of the UV cross-linked products with an IBI Gel Reader (Kodak Co.) using the least-squares method (45).

Northwestern (RNA-protein) analysis of RNA-binding proteins. BHK proteins were denatured and renatured on a solid support membrane and then probed with ^{32}P -WNV (+)3'SL RNA as described elsewhere (29), with some

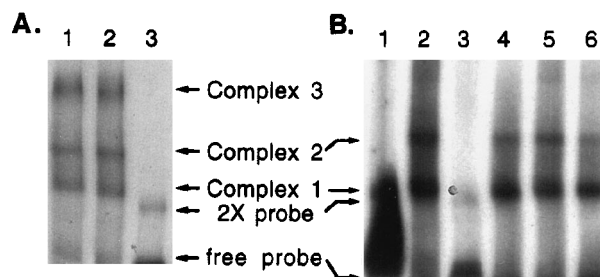


FIG. 2. Identification and specificity analysis of WNV (+)3'SL RNA-protein complexes. (A) Gel mobility shift assay of BHK S100 cytoplasmic extracts. Lanes: 1, probe plus WNV-infected BHK extract; 2, probe plus uninfected BHK extract; 3, free WNV (+)3'SL RNA probe. The position of a dimer of the WNV (+)3'SL RNA is indicated (2X probe). (B) Competitor gel shift analysis of BHK cytoplasmic extracts. Lanes: 1, free WNV (+)3'SL RNA probe; 2, RNA-protein complex formation with no competitor added; 3, complex formation in the presence of specific competitor WNV (+)3'SL RNA; 4 to 6, complex formation in the presence of nonspecific competitor D10 RNA, poly(I)-poly(C), or rubella virus (+)3'SL RNA, respectively. Each competitor (225 ng per reaction mixture) was preincubated with the cytoplasmic extract for 5 min before the addition of the WNV (+)3'SL RNA probe. Computer imagings were generated by Adobe Photoshop, version 2.5, after the X-ray films were scanned with an Agfa Arcus II flatbed scanner.

modifications. Briefly, the proteins from BHK S100 cytoplasmic extracts were separated by SDS-12% polyacrylamide gel electrophoresis (PAGE) and then electrophoretically transferred to a nitrocellulose membrane (0.22- μm pore size; Micron Separations, Inc.). The membrane was blocked with PBS containing 5% dry milk and 1 mM DTT for 1 h and then washed with $1 \times$ HBB (25 mM HEPES-KOH [pH 7.5], 25 mM NaCl, 5 mM MgCl_2 , and 1 mM DTT). Proteins were denatured and slowly renatured by consecutive washes (10 min each) with 6 (twice), 3, 1.5, 0.75, 0.375, and 0.187 M guanidine hydrochloride in $1 \times$ HBB. The membrane was washed once with $1 \times$ HBB and twice with HYB100 (20 mM HEPES-KOH [pH 7.5], 100 mM KCl, 2.5 mM MgCl_2 , 0.1 mM EDTA, 0.05% Nonidet P-40, and 1 mM DTT). ^{32}P -WNV (+)3'SL RNA in HYB100 ($\sim 10^5$ cpm/ml) was incubated with the renatured membrane-bound proteins for 1 h at 25°C and then for 6 h at 4°C . Unbound probe was removed by three washes with HYB100 followed by autoradiography at -70°C for 12 to 24 h.

Computer prediction of RNA secondary structure. The optimal secondary structures for wild-type and truncated RNA molecules were predicted by the method of Zuker and Stiegler (49) with the Fold program in the Genetics Computer Group sequence analysis software package.

RESULTS

BHK cell proteins which bind to the (+)3'SL of WNV. The gel mobility shift assay was used to identify proteins in BHK cells that bind specifically to the 3'-terminal structure of WNV genomic RNA. A 109-nt ^{32}P -labeled riboprobe, designated WNV (+)3'SL RNA, was transcribed in vitro from linearized pWNV(+3'SL DNA and incubated with BHK cytoplasmic extracts in binding buffer as described in Materials and Methods. Three distinct RNA-protein complexes were observed with extracts from both uninfected (Fig. 2A, lane 2) and WNV-infected (Fig. 2A, lane 1) BHK cells. No obvious differences were observed in the number or the electrophoretic mobilities of the RNA-protein complexes that formed with extracts from uninfected (Fig. 2A, lane 2) or WNV-infected (Fig. 2A, lane 1) BHK cells. In two other virus systems in which differences in the proteins that bound to the viral RNA probe from infected- and uninfected-cell extracts were observed, an altered gel shift pattern due to the binding of additional proteins or an increased binding affinity was observed (1, 34). Although our gel shift results do not rule out the possibility that different or additional proteins may be present in the RNA-protein complexes from WNV-infected-cell extracts, it seems unlikely, since no differences in the gel shift patterns were observed. However, since our goal was to identify cellular proteins that specifically interact with the WNV (+)3'SL RNA, we chose to

first analyze the proteins present in uninfected-cell extracts. WNV-infected-cell extracts will be analyzed further in future studies.

