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RNA-Protein Interactions: Involvement of NS3, NS5, and 3′ Noncoding Regions of Japanese Encephalitis Virus Genomic RNA

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The mechanism of replication of the flavivirus Japanese encephalitis virus (JEV) is not well known. The structures at the 3′ end of the viral genome 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. UV cross-linking experiments were performed to identify the proteins that bind with the JEV plus-strand 3′ noncoding region (NCR). Two proteins, p71 and p110, from JEV-infected but not from uninfected cell extracts were shown to bind specifically to the plus-strand 3′ NCR. The quantities of these binding proteins increased during the course of JEV infection and correlated with the levels of JEV RNA synthesis in cell extracts. UV cross-linking coupled with Western blot and immunoprecipitation analysis showed that the p110 and p71 proteins were JEV NS5 and NS3, respectively, which are proposed as components of the RNA replicase. The putative stem-loop structure present within the plus-strand 3′ NCR was required for the binding of these proteins. Furthermore, both proteins could interact with each other and form a protein-protein complex in vivo. These findings suggest that the 3′ NCR of JEV genomic RNA may form a replication complex together with NS3 and NS5; this complex may be involved in JEV minus-strand RNA synthesis.

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* of the family *Flaviviridae* and contains an ~11-kb single-stranded positive-sense RNA genome. The genome has a type 1 cap at its 5′ end but lacks a poly(A) tail at its 3′ end (64). It serves as the only viral mRNA and encodes a single open reading frame of about 10 kb (55). A large polyprotein is translated from this open reading frame, which is subsequently processed by both host and viral proteases into three structural (C, pre-M, and E) and seven nonstructural (NS1 to NS5) proteins (11). The functions of the seven nonstructural proteins are not currently well understood (55). Both NS5 and NS3 proteins are likely to be components of the viral RNA replicase involved in RNA replication (2, 55). The coding region of the genome is flanked by 5′ and 3′ noncoding regions (NCRs) that are 95 and 585 nucleotides (nt) long, respectively.

The RNA replication of a positive- or negative-strand RNA virus is a consequence of specific RNA-RNA, RNA-protein, and protein-protein interactions. RNA-RNA interactions manifest themselves both in intermolecular forms such as base complementarity in the replicative-intermediate and replication-form RNAs and in intramolecular RNA secondary structures. The role of RNA-protein and protein-protein interactions involved in RNA virus replication has not been well defined. These interactions may play important roles in viral RNA and protein synthesis and also viral assembly (12–14, 17, 20, 38, 40, 44).

Evidence for specific binding of cellular proteins to either cellular or viral RNAs has been obtained for a number of systems (41). While in some cases proteins recognize RNA in a sequence-specific manner (20, 41), in other cases the specificity of the protein binding depends on the RNA secondary or tertiary structures (21, 24, 31, 46, 47, 57).

Data indicating that host proteins are components of the RNA virus replicase complex have been reported for phage Qβ, influenza virus, Sindbis virus, and cucumber mosaic virus (3, 30, 37, 48). Viral gene products, including RNA-dependent RNA polymerase, are also important for viral replication (17, 19, 35). Conceivably, both cellular and viral proteins are involved in the synthesis of both plus- and minus-strand RNAs.

It has been suggested that JEV genomic RNA has a specific secondary structure at the 3′ end and that this structure is highly conserved among divergent flaviviruses (7, 8, 55, 60, 62). Both sequence and structural elements present within the 3′ end of RNA may function as *cis*-acting signals for the initiation of viral RNA synthesis and may play a role in the regulation of viral RNA synthesis; however, this potential function has not been demonstrated directly.

In this study, we used a UV-induced cross-linking assay to investigate RNA-protein interactions at the 3′ end of JEV genomic RNA. Two proteins from JEV-infected cell extracts were found to bind specifically to the JEV plus-strand 3′ NCR. We further demonstrated that these proteins were the viral nonstructural proteins NS5 and NS3. In addition, both proteins could bind to each other and form protein-protein complexes. These results suggest that the plus-strand 3′ NCR can form a complex with the viral replicase proteins, NS3 and NS5, which may be involved in the initiation of flavivirus RNA synthesis.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were used as the source of uninfected and infected cytoplasmic extracts. Confluent monolayers of BHK cells in a T-75 flask were infected with JEV (Taiwanese strain, JEV NT113, graciously donated by the National Institute of Preventive Medicine, Department of Health) at a multiplicity of infection of 5, and the cell extracts were prepared at 24 h or various other times after infection.

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Preparation of cell extracts. Cell extracts were prepared as described previously (15). Briefly, the cells were pelleted by centrifugation and resuspended in 10 mM Tris-HCl (pH 8.0)–10 mM sodium acetate at 2.5×10^7 cells/ml. The resultant cell suspensions were disrupted by 20 passages through a 21-gauge needle followed by 20 through a 27-gauge needle, and aliquots were stored at −70°C. For experiments, frozen aliquots were thawed and centrifuged at 800 × *g* for 7 min, and the supernatants were used for all of the assays.

