4B Fast Flow beads (GE Healthcare) and 1 μg of normal mouse IgG for 1 h at 4°C. After centrifugation, the supernatants were further incubated with 1 μg of anti-HA mouse MAb or normal mouse IgG for 2 h at 4°C, and 25 μl of protein G-Sepharose beads was added. After 1 h of incubation at 4°C, the beads were washed five times with RNA-protein binding buffer without yeast tRNA, and RNA was isolated by use of TRIzol reagent (Invitrogen). RT-PCR was carried out using random hexamers and PrimeScript II reverse transcriptase (Takara Bio, Shiga, Japan), followed by PCR with PrimeSTAR GXL DNA polymerase (Takara Bio) and primers (5'-TCTGTCACTAGACTGGAGCA-3' and 5'-CCA GAAACATCACCAGAAGG-3') targeted to a fragment consisting of nucleotides 2652 to 3589 in the JEV NS1 gene.

***In vitro* transcription.** cDNA fragments encoding either the positive or negative strand of the 5' and 3' UTRs of JEV under the control of the T7 promoter were amplified by PCR. RNA transcripts were synthesized using a MEGAscript T7 kit (Ambion, Austin, TX). Biotinylated RNA was synthesized by adding 20 pmol of biotinylated UTP (biotin-16-UTP; Roche) to a 20-μl MEGAscript transcription reaction mix. Synthesized RNAs were purified using phenol-chloroform extraction and isopropanol precipitation and were analyzed in a 2% agarose gel.

RNA pulldown assay. Cell lysates (200 μg) extracted from 293T cells expressing HA-hnRNP A2 were incubated for 15 min at 30°C with the biotinylated JEV UTR RNA (10 pmol) in RNA-protein binding buffer and further incubated for 10 min at room temperature after addition of 250 μl streptavidin-conjugated MagneSphere paramagnetic particles (Promega). The RNA-protein complexes were washed five times with RNA-protein binding buffer without yeast tRNA and then subjected to SDS-PAGE and immunoblotting after boiling in 25 μl 2× SDS-PAGE sample buffer.

Preparation of recombinant hnRNP A2 and GST pulldown assay. GST-fused hnRNP A2 was expressed in *Escherichia coli* BL21(DE3) cells transformed with pGEX-hnRNP A2. Bacteria grown to an optical density at 600 nm of 0.6 were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside, incubated for 4 h at 37°C with shaking, collected by centrifugation at 6,000 × *g* for 10 min, lysed in 10 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) by sonication on ice, and centrifuged at 10,000 × *g* for 20 min. The supernatant was mixed with 200 μl of glutathione-Sepharose 4B beads (GE Healthcare) equilibrated with lysis buffer for 1 h at room temperature, and the beads were washed five times with lysis buffer and then replaced with RNA-protein binding buffer. Ten micrograms of GST or GST-hnRNP A2 was mixed with 10 pmol of the biotinylated positive or negative strand of the 3' or 5' UTR of JEV RNA for 15 min at 30°C with gentle agitation. The beads were washed five times with RNA-protein binding buffer without yeast tRNA.

Northern blotting. RNAs interacting with proteins were isolated by use of TRIzol reagent, separated by use of a formaldehyde-free RNA gel electrophoresis system (Amresco, Solon, OH), and transferred to a positively charged nylon membrane (Roche). The biotinylated RNA was detected with streptavidin conjugated with alkaline phosphatase (Roche) and visualized by chemiluminescence using CSPD (Roche).