A higher-molecular-weight band, designated 2X probe (Fig. 2A, lane 3), was sometimes observed in the free-probe lane. This probably represents a dimer formed by the interaction between two (+)3'SL RNA molecules in the complementary regions of their stems. Subsequently, it was found that heating and quick cooling of dilute solutions of the probe eliminated this band. Therefore, the 2X band was not detected on the rest of the gels shown. No complexes were observed when cytoplasmic extracts were preincubated with proteinase K (20 μ g/ml) in the binding reaction mixture at room temperature for 15 min prior to the addition of the 32 P-WNV (+)3'SL RNA, indicating that complex formation is dependent on the presence of protein (data not shown).

Unlabeled WNV (+)3'SL RNA, used as a specific competitor, was titrated to optimize the amount of competitor required to consistently give complete inhibition of complex formation. Although 100 ng of the WNV (+)3'SL RNA completely inhibited complex 2 formation, 225 ng was required for complete inhibition of complex 1 (data not shown). The amounts of specific competitor RNA determined in these titration experiments were used in the subsequent experiments. The difference in the amount of specific competitor required to completely inhibit complexes 1 and 2 may be due to differences in the binding affinities and/or concentrations of the individual host proteins forming these two complexes.

Gel shift competitor analyses with a number of competitor RNAs were carried out to evaluate the specificity of host protein binding to the WNV (+)3'SL RNA. With the specific competitor, WNV (+)3'SL RNA, the formation of RNA-protein complexes 1 and 2 was inhibited (Fig. 2B, lane 3), whereas no inhibition of the formation of these complexes was detected when D10 RNA, poly(I)-poly(C), or rubella virus (+)3'SL RNA (Fig. 2B, lanes 4 to 6) was used as a competitor. These results indicated that the host protein(s) that forms complexes 1 and 2 specifically recognizes the WNV (+)3'SL RNA. Complex 3 formation was often not detectable in extracts that had been stored at -20°C (Fig. 2B). Competitor gel mobility shift analyses using fresh S100 extracts indicated that the formation of complex 3 is probably due to nonspecific interactions (data not shown).

Determination of the molecular masses of the WNV (+)3'SL RNA-binding proteins in BHK cytoplasmic extracts. Two techniques were used to estimate the sizes of the WNV (+)3'SL RNA-binding proteins (Fig. 3). All of the initial attempts to detect WNV RNA-binding proteins by UV-induced cross-linking with a WNV (+)3'SL RNA probe that had either one or two labeled ribonucleotides incorporated during *in vitro* synthesis were unsuccessful. Subsequently, WNV (+)3'SL RNA that was prepared with the photoactive nucleotide analog of UTP, $5\text{N}_3\text{-UTP}$, was used in UV-induced cross-linking analyses (Fig. 3A). Although this analog has been shown to have higher photoactivity, it was incorporated with kinetics similar to those of the natural substrate, UTP, in *in vitro* polymerase studies (21). When BHK cytoplasmic extracts were UV cross-linked in a binding reaction with the WNV (+)3'SL RNA probe containing the photoactive analog, two strong bands estimated to be approximately 56 and 105 kDa were detected after SDS-10% PAGE by autoradiography (Fig. 3A, lane 2).

A Northwestern blotting technique was also used to detect proteins that interact with the WNV (+)3'SL RNA. BHK cytoplasmic proteins were resolved by SDS-12% PAGE, transferred to a nitrocellulose membrane, renatured, and then probed with 32 P-WNV (+)3'SL RNA. Following autoradiog-

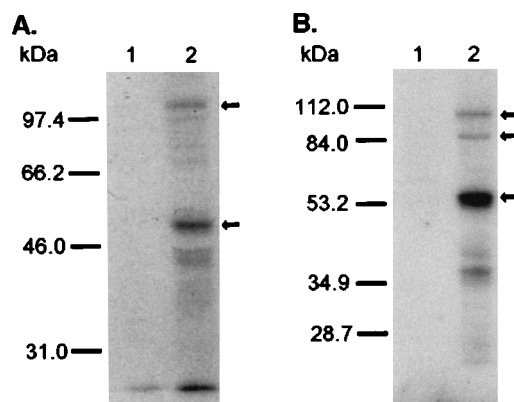


FIG. 3. Detection of BHK cell proteins that bind to the WNV (+)3'SL RNA. (A) Proteins in an S100 cytoplasmic extract were UV cross-linked to 32 P-WNV (+)3'SL RNA and analyzed by SDS-10% PAGE. Lanes 1 and 2, binding reaction mixtures not exposed and exposed to UV light, respectively. (B) Northwestern blotting analysis of WNV (+)3'SL RNA-binding proteins. BHK cytoplasmic proteins were separated by SDS-12% PAGE, transferred to a nitrocellulose membrane, denatured, renatured, and probed with 32 P-WNV (+)3'SL RNA. Lane 1, BHK cytoplasmic proteins preincubated with proteinase K (2 μ g/ml, 37°C , 15 min) prior to SDS-PAGE; lane 2, BHK cytoplasmic proteins. Computer images were generated by Adobe Photoshop, version 2.5, after the X-ray films were scanned with an Agfa Arcus II flatbed scanner. Molecular masses are indicated on the left (panel A, Bio-Rad low-range molecular mass standards; panel B, Bio-Rad prestained low-range standards).

