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

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Received 6 June 1996/Accepted 15 January 1997

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 Flavi- virus 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, 34, 37, 47).

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.
Preparation of cell extracts. Cell extracts were prepared as described previ- ously (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 × 10⁷ cells/ml. The resultant cell suspensions were disrupted by 20 passages through a 27-gauge needle (15). One half of the supernatants were added with 1.5% SDS and incubated for an additional 2 h at 37°C. Protein A–agarose beads 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-polyacryl- amide 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 con- firmed 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 32P-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, were found in the JEV-infected cell lysates 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 kinet- ics 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 protein A–agarose beads was used with the 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 and bound proteins, protein A–agarose beads were added with fresh binding buffer 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-polyacryl- amide gels.

Immunoprecipitation. Protein A–agarose beads were bound 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 and bound proteins, protein A–agarose beads were added with fresh binding buffer 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-polyacryl- amide gels.
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,
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-
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. 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.

![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.
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 non-structural 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 3Dpol 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 recognizes 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 communoprecipitation 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 communoprecipitation and affinity column chromatography. This interaction between NS3 and NS5 supports the notion that they are components of the putative
viral replication 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 replication 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 initiates 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 replication 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.

ACKNOWLEDGMENTS

We are grateful to M. M. C. Lai for helpful comments and critical reading of the manuscript. This work was supported by the Department of Health (grant DOH 85-HR–406) and the Institute of Preventive Medicine of the National Defense Medical Center (grant 850603).

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