In vitro RNA-dependent RNA polymerase assay. The RNA-dependent RNA polymerase activity in the cell extract was assayed as described previously (15). In brief, the assay was carried out in a 50-μl reaction volume containing 50 mM Tris-HCl (pH 8.0), 10 mM magnesium acetate, 7.5 mM potassium acetate, 10 mM 2-mercaptoethanol, 6 μg of actinomycin D, 5 mM phosphoenolpyruvate, 3 U of pyruvate kinase per μl, 0.5 mM each ATP, CTP, GTP, and UTP, 5 μCi of [α-^{32}P]UTP (Amersham), 20 U of RNasin, and 300 μg of cell extract. The reaction was stopped after 2 h at 37°C by the addition of EDTA at a final concentration of 10 mM. An equal volume of TNE-SDS (50 mM Tris-acetate [pH 7.6], 0.1 M sodium acetate, 1 mM EDTA, 2% sodium dodecyl sulfate [SDS]) was added to disrupt the membranes. The RNAs were then extracted with phenol-chloroform and precipitated with 2-propanol.

Construction of plasmids. A cDNA fragment obtained from reverse transcription-PCR amplification with RNA isolated from a JEV-infected cell extract as a template and primer pairs, including one synthetic oligonucleotide, 10392-TAG TGTGATTTAAAGTAGAAAAG-10414, and another complementary oligonucleotide with an extra extension containing a *Sal*I cutting site immediately after the 3′ terminus of the JEV genome, aaatctagagtcgacAGATCCTGTGTTCTTC CTCA-10957 (the nucleotides in lowercase letters are the extension sequences, the underlined nucleotides denote the *Sal*I site, and the nucleotides in capital letters represent the JEV sequences), was subcloned into pGEM-3Zf(+) (Promega) at *Sma*I and *Sal*I sites. The resulting recombinant plasmid, p5-3, under the control of the T7 promoter, was able to synthesize 585 nucleotides of the JEV 3′ genomic RNA. Plasmid p5-3 was cut with *Hpa*I and *Sac*I and treated with T4 DNA polymerase before self-ligation to generate plasmid p3NCR, which could synthesize the JEV 3′-terminal 442 nt in vitro. Plasmid p3NCR was digested with *Eco*RI and *Bgl*II and treated with Klenow fragment before self-ligation. The resultant plasmid, p3SL, could transcribe the 3′-terminal 83 residues of the JEV genome. Two other plasmids were also constructed by PCR amplification and used to synthesize RNA-256 (nt 10392 to 10647) and RNA-429 (nt 10392 to 10820).

Preparation of RNA transcripts. To synthesize RNA in vitro, purified plasmid DNA was first linearized. Plasmid p5-3 was cut with *Hpa*I and used to transcribe a JEV 3′ RNA fragment, RNA-143 (nt 10392 to 10534). RNA-362 (nt 10535 to 10897) was generated by cutting plasmid p3NCR with *Bgl*II. A radiolabeled RNA probe was produced by in vitro transcription with T7 or Sp6 RNA polymerase (Promega) in a 20-μl reaction volume containing 40 mM Tris-HCl (pH 7.9), 6 mM magnesium chloride, 10 mM sodium chloride, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM nucleoside triphosphates (ATP, CTP, and GTP), 12 μM UTP, 50 μCi of [α-^{32}P]UTP (Amersham), 1 μg of linearized DNA template, and 20 U of RNasin. UTP (0.5 mM) was substituted for the radiolabeled nucleotides for synthesis of nonlabeled RNA. The reaction mixtures were incubated for 2 h at 37°C. RNase-free DNase (20 U; Promega) was added, the incubation was continued for 10 min at 37°C, and the mixture was extracted with phenol and precipitated with 2-propanol.

RNase protection assay. An RNase protection assay was carried out as specified by the manufacturer (Boehringer Mannheim). Briefly, two radiolabeled riboprobes (minus probe for detection of the plus strand [nt 10647 to 10534]; plus probe for detection of the minus strand [nt 1 to 118]) were annealed to RNAs derived from an in vitro RNA-dependent RNA reaction. Annealing was carried out for 6 h at 45°C in a buffer containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 400 mM sodium chloride, 1 mM EDTA, and 80% deionized formamide. The reaction mixture was further treated by RNase A and RNase T_1 digestion followed by proteinase K digestion. The products were precipitated and analyzed in 6% polyacrylamide–8 M urea gels.

UV cross-linking assay. UV cross-linking was performed essentially as described in reference 66, with some minor modifications. Briefly, the cytoplasmic extract was first mixed with nonlabeled competitor RNAs (if needed in competition experiments) in 20 μl of the binding buffer containing 10 mM HEPES (pH 7.6), 0.3 mM magnesium chloride, 40 mM potassium chloride, 1 mM dithiothreitol, 5% glycerol, 20 μg of tRNA, and 40 U RNasin and incubated at 30°C for 10 min. Subsequently, 0.25 pmol of [^{32}P]RNA was added, and the reaction mixture incubated for an additional 30 min. The reaction mixture was then placed on ice and irradiated with UV light (254 nm) at a 3-cm distance for 30 min. After irradiation, RNase A was added to a final concentration of 1 mg/ml, and the mixture was incubated for 30 min at 37°C and then separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide). The results were analyzed by autoradiography or, alternatively, with a Bas-1500 bio-imaging analyzer (Fuji Photo Film Co.).

Immunoblot analysis. Proteins were separated by SDS-PAGE (8% polyacrylamide), transferred to an Immobilon-P transfer membrane (Millipore), and processed as described previously (13). Filters were probed first with mouse polyclonal antibody against the viral protein (1:2,000), and the bound antibodies were detected by alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin.

Immunoprecipitation. Protein A-agarose beads were washed with binding buffer containing 100 mM sodium chloride, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.05% SDS and then incubated with antibody for 1 h at room temperature. After removal of the unbound antibodies, protein mixtures were added with gentle shaking and incubated for an additional 4 h at room temperature. Immunoprecipitates were washed twice with binding buffer and then three times with washing buffer containing 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.6), and 0.1 mM EDTA, and the immunoprecipitated proteins were separated on SDS-polyacrylamide gels.