raphy, bands at approximately 56, 84, and 105 kDa were observed (Fig. 3B, lane 2). Although other, lower-molecular-weight bands were also observed, the RNA binding activity of these bands could be selectively reduced with higher-stringency washes (i.e., three washes with HYB100 containing 0.5 M KCl; data not shown), indicating that these other proteins bound nonspecifically to the WNV (+)3'SL RNA. No bands were observed when the BHK cytoplasmic extract was preincubated with proteinase K prior to SDS-PAGE (Fig. 3B, lane 1), demonstrating that the RNA binding activity observed in this experiment was due to proteins in the BHK cytoplasmic extract.

It is unclear why the 84-kDa protein was detected by Northwestern analysis but not by UV-induced cross-linking. One possibility is that another factor(s) present in the BHK cytoplasmic extract was complexed with the 84-kDa protein, so little of the 84-kDa protein was free to be cross-linked to the WNV (+)3'SL RNA. However, after the proteins in the BHK cytoplasmic extract had been separated from these competing factors by SDS-PAGE, the 84-kDa protein was free to interact efficiently with the WNV (+)3'SL RNA. Furthermore, the results from the Northwestern analysis indicated that the 56-, 84-, and 105-kDa proteins were capable of directly interacting with the WNV (+)3'SL RNA without the presence of cofactors.

Partial purification of the WNV (+)3'SL RNA-binding proteins. As a means of further characterizing the proteins that were detected by the UV-induced cross-linking and Northwestern analyses, proteins present in the S100 cytoplasmic extract were subjected to anion-exchange chromatography using a MonoQ HR 5/5 column as described in Materials and Methods. A sample from each fraction was assayed for its binding activity with the WNV (+)3'SL RNA riboprobe in a gel mobility shift assay. Eight fractions which contained a single, predominant gel shift band were detected, and they were further assayed by competition gel shift analysis using poly(I)-poly(C) and tRNA as the nonspecific competitors and WNV (+)3'SL

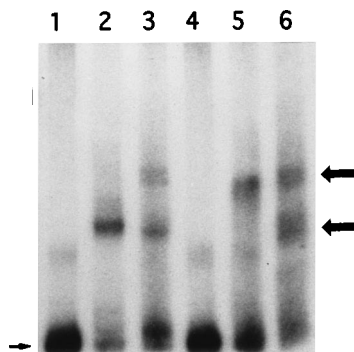


FIG. 4. Comparison of the RNA-protein complexes formed by BHK S100 cytoplasmic extract proteins with those formed by fraction 17 or fraction 19 proteins. The WNV (+)3'SL RNA was used as a probe. Lanes: 1 and 4, free probe; 2 and 5, complex formation in the presence of the fraction 19 and 17 proteins, respectively; 3 and 6, complex formation in the presence of the unfractionated cytoplasmic extract. The positions of the free probe (arrow on left), complex 2 (top arrow on right), and complex 1 (bottom arrow on right) are indicated.

RNA as the specific competitor (data not shown). Only fractions 17 and 19 contained proteins with WNV (+)3'SL RNA-specific binding activity. The electrophoretic mobilities of the complexes formed by fractions 17 and 19 were compared with those formed by the unfractionated BHK S100 cytoplasmic extract in a gel shift assay (Fig. 4). The fraction 19 complex (Fig. 4, lane 2) migrated to approximately the same position as complex 1 in the cytoplasmic extract (Fig. 4, lane 3), whereas the fraction 17 complex comigrated with complex 2 (Fig. 4, lanes 5 and 6). Only one of the two gel shift bands was detected in each of the fractions. However, because the proteins of fractions 17 and 19 eluted very closely on the NaCl gradient (i.e., 425 and 475 mM, respectively), small amounts of proteins present in one fraction could have been present in the other.

Determination of the binding specificity of the partially purified RNA-binding proteins. The proteins in fractions 17 and 19 were concentrated into storage buffer as described in Materials and Methods. Next, different amounts of WNV (+)3'SL RNA were titrated into the binding reaction mixture to determine the range of specific competitor that was required to inhibit RNA-protein complex formation with the fraction 17 and 19 proteins (data not shown). As was found with complex 2 in the unfractionated extract, at least 75 ng of specific competitor was required for complete inhibition of the fraction 17 complex. The fraction 19 complex was similar to complex 1 from the unfractionated extract in that a minimum of 175 ng of specific competitor was required for complete inhibition of complex formation. The amounts of competitor RNAs used in the following competition gel mobility shift assays were chosen on the basis of the results of these titration experiments.

The binding specificities of the fraction 17 and 19 RNA-binding proteins were more extensively analyzed by competition gel mobility shift assays (Fig. 5). No inhibition of complex formation was observed when the fraction 17 protein was incubated with the ³²P-WNV (+)3'SL RNA riboprobe in the presence of 100 ng of one of the nonspecific competitors, D10 RNA, poly(I)-poly(C), or rubella virus (+)3'SL RNA (Fig. 5A, lanes 4 to 6). In contrast, complete inhibition of complex formation was observed when the same amount of specific competitor, WNV (+)3'SL RNA, was used (Fig. 5A, lane 3). Since the probe was not in excess in the fraction 17 binding reactions the results of which are shown in Fig. 5A, all of the probe was bound and shifted. Fraction 19 was also assayed by competition

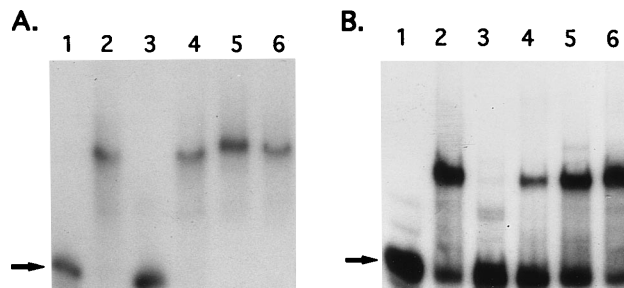


FIG. 5. Competitor gel shift analysis of partially purified host cell proteins in fraction 17 (A) and fraction 19 (B) to assess their binding specificities. Lanes: 1, free WNV (+)3'SL RNA probe (arrow); 2, complex formation with no competitor added; 3, complex formation in the presence of specific competitor WNV (+)3'SL RNA; 4 to 6, complex formation in the presence of nonspecific competitor D10 RNA, poly(I)-poly(C), or rubella virus (+)3'SL RNA, respectively. Fraction 17 and 19 reaction mixtures contained 100 and 225 ng of competitor RNA, respectively.

gel shift analysis, except that 225 ng of each competitor was used in the binding reaction. Fraction 19 complex formation was found to be inhibited only by the specific competitor (Fig. 5B, lane 3) and not by the nonspecific competitors (Fig. 5B, lanes 4 to 6). The binding characteristics of the complexes formed by the proteins in fractions 17 and 19 correlated with those of complexes 1 and 2 in the S100 cytoplasmic extracts (Fig. 2).

Strand specificity of the cell proteins. The 3'-terminal sequence of the WNV minus-strand RNA has also been predicted to form an SL structure (9). Although this structure is also conserved among flaviviruses, it is not as stable as the WNV (+)3'SL (10). The 3'-terminal position of the minus-polarity SL suggests that it may play a role in the initiation of plus-strand RNA synthesis. Therefore, the abilities of the fraction 17 and 19 proteins to interact with the WNV (-)3'SL RNA were tested in a gel mobility shift assay in which the WNV (+)3'SL RNA was used as the probe and the WNV (-)3'SL RNA was used as a competitor. The competitor WNV (-)3'SL RNA was added to the fraction 17 assays at 100 ng and to the fraction 19 assays at 225 ng. Fraction 19 complex formation, but not fraction 17 complex formation, was inhibited by the WNV (-)3'SL RNA competitor (data not shown).

Additional competition gel mobility shift assays were performed to compare the affinity of the fraction 19 proteins for the WNV (+)3'SL RNA with their affinity for the WNV (-)3'SL RNA in more detail. For these experiments, the WNV (+)3'SL RNA was used as the probe and either WNV (+)3'SL or WNV (-)3'SL RNA was used as the competitor. Prior to these experiments, very small amounts of the WNV (+)3'SL RNA were titrated into the fraction 19 binding reaction mixtures. Over the ranges of 5 to 50, 60 to 75, and 80 to 100 ng of WNV (+)3'SL RNA, complex formation was inhibited by approximately 25, 50, and 100%, respectively, compared with complex formation in the absence of competitor (data not shown).