RESULTS

Kinetics of viral RNA synthesis. To characterize the requirements for viral RNA synthesis, we first determined the kinetics of viral RNA synthesis with an in vitro replication system (10, 15, 16, 28, 54, 61, 67). JEV-infected BHK-21 cells were harvested at various time points postinfection (p.i.), and lysates were prepared as described in Materials and Methods. RNA synthesis was then carried out with the endogenous RNA template and proteins. The radiolabeled RNA products were separated by agarose gel electrophoresis (1% agarose). Low levels of RNA synthesis were detected as early as 6 h p.i., and these levels increased markedly until they peaked at 24 h p.i. (Fig. 1A). The identities of these in vitro-labeled RNAs were confirmed to be JEV specific by an RNase protection assay, and the majority of them were found to be plus-strand RNA, whereas minus-strand RNA was hardly detectable (Fig. 1B). These results showed that the lysate contained all the essential factors including RNA for viral RNA synthesis.

Detection of proteins binding to the 3′ NCR of JEV RNA. Next, we tried to identify any cellular and/or viral proteins involved in viral RNA synthesis in the replication-competent cellular lysates. Since viral RNA replication is expected to start with minus-strand RNA synthesis from the 3′ end of genomic RNA, we expected that a replication complex would be formed at the 3′ end of viral RNA. We thus attempted to detect proteins bound to the 3′ end of RNA by using UV cross-linking.

For this purpose, a 585-nt ^{32}P-labeled riboprobe, designated plus-strand 3′ NCR, was used to probe the specific binding proteins. When cell extracts derived from JEV-infected cells or control cells at different time points p.i. were UV cross-linked in a binding buffer, several radiolabeled protein species were detected, some of which were seen only in virus-infected cell lysates (Fig. 1C). Interestingly, two distinct bands, with molecular masses estimated to be approximately 110 and 71 kDa (designated p110 and p71, respectively), appeared in infected cell extracts but not in uninfected cell extract, and their amounts increased as the infection progressed (Fig. 1C). Another finding was that in some cell extract preparations, the amounts of some of the cellular proteins were significantly reduced as the infection progressed (Fig. 1D). It seems likely that JEV infection affects cellular protein synthesis. The kinetics of the appearance of the bound proteins paralleled the activities of the RNA synthesis in the extracts (Fig. 1A and C). This result suggests that the bound proteins, p71 and p110, may be involved in RNA synthesis.

Characterization of the binding specificity of the RNA-binding proteins. The specificity of the proteins cross-linked to the plus-strand 3′ NCR was further characterized. The amounts of the proteins bound increased when an increasing amount of lysate was used (Fig. 2, lanes 1 to 3). The binding specificity was then tested by using unlabeled 3′-NCR RNA and unrelated RNA sequences as specific and nonspecific competitors, respectively. Since the transcripts used in this study contain parts of the pGEM-3Zf(+) sequence, the transcript from this vector was selected as a nonspecific competitor. The two major ra-