Various amounts (6.25, 12.5, 25, or 50 ng) of the WNV (+)3'SL RNA competitor, each of which inhibited complex formation by only 25% or less, were then used in the following competition gel shift assays. Unlabeled WNV (-)3'SL RNA competitor was also used in the binding reactions in the same amounts. The degree of inhibition of fraction 19 complex formation was determined by densitometric quantitation (Fig. 6). Over this range of competitor concentrations, the WNV (-)3'SL RNA inhibited fraction 19 complex formation ap-

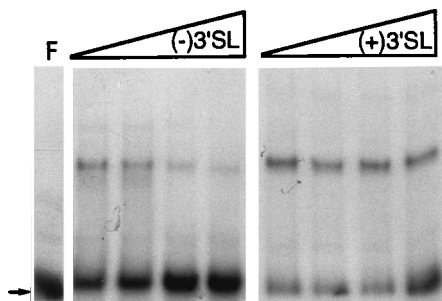


FIG. 6. Inhibition of fraction 19 complex formation by WNV (+)3'SL and (-)3'SL RNAs. Competition mobility shift assays were performed in the presence of increasing amounts of the competitor RNAs (from left to right, as indicated above the lanes): 6.25, 12.5, 25, and 50 ng. On the basis of previous experiments (see text), these small amounts of specific competitor had been determined to inhibit complex formation by 25% or less. Free WNV (+)3'SL RNA in lane F is indicated (arrow). The amounts of both unbound RNA and RNA-protein complex were densitometrically quantified as described in Materials and Methods.

proximately 4.5 times more efficiently than did the WNV (+)3'SL RNA.

Preliminary mapping of the protein binding sites on the WNV (+)3'SL RNA. In order to begin to define the regions on the WNV (+)3'SL RNA recognized by each of the cell proteins, a truncated version of the full-length WNV (+)3'SL RNA, designated WNV (+)3'SL-trunc1 RNA, was synthesized. This RNA had the top half of the SL structure deleted and four U residues added to allow a new top loop to form as shown in Fig. 7C. This truncated WNV RNA and the full-length WNV (+)3'SL RNA were used as competitors in gel shift assays with the full-length WNV (+)3'SL probe (Fig. 7A and B). Equimolar amounts of the two competitor RNAs were used. The WNV (+)3'SL-trunc1 RNA (Fig. 7A, lane 5) and the full-length WNV (+)3'SL RNA (Fig. 7A, lane 4) inhibited fraction 17 complex formation to similar extents. The amounts of inhibition caused by these two competitors were determined by densitometric quantitation. Relative to the reaction with no

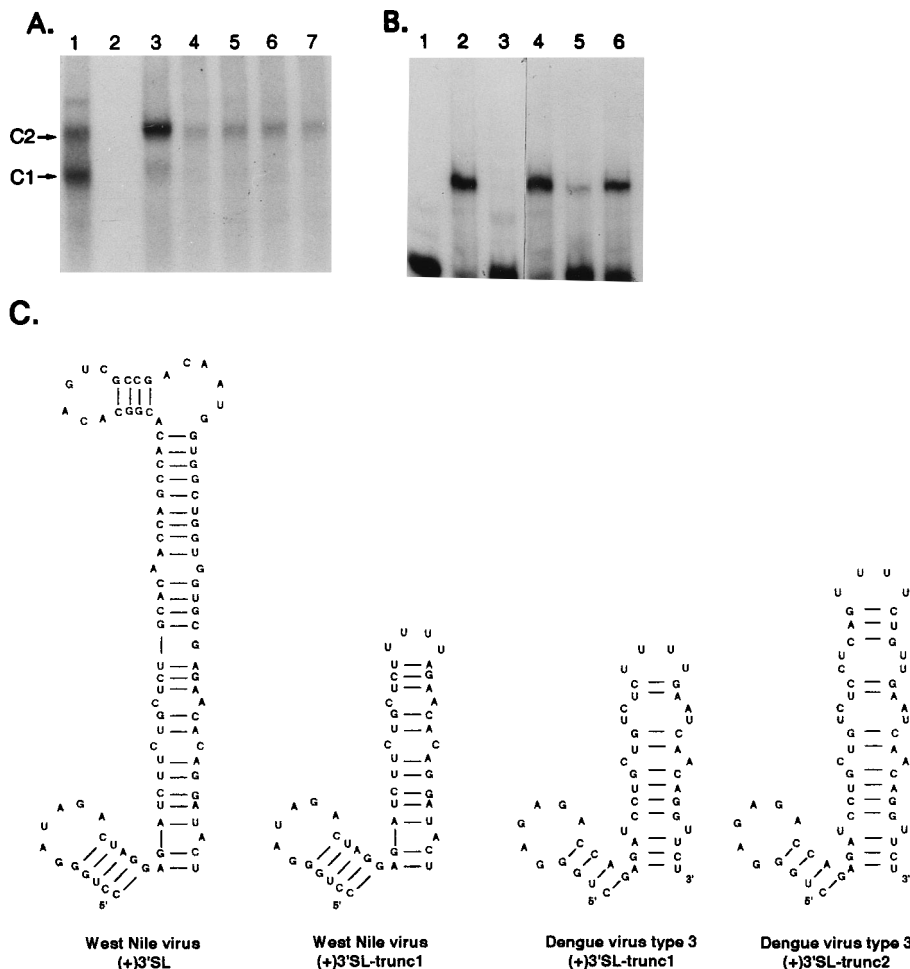


FIG. 7. Preliminary mapping of the fraction 17 and 19 binding regions on the WNV (+)3'SL RNA and analysis of the flavivirus specificity of the partially purified WNV RNA-binding proteins. (A) Analysis of the fraction 17 protein(s). Lanes: 1, RNA-protein complexes 1 (C1) and 2 (C2) in unfractionated BHK cytoplasmic extracts; 2, free probe (not visible in this lane because it was electrophoresed off the gel); 3, fraction 17 complex formation in the absence of competitor RNA; 4 to 7, fraction 17 complex formation in the presence of 1.8×10^{-2} pmol of WNV (+)3'SL RNA, WNV (+)3'SL-trunc1 RNA, Den3 (+)3'SL-trunc2 RNA, or Den3 (+)3'SL-trunc1 RNA, respectively. (B) Analysis of the fraction 19 protein(s). Lanes: 1, free probe; 2, fraction 19 complex formation in the absence of competitor RNA; 3 to 6, fraction 19 complex formation in the presence of 7.5×10^{-2} pmol of WNV (+)3'SL RNA, WNV (+)3'SL-trunc1 RNA, Den3 (+)3'SL-trunc2 RNA, or Den3 (+)3'SL-trunc1 RNA, respectively. (C) Computer-predicted secondary structures of WNV and Den3 truncated (+)3'SL RNAs that were utilized in the mobility shift assays. The full-length WNV (+)3'SL RNA is shown on the left. The WNV (+)3'SL-trunc1 and Den3 (+)3'SL-trunc1 RNAs are truncated at approximately the same positions. Den3 (+)3'SL-trunc2 is 5 bp longer than the trunc1 RNAs. Four uridine (U) residues were added to each of the truncated RNAs to allow a new loop to form at the top of the truncated stems.

competitor RNA (Fig. 7A, lane 3), the WNV (+)3'SL-trunc1 RNA and the WNV (+)3'SL RNA inhibited complex formation by 77.9 and 85.5%, respectively. The efficient inhibition of fraction 17 complex formation by the WNV (+)3'SL-trunc1 RNA indicates that this RNA contains the fraction 17 protein binding site.

Competitor gel shift analysis of the fraction 19 RNA-binding protein indicated that the WNV (+)3'SL-trunc1 RNA had no effect on complex formation (Fig. 7B, lane 4), whereas the same amount of specific competitor completely inhibited fraction 19 complex formation (Fig. 7B, lane 3). These results suggest that the WNV (+)3'SL-trunc1 RNA does not contain the binding site(s) for the fraction 19 protein. Therefore, the critical binding region(s) for this protein must be located in the top portion of the SL structure, which was deleted in the WNV (+)3'SL-trunc1 RNA.

Flavivirus binding specificity of the cell proteins. Two truncated (+)3'SL RNAs (Fig. 7C) from Den3 that had been produced for ongoing biophysical studies in our laboratory were also used as competitors in gel shift analyses to test the flavivirus specificity of the fraction 17 and fraction 19 RNA-binding proteins. Like the WNV (+)3'SL-trunc1 RNA, these two truncated Den3 (+)3'SL RNAs are identical to the wild-type 3' SL structure except that various amounts of the top half of the 3'-terminal SL were deleted and four U residues were added to form a new top loop. The Den3 (+)3'SL-trunc1 RNA was deleted at the same location as the WNV (+)3'SL-trunc1 RNA, whereas the Den3 (+)3'SL-trunc2 RNA included an additional 5 bp of the wild-type Den3 (+)3'SL stem region (Fig. 7C).

The truncated Den3 (+)3'SL RNAs were separately assessed for their effects on fraction 17 complex formation in a competition gel shift assay. Equimolar amounts of each competitor were used. The degree of inhibition observed with the two truncated Den3 RNAs (Fig. 7A, lanes 6 and 7) was similar to that observed with the full-length and truncated WNV RNA competitors (Fig. 7A, lanes 4 and 5). Densitometric quantitation showed that the Den3 (+)3'SL-trunc1 and Den3 (+)3'SL-trunc2 RNAs inhibited complex formation by 86.0 and 74.8%, respectively, relative to the amount of complex formed when no competitor was present (Fig. 7A, lane 3). These results indicate that fraction 17 complex formation is flavivirus specific, since the WNV and the divergent Den3 3'-terminal SL structures inhibit complex formation with approximately equal efficiencies.