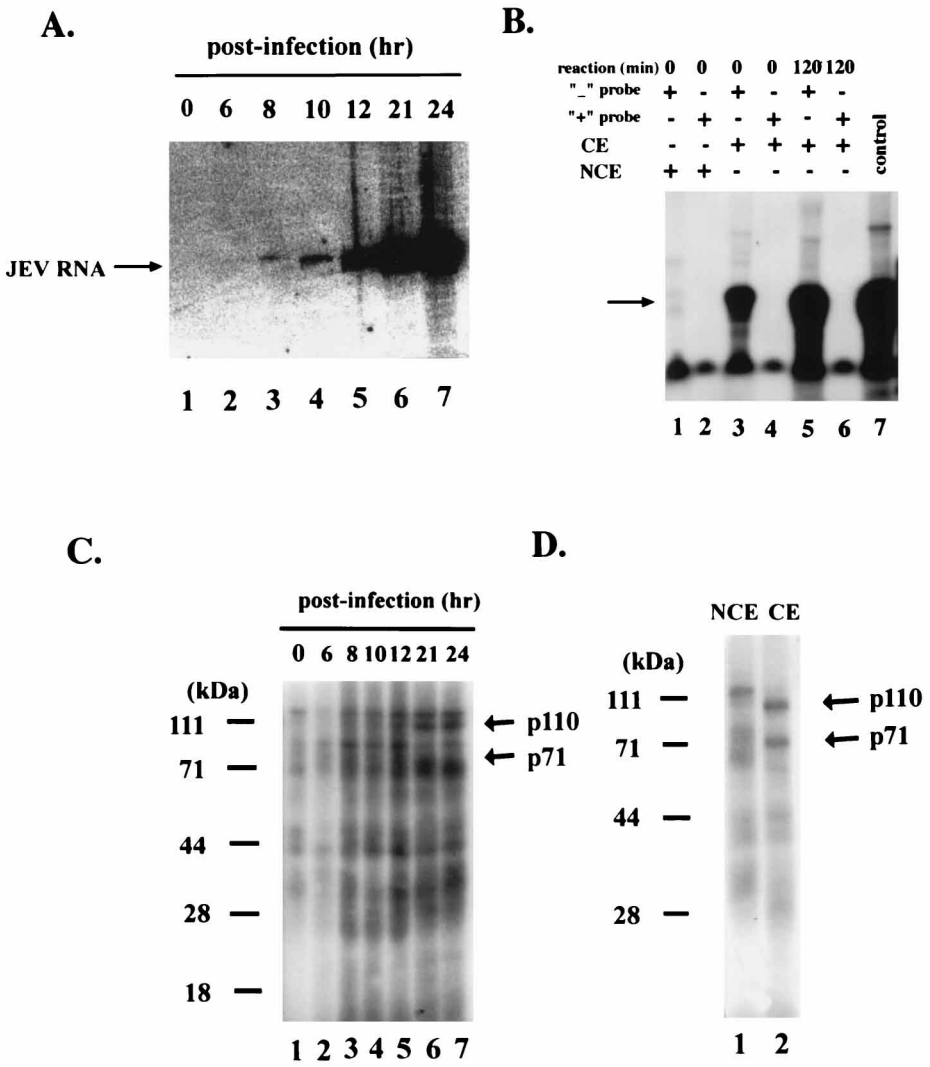

FIG. 1. UV cross-linked proteins that interact with the JEV plus-strand 3′ NCR during viral RNA synthesis. (A) Analysis of JEV RNA synthesized by an in vitro RNA-dependent RNA polymerase assay in the course of viral infection. Cell extracts were prepared from cells infected with JEV NT113 for various times (0, 6, 8, 10, 12, 21, and 24 h) and subjected to in vitro RNA synthesis reactions in the presence of [α-^{32}P]UTP, with the RNA products being separated by agarose gel electrophoresis (1% agarose) followed by autoradiography. (B) Analysis of RNA products by RNase protection. The detailed procedure is described in Materials and Methods. Probes (− for detection of plus strand [lanes 1, 3, and 5]; + for detection of minus strand [lanes 2, 4, and 6]) were used to determine the polarity of RNAs harvested at various reaction times (0 min [lanes 1 to 4] and 120 min [lanes 5 and 6]). CE represents the JEV-infected cell extract (lanes 3 to 6), and NCE represents the uninfected cell extract (lanes 1 and 2). The RNA size marker (lane 7) and protected species are marked by an arrow. (C) Identification of RNA-binding proteins by UV cross-linking. The cell extracts as described in panel A were mixed with the radiolabeled JEV plus-strand 3′ NCR (0.25 pmol), subjected to UV irradiation and RNase A digestion, and finally analyzed by SDS-PAGE (10% polyacrylamide). The autoradiographic results are also shown. The distinct proteins formed only in the infected cells and bound to the JEV plus-strand 3′ NCR are marked by arrows. (D) Uninfected (lane 1) and infected (24 h p.i.) (lane 2) cell extracts obtained from other preparations were analyzed by a UV cross-linking assay.

diolabeled species, p71 and p110, were almost completely competed out by a 100-fold excess of the unlabeled 3′-NCR RNA (lane 6). In contrast, the binding of these two proteins was not blocked completely in the presence of vector RNA at the same concentration (lane 9) or by 20 μg of poly(I-C) (lane 12). These results indicate that the binding proteins, p71 and p110, preferentially recognize the JEV plus-strand 3′ NCR.

Mapping of the protein-binding sites on the JEV (+) strand 3′ NCR. To define the regions on the JEV plus-strand 3′ NCR recognized by each of the binding proteins, a competition experiment was conducted with unlabeled RNA fragments representing different regions of the JEV plus-strand 3′ NCR (nt 10392 to 10976). Equimolar amounts of the competitor RNAs were used. The results showed that p110 binding was competed out completely only by RNAs containing the 3′-terminal 80 nt, RNA-585 and RNA-442 (Fig. 3A, lanes 2, 3, 14, and 15), but not other RNAs (lanes 4 to 13), suggesting that its binding site is in the C-terminal 80 nt. In contrast, the binding site of p71 could not be precisely determined, because all the RNA fragments used competed with the full-length plus-strand 3′ NCR for p71 by binding to different extents (lanes 2 to 15). The protein-binding regions are summarized in Fig. 3B. The binding site of p110 was further confirmed by using an RNA-83 representing the 3′-terminal 83 nt of genomic RNA (Fig. 4A). The results demonstrated that the RNA-83 was still capable of binding p110 (Fig. 4B, lane 7), and this 83-nt RNA competed with the full-length labeled 3′-NCR RNA (lanes 1 to 5), and RNA-83 (lanes 6 to 10) for p110 binding. Taken together,

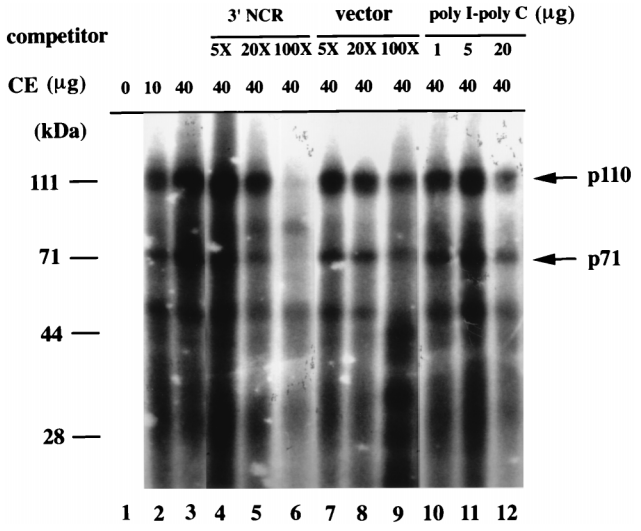

FIG. 2. Dose and competition assays for UV cross-linking. Binding and competition with unlabeled RNAs at 5-, 20-, and 100-fold molar ratios with respect to the labeled plus-strand 3′ NCR probe (0.25 pmol) is shown. Lanes: 1 to 3, cross-linking with labeled plus-strand 3′ NCR and different amounts of extracts (0, 10, and 40 μg) from infected cells; 4 to 6, cross-linking competitions with unlabeled JEV plus-strand 3′ NCR RNA; 7 to 9, vector RNA at 5-, 20-, and 100-fold molar excess; 10 to 12, poly(I-C) (1, 5, and 20 μg).

these results indicate that the C-terminal 83 nt is the critical determinant for p110 protein binding. It is interesting that the 3′-terminal 83 nt contains a stem-loop structure, but it is not clear whether this structure is responsible for p110 binding.

Characterization of the RNA-binding proteins. The binding studies described above showed that p71 and p110 were induced in virus-infected cells and that the time course of their appearance paralleled the kinetics of RNA replication. These proteins were not detected in extracts from uninfected cells (Fig. 1C and D, lanes 1). These results suggest that these proteins are either cellular proteins induced by virus infection or viral gene products. Because their molecular masses are similar to the predicted molecular masses (69 and 105 kDa) of the JEV nonstructural proteins NS3 and NS5, respectively, we next determined whether these two proteins were actually NS3 and NS5.

The time course of the appearance of the NS5 protein coincided with the kinetics of viral RNA synthesis (Fig. 1A and 5A) and the appearance of the RNA-binding proteins (Fig. 1C and 5A). When the UV-cross-linked, RNase-digested products were analyzed, the NS5 protein appeared to have a lower migration rate (Fig. 5A, lanes 10 to 12) and colocalized with the ^{32}P-radiolabeled protein after autoradiography (Fig. 5C, lanes 2 to 4). These results suggest that the RNA-binding protein p110 is the viral nonstructural protein NS5, and the slightly different migration mobility was caused by the nucleotides bound covalently to the NS5 protein after UV cross-linking. After detection of the NS5 protein, the same membrane was rehybridized with NS3 antibody. The results showed that the RNA-binding protein p71 coincided with the nonstructural protein NS3 (Fig. 1 and 5B and C). Furthermore, the ^{32}P-labeled, cross-linked p110 and p71 could be precipitated by antibodies against NS3 or NS5, suggesting that both of them form a complex with viral RNA (Fig. 6A) (see below). All the results together demonstrate that the NS5 and NS3 proteins of JEV are the identified RNA-binding proteins p110 and p71 which bind to the JEV plus-strand 3′-NCR.

The RNA-binding proteins NS5 and NS3 of JEV form a protein-protein complex in vivo. The results described above suggest that the JEV nonstructural proteins NS3 and NS5 could form a ribonucleoprotein complex with the JEV plus-strand 3′ NCR. Since NS5 and NS3 have been speculated to be RNA-dependent RNA polymerase and RNA helicase, respectively (27, 55, 65), they are probably components of the putative viral RNA replicase complex. Therefore, we propose that both proteins might interact with each other to effect viral RNA synthesis. Two approaches were used to test this hypothesis. First, proteins present in the infected cell extract were subjected to immunoprecipitation, and then the precipitates were analyzed by Western blotting with NS3 and NS5 antibodies. No NS3 and NS5 proteins were precipitated by protein A-agarose beads only (Fig. 6B, lane 1) or preimmune antiserum (lane 2). However, antibodies against either NS3 or NS5 precipitated both proteins (lanes 3 and 4). No cross-reactivity between these two antibodies was found (data not shown). When infected cell extracts cross-linked with the radiolabeled plus-strand 3′ NCR were subjected to immunoprecipitation by either NS3- or NS5-specific antibody, both RNA-binding pro-