The truncated Den3 (+)3'SL RNAs were also used as competitors in binding reactions with the fraction 19 protein (Fig. 7B). The shorter Den3 (+)3'SL-trunc1 RNA had little effect on complex formation (Fig. 7B, lane 6); however, the slightly longer Den3 (+)3'SL-trunc2 RNA strongly inhibited fraction 19 complex formation (Fig. 7B, lane 5). Densitometric quantitation showed that the Den3 (+)3'SL-trunc2 RNA inhibited fraction 19 complex formation by 84.5%. These results suggest that the additional 5 bp of the stem region in the Den3 (+)3'SL-trunc2 RNA contains at least one of the contact sites utilized by the fraction 19 protein(s) for binding to the flavivirus (+)3'SL RNA.

Determination of the molecular masses of the partially purified RNA-binding proteins. UV-induced cross-linking was performed to determine the molecular masses of the proteins that specifically bind to the WNV (+)3'SL RNA. SDS-PAGE of the UV cross-linked products identified bands with molecular masses of approximately 84 and 105 kDa in samples of fractions 17 and 19, respectively (Fig. 8A and B, respectively). In addition, four RNase-resistant bands that migrated to the position of the 31-kDa marker or below it were routinely de-

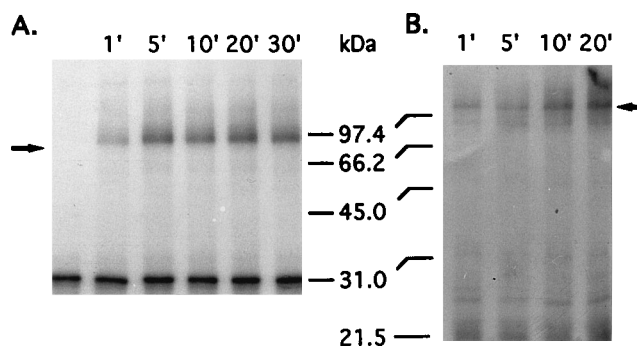


FIG. 8. Detection of proteins that bind to the WNV (+)3'SL RNA in fast protein liquid chromatography fractions 17 and 19. Proteins in fraction 17 (A) or fraction 19 (B) were UV cross-linked to ^{32}P -WNV (+)3'SL RNA, analyzed by SDS-10% PAGE, and detected by autoradiography. The length of time (in minutes) that the samples were exposed to UV light is indicated above each lane. The first lane in panel A contains only free probe. The arrows indicate the predominant cross-linked product. The electrophoretic mobilities and molecular masses of the protein standards (Bio-Rad, low range) are indicated.

tected (Fig. 8). However, the 84- and 105-kDa proteins were the preferred cross-linked products, since the intensity of only these two cross-linked proteins increased as the time of irradiation was extended (Fig. 8). In the fraction 19 samples, an additional band that could be some copurified 84-kDa protein was observed below the 105-kDa band.

It is worth noting that for these UV-induced cross-linking analyses the normal ribonucleotide, UTP, was used instead of the photoactive analog, 5N_3 -UTP. The ability to UV cross-link the fractionated RNA-binding proteins without the aid of 5N_3 -UTP suggests that the fractionation procedure enriched for the 84- and 105-kDa proteins and/or removed other proteins or RNAs that interfered with the ability of the 84- and 105-kDa proteins in unfractionated extracts to interact efficiently with the WNV (+)3'SL RNA.

A heterogeneous mixture of proteins was observed when the fraction 17 and 19 proteins were silver stained after SDS-PAGE. However, neither the 84-kDa nor the 105-kDa protein could be detected by silver staining. Although the total protein concentrations for fractions 17 and 19 were 0.6 and 1.3 $\mu\text{g}/\mu\text{l}$, respectively, the 84- and 105-kDa proteins detected by UV cross-linking were minor components of their respective fractions.

The 56-kDa protein that was detected by UV-induced cross-linking and Northwestern analyses of the BHK cytoplasmic extract could not be detected in either fraction 17 or fraction 19. Further study of the 56-kDa protein is under way.

The electrophoretic mobilities of the fraction 17 and 19 RNA-protein complexes did not correlate with the estimated molecular masses of the UV cross-linked proteins that formed them, since the smaller (84-kDa) protein formed the complex with the slower mobility and the larger (105-kDa) protein formed the complex with the faster mobility. Similar discrepancies have been observed with other nucleic acid-protein complexes (27) and have been attributed to such factors as the globular characteristics of the protein, the charge of the protein, the oligomerization state of the protein, and the conformational changes in the nucleic acid due to interaction with a protein.

DISCUSSION

We have demonstrated that BHK cytoplasmic proteins bind specifically to the 3'-terminal SL structures of the genomic

RNAs of both WNV and Den3. UV-induced cross-linking and Northwestern blot analyses of BHK cytoplasmic extracts showed that the sizes of the 3'SL RNA-binding proteins are 56, 84, and 105 kDa. The 84- and 105-kDa proteins, but not the 56-kDa protein, were subsequently reidentified when the BHK cytoplasmic extract was fractionated by ion-exchange chromatography. It is unclear why the 56-kDa protein could not be detected following partial purification by liquid chromatography, but the ability to efficiently detect the 56-kDa protein by UV-induced cross-linking and Northwestern blotting analyses (Fig. 3A and B, lanes 2) makes it an attractive candidate for future studies.