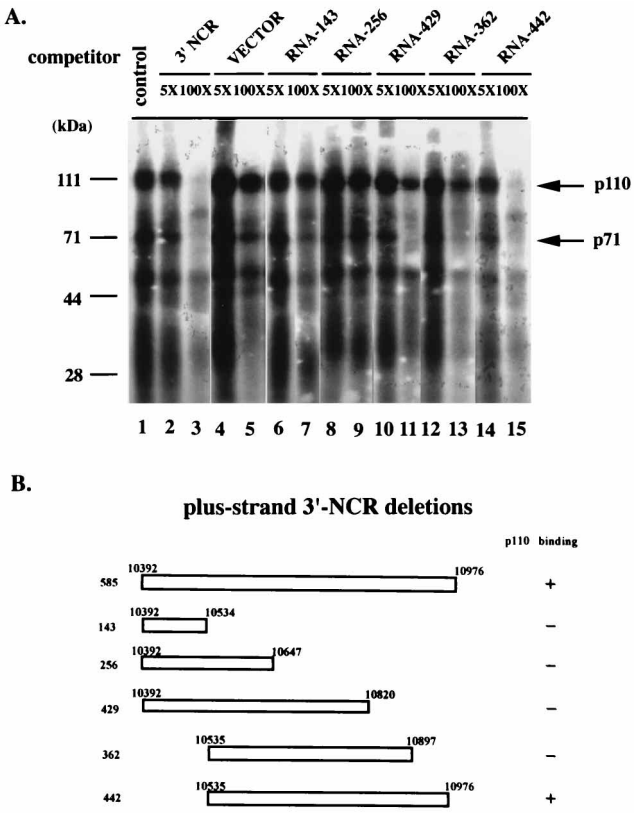

FIG. 3. Mapping of the binding sites of p71 and p110 on the JEV plus-strand 3′ NCR. (A) The UV cross-linking competition was conducted with different unlabeled RNAs at 5-fold (lanes 2, 4, 6, 8, 10, 12, and 14), and 100-fold (lanes 3, 5, 7, 9, 11, 13, and 15) molar excess with respect to the labeled plus-strand 3′ NCR probe (0.25 pmol). The results shown are no competitor RNA (lane 1), competed with unlabeled, plus-strand 3′ NCR (lanes 2 and 3), vector RNA (lanes 4 and 5), RNA-143 (lanes 6 and 7), RNA-256 (lanes 8 and 9), RNA-429 (lanes 10 and 11), RNA-362 (lanes 12 and 13), and RNA-442 (lanes 14 and 15), respectively. The detailed RNA-covering regions are described in Materials and Methods. (B) Structure of JEV plus-strand 3′ NCR deletions and their characteristics in protein binding. The JEV 3′ noncoding region contained 585 residues, from nt 10392 to 10976 without a poly(A) tail. Numbers denote the positions of nucleotide residues flanking the RNA terminus. The ability of each RNA to bind p110 is indicated by + and −.

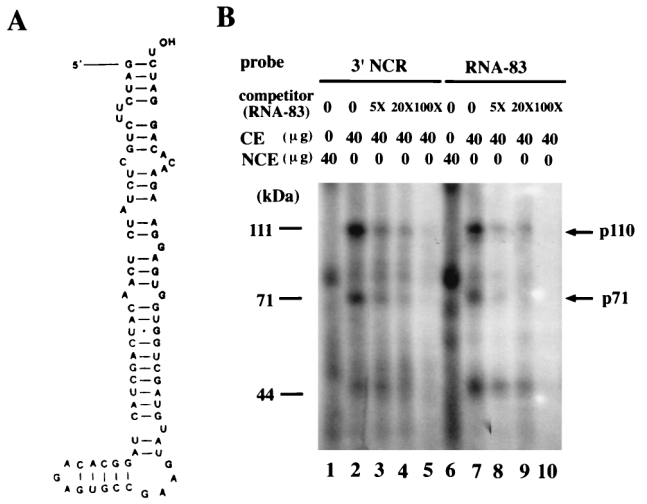

FIG. 4. The 3′-terminal stem-loop structure is critical for p110 binding in a UV cross-linking assay. (A) RNA-83 representing the 83 nt from the 3′ terminus of JEV genomic RNA may form a stem-loop structure. The secondary structure was modified from that in reference 60. (B) UV cross-linking competition assay with the plus-strand 3′ NCR and RNA-83. A UV cross-linking competition assay was performed with labeled plus-strand 3′ NCR (lanes 1 to 5) and RNA-83 (lanes 6 to 10) in the presence of various amounts of unlabeled RNA-83 competitor. CE denotes the JEV-infected cell extract, and NCE denotes the uninfected cell extract.

teins p71 and p110 were detected by autoradiography (Fig. 6A, lanes 4 and 5). Preimmune antiserum did not precipitate any protein (lane 3). All the results combined imply that the JEV nonstructural proteins NS3 and NS5 can form a protein-protein complex in vivo. However, the requirement for the involvement of other factors or the JEV genome in this protein interaction is still unclear.

DISCUSSION

In an effort to better understand the molecular mechanism involved in JEV RNA replication, a UV-induced cross-linking assay was undertaken to investigate the specific interactions between cellular and/or viral proteins and the JEV plus-strand 3′ NCR. Two distinct viral proteins were identified which interact with the 3′ NCR of JEV genomic RNA (Fig. 1C and D), and these two bound proteins were further shown to be the viral proteins NS3 and NS5. NS5 protein specifically bound to the plus-strand 3′ NCR within the extreme 3′ end, which has a conserved stem-loop structure; however, the binding of NS3 was less specific (Fig. 3 and 4). Our data suggest that this viral protein complex is important for molecular recognition of the JEV genomic RNA to direct the replication of viral genomic RNAs in the infected cell.

Several cellular and/or viral proteins that interact with virus-encoded *cis*-acting elements located within the plus-strand 3′ NCR were detected (Fig. 1C). Two of the distinct proteins are induced by JEV infection. Further experiments were per-

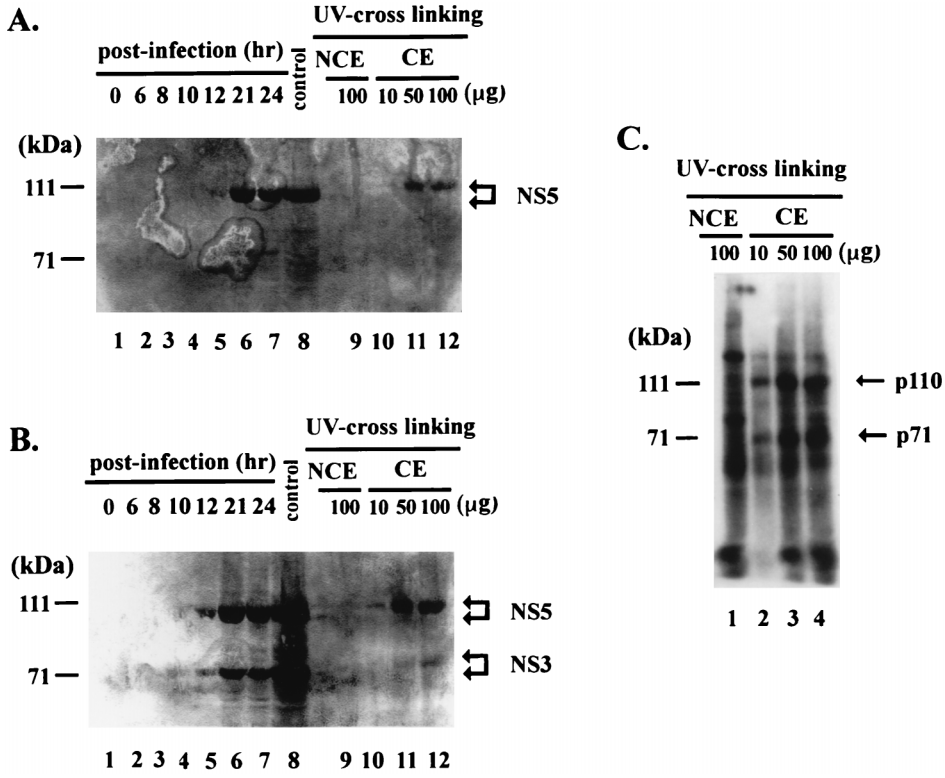

FIG. 5. Identification of UV cross-linked proteins by immunoblot analysis. (A) Immunoblot analysis of the NS5 protein from infected cell extracts and UV cross-linking mixtures. Extracts (300 μg) prepared from infected cells at various times p.i. (0, 6, 8, 10, 12, 21, 24 h) were separated by SDS-PAGE (8% polyacrylamide), transferred onto Immobilon-P membrane, and probed with NS5 antibody. The bound antibodies were detected by treatment with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (lanes 1 to 7). A JEV persistent-infection cell extract is shown in lane 8 as a control. UV cross-linking mixtures from uninfected (lane 9) and infected (10, 50, and 100 μg) (lanes 10 to 12) cells are shown. (B) After detection of the NS5 protein, the above membrane was reprobed with NS3 antibody. (C) Autoradiography of UV cross-linking products. The above membrane was analyzed by autoradiography. The content and order of samples (lanes 1 to 4) are the same as in panels A and B, lanes 9 to 12.

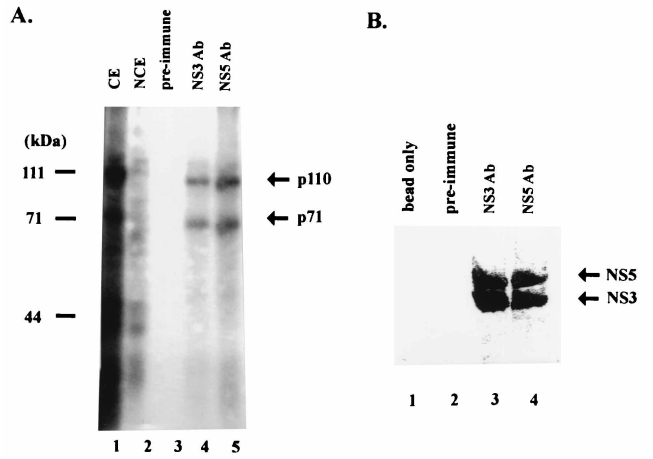

FIG. 6. Protein-protein complex formation between NS3 and NS5 in vivo. (A) Analysis of UV cross-linked proteins by immunoprecipitation. UV cross-linking mixtures were subjected to immunoprecipitation with antibodies against NS3 (lane 4) or NS5 (lane 5) or with preimmune antiserum (lane 3). The reaction mixture untreated with antibody is shown in lane 1, and the extract from uninfected cells untreated with antibody is shown in lane 2. CE, infected cell extract; NCE, uninfected cell extract. (B) Immunoblot analysis of NS3 and NS5 proteins derived from precipitates with either NS3 or NS5 antibodies. Extracts from infected cells were first treated with antibodies against NS3 (lane 3) or NS5 (lane 4) or with preimmune antiserum (lane 2) or protein A-agarose beads only (lane 1). The precipitates were then separated by SDS-PAGE, transferred onto a membrane, and finally detected by immunoblot with NS3 and NS5 antibodies.

formed by immunoprecipitation and immunoblotting coupled with a UV cross-linking assay with antibodies against NS3 and NS5, which clearly identified these proteins as the viral nonstructural proteins NS3 and NS5. This is the first report demonstrating that JEV genomic RNA forms a ribonucleoprotein complex with its nonstructural proteins from infected cell extracts. Together with the results from the antibody inhibition experiment (2), these data support the idea that viral NS3 and NS5 proteins form parts of the viral replication complex and are involved in viral RNA replication.