The WNV and Den3 (+)3'SL RNAs can be folded into very similar secondary structures (Fig. 7C) (10, 31), even though the sequences of these two RNAs show only a 42% nucleotide identity. The divergence of the Den3 and WNV (+)3'SL RNA sequences suggests that the conserved structural elements play an important role in the recognition of these RNAs by the 84- and 105-kDa cellular proteins. Initial mapping studies of the binding sites for the two proteins indicated that these two proteins may bind at different sites on the flavivirus (+)3'SL RNA. A possible contact site for the 105-kDa protein was detected in a base-paired region located just above the center of the first 3' SL.

The second 3' SL (Fig. 7C) was included in the (+)3'SL RNA probe used in this study because the sequence in this region is relatively well conserved among flaviviruses (10, 11) and because preliminary structure probing data show that it is possible for the loop of this second SL structure to form a tertiary structure with nucleotides on the back side of the first SL (45b). Modeling studies have also shown that such a tertiary structure is thermodynamically feasible. Additional mapping studies using various deleted and substituted WNV (+)3'SL RNAs are under way to further define the RNA structural and sequence elements that are critical for the binding of each of the two cell proteins.

Under natural conditions, as well as in the laboratory, the host range of flaviviruses is very broad. Flaviviruses can replicate efficiently in mammalian, avian, insect, reptile, and amphibian hosts as well as in cell cultures from these various hosts (8). If the 84- and 105-kDa proteins found in BHK cells provide functions which are critical to flavivirus RNA replication, it would be expected that these proteins would be highly conserved and therefore present in the cells of all species that are susceptible to flavivirus replication. Studies are under way to test this hypothesis.

Although a number of other investigators have utilized binding assays to probe for cellular proteins which specifically recognize viral RNA promoter sequences as a means of studying the mechanism of transcription and asymmetric production of plus and minus RNA strands (19, 28, 33, 34, 36, 37), in all cases the functional contribution of these viral RNA-binding proteins to viral replication remains to be demonstrated. The identities of a few of the cell proteins which have been shown to bind specifically to particular viral RNAs or to form complexes with viral polymerase proteins have been discovered. The three cellular proteins found in the Q β replication complex were identified as S1 ribosomal protein and translation elongation factors EF-Tu and EF-Ts (6, 26). One of the proteins binding specifically to the 3' NCR of rubella virus RNA has been identified as calreticulin (3). Mapping studies carried out with the calreticulin protein have shown that the rubella virus RNA binding domain in this protein does not overlap either of the two functional domains that provide the known cell activities (3).

In only a few instances (4, 22, 26, 35) has it been possible to

directly demonstrate viral replication complexes composed of cellular and viral proteins. This may indicate that for the majority of RNA viruses the initial interaction of the cellular proteins may be with the viral template RNA rather than with the viral polymerase. This initial RNA-protein interaction may result in conformational changes in both the viral RNA and the cellular proteins. It is possible that some of the viral RNA-dependent RNA polymerases can recognize their templates only after the templates have first bound to cellular proteins.

Although the functional role of the fraction 19 RNA-binding protein (i.e., the 105-kDa protein) remains to be determined, the finding that it recognizes both the WNV (+)3'SL and the (-)3'SL RNAs suggests that this protein may play a role in the initiation of transcription from both the flavivirus plus- and minus-strand RNA templates. Similarly, it has been reported that one of the rubella virus RNA-binding proteins interacts with both minus- and plus-strand 3' SL structures (34). In contrast, the 84-kDa protein binds only to the WNV (+)3'SL RNA. Preliminary evidence indicates that there are additional BHK cell proteins which bind only to the (-)3'SL RNA (45a). In infected cells, the level of flavivirus plus-strand RNA synthesis is 10 to 100 times greater than that of minus-strand synthesis (12, 46). The use of different sets of cell proteins for the initiation of transcription from the two viral template RNAs could contribute to the differential regulation of the rates of RNA transcription from these two templates. The 105-kDa protein, which binds to both the WNV 3' plus- and minus-strand RNAs, was shown to have a higher affinity for the WNV (-)3'SL RNA than for the WNV (+)3'SL RNA. It is possible that this difference in protein binding affinity could also play a role in regulating viral RNA transcription rates. However, because of the requirement for reversibility in the binding of proteins involved in RNA transcription initiation, the effect of a higher-affinity interaction may be to reduce rather than stimulate transcription rates. This was shown to be the case for a mutant Sindbis virus RNA which interacted with a cellular protein with a greater affinity than did the wild-type viral RNA (37). The significantly greater stability of the flavivirus (+)3'SL RNA compared with the flavivirus (-)3'SL RNA may also be an important, if not the most important, factor in regulating the rate of flavivirus RNA transcription.

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