Stem-loop structures have been implicated in the processes of RNA virus translation, RNA replication, and encapsidation (1, 25, 29, 42–44, 46, 47, 49, 51, 58). Indeed, binding determinants of several cellular and virus-encoded RNA-binding proteins have been demonstrated to be contained within stem-loop structures (1, 5, 23, 24, 33, 42, 44–47, 49). Computer folding predictions and RNase cleavage studies have indicated that the 3′-terminal nucleotides of the flavivirus genome form a stable stem-loop structure, especially the last 100 nt (Fig. 4A) (7, 8, 55, 60, 62), and this secondary structure has been shown to be a target for cellular protein binding (5). Blackwell and Brinton (5) has demonstrated that BHK cell proteins from the cytoplasmic S100 fraction bind to the 3′ stem-loop structure of the West Nile virus genome RNA. For the most distal regions of either RNA terminus, higher-order structures which are essential as recognition signals for the viral polymerase have been proposed (1, 19, 31). Although recognition of the 3′ end of the viral RNA by the viral polymerase must be one of the first steps in RNA amplification, the functional roles of the 3′-terminal region and *cis*-acting recognition signals therein have not been studied in detail. Recently, the $3D^{pol}$ of encephalomyocarditis virus has been shown to interact specifically with the 3′ NCR-poly(A) fragment of encephalomyocarditis virus RNA (19), and from our studies, the JEV plus-strand 3′-terminal sequences containing a stem-loop structure were shown to be capable of binding to viral polymerase (Fig. 3 and 4). The other terminal 3′ NCR of the minus strand has also been predicted to form a stem-loop structure, but this structure is much less stable than the plus-strand 3′ stem-loop structure (9). The JEV minus-strand 3′-NCR still possesses the property of binding to viral polymerase (data not shown). The detailed binding determinants and the contribution of the stem-loop structure to the binding of these proteins remain to be elucidated, whereas the other identified binding protein, NS3, seemed to be a random single-stranded RNA-binding protein with little preference for JEV RNA (Fig. 3). Actually, the bacterially expressed recombinant NS3 protein is able to bind to and unwind an artificial RNA duplex template (36). Distinct binding characteristics between these two proteins, which have been proposed as cooperating, have been found (34). In infected cells, the level of flavivirus plus-strand RNA synthesis is 10 to 100 times greater than that of minus-strand synthesis (18, 59). It would certainly be a fascinating feature of virus replication strategy to achieve differential regulation of the rates of RNA transcription from these two templates. The requirements of different sets of cell proteins, other unknown factors, or intrinsic characteristics of templates in contributing to the regulation of the initiation of transcription remain to be determined.

In only a few instances has it been possible to directly demonstrate viral replication complexes composed of cellular and viral proteins (3, 13, 14, 30, 37, 38, 48). For picornaviruses, the virus-encoded enzyme provides the elongation function, but cellular proteins recognize, bind, and initiate the viral RNA template (22, 56). However, in most cases, the functional contribution of those viral RNA-binding proteins to viral replication remains to be elucidated (26, 39, 46, 47, 49, 50). Despite the difficulty in identifying the nature and function of those RNA-binding proteins, cellular proteins that regulate viral RNA replication by binding to the noncoding regions of the viral RNA genome have been demonstrated in Sindbis virus, poliovirus, brome mosaic virus, coronavirus, and other plant viruses (1, 26, 49, 51). One of the possible functions of cellular proteins involved in viral RNA synthesis is to correctly direct and initiate the specific target template. Recently, two reports have demonstrated that the recombinant RNA-dependent RNA polymerase from dengue virus type 1 and hepatitis C virus contain polymerization activity but without template selectivity in vitro (4, 63). In contrast, the flavivirus replicase appears to selectively replicate virus-specific RNAs in vivo (15, 16, 28, 61, 67). From our studies, the putative RNA-dependent RNA polymerase of JEV recognized its target viral template with high specificity in infected cell extracts. This specific recognition may be mediated by those as yet uncharacterized cellular proteins binding to the plus-strand 3′ NCR observed in the above studies (Fig. 1C).

Many positive-strand RNA viruses of plants and animals have similarities in replication and transcription of their genome. In addition to the likely participation of some host factors (32, 52, 53), these RNA-dependent RNA synthesis events require viral proteins implicated in polymerase and helicase functions. The bacterial phage Qβ replicase is a stable complex consisting of three cellular proteins and one viral protein (3, 6), whereas it is unclear whether JEV RNA synthesis requires any direct in vivo interactions between these protein factors or other cooperative relationships. The results of the coimmunoprecipitation experiments showed an association between NS3 and NS5 in vivo (Fig. 6). The same characteristic has also been found in dengue virus type 2 (34), demonstrated by using coimmunoprecipitation and affinity column chromatography. This interaction between NS3 and NS5 supports the notion that they are components of the putative

viral replicase postulated in earlier studies (2, 55) and is based on the presence of conserved motifs of an RNA helicase in NS3 and the G-D-D motif in NS5, characteristic of RNA-dependent RNA polymerase (27, 55, 65). However, the requirement of a cellular protein(s) for this interaction and the participation of other viral and cellular proteins in the stabilization of this complex are still unclear. Prior to RNA synthesis, the RNA duplexes and/or secondary structures must be unwound, and the responsible polymerase and helicase enzymes might function in cooperation. Indeed, the NS3 protein contains unwinding activity and binds to single-stranded RNA in vitro (36), and NS5 exhibits RNA-dependent RNA polymerase activity in vitro (4, 63), although neither function shows template specificity. However, both enzymes exhibited a high preference for the viral template in vivo (15, 16, 28, 61, 67). Taken together, the functional replicase complex containing at least two activities formed in infected cells could modulate each function to improve template selectivity. In addition, it is likely that the virus uses host cellular factors, presently unidentified, to regulate and facilitate the replication of the virus genome.

It is interesting that the viral NS3 and NS5 proteins were found to bind to the 3′ end of the viral genomic RNA. In JEV-infected cells, the incoming viral genomic RNA is expected to be replicated from the 3′ end of positive-strand RNA into negative-strand RNA by virus-specific RNA polymerase. Thus, single-stranded, positive-sense genomic RNA probably serves directly as the template for the synthesis of negative-strand RNA. At the beginning of infection, the simplicity of this template and strong specific binding to RNA polymerase may simplify the process for minus-strand RNA synthesis. However, the full processes of JEV RNA synthesis and the participation of other cellular and/or viral proteins involved in viral replication regulation remain to be determined.

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