RESULTS

Identification of hnRNP A2 as a binding partner of the JEV core protein. To identify cellular proteins associated with the JEV core protein, we employed an MEF affinity tag purification method. The MEF-Core protein was expressed in 293T cells and purified together with associated proteins as described previously (62). The silver-stained proteins were excised from an SDS-PAGE gel and analyzed by use of a nanoflow LC-MS/MS system. This procedure identified the amino acid sequences QEMQEVQSSR and GGGGNFGPGPGS NFR, which respond to amino acid residues 179 to 188 and 202 to 206 of human hnRNP A2, respectively. To confirm the interaction between the JEV core protein and hnRNP A2 in cells, 293T cells expressing FLAG-Core and HA-hnRNP A2 were examined by immunoprecipitation analyses. FLAG-Core and HA-hnRNP A2 were shown to be immunoprecipitated with each other (Fig. 1A). Furthermore, endogenous hnRNP A2 was coprecipitated with the JEV core protein in 293T cells infected with JEV but not in mock-infected cells (Fig. 1B). These results indicate that hnRNP A2 interacts with the JEV core protein.

Next, to determine the regions responsible for the interaction between JEV core and hnRNP A2, series of deletion mutants of the JEV core protein and hnRNP A2 were generated and examined by immunoprecipitation analyses. FLAG-Core (full length) and mutants lacking the N-terminal 20 and 40 amino acid residues (ΔN20 and ΔN40), but not those lacking the C-terminal 20 and 40 amino acid residues (ΔC65 and ΔC85), were immunoprecipitated with HA-hnRNP A2 (Fig. 1C). The flavivirus core protein has been shown to form homodimers via the central and C-terminal regions (26, 39). Therefore, to exclude the possibility that C-terminal deletion of the core protein abrogates dimerization, FLAG-Core (full length or ΔC85) and Core-HA (full length or ΔC85) were coexpressed and immunoprecipitated. As shown in Fig. 1D, the C-terminal deletion exhibited no effect on the homotypic interaction of the core protein, consistent with previous data showing that deletion of the C-terminal amino acid residues (aa 73 to 100) did not abolish the homotypic interaction of DEN core protein (64). These results indicate that the C-terminal region (aa 85 to 104) of the JEV core protein is responsible for the protein-protein interaction with hnRNP A2. hnRNP A2 is composed of two N-terminal RNA recognition motifs (RRM) followed by a Gly-rich C-terminal domain (GRD) (29). FLAG-hnRNP A2 (full length) and a mutant lacking the RRM1 domain (ΔRRM1), but not mutants lacking either RRM2 (ΔRRM2) or GRD (ΔGRD), were immunoprecipitated with Core-HA (Fig. 1E). The results indicated that the C-terminal residues from positions 85 to 104 of the JEV core protein and RRM2 and GRD in hnRNP A2 are responsible for the interaction.

hnRNP A2 translocates from the nucleus to the cytoplasm upon infection with JEV. To examine the intracellular localization of hnRNP A2 in cells infected with JEV, Vero cells expressing HA-hnRNP A2 were infected with JEV because an anti-hnRNP A2 antibody capable of detecting endogenous hnRNP A2 by immunofluorescence analysis was not available. We employed Vero cells, which exhibit a wider cytoplasm space than 293T cells, to investigate the cellular localization of each protein. Although HA-hnRNP A2 was detected mainly in the nucleus in mock-infected cells, as previously described (19), translocation of HA-hnRNP A2 into the nucleolus and cytoplasm and colocalization with the core protein were observed upon infection with JEV (Fig. 2A). HA-hnRNP A2 was detected in both the nucleus and cytoplasm in <60% of cells infected with JEV, while only 5% of mock-infected cells exhibited cytoplasmic localization of HA-hnRNP A2 (Fig. 2B). To further confirm the subcellular localization of hnRNP A2, the cytoplasmic and nuclear fractions of JEV-infected cells were analyzed by immunoblotting (Fig. 2C). hnRNP A2 was detected in both the cytoplasmic and nuclear fractions of the JEV-infected cells, while it was detected mainly in the nuclei of the mock-infected cells. These results indicate that infection of JEV induces translocation of hnRNP A2 from the nucleus to the cytoplasm.

Knockdown of hnRNP A2 decreases propagation of JEV. To determine the role of hnRNP A2 in the propagation of JEV, JEV was inoculated into 293T cells transfected with two siRNAs targeted to hnRNP A2/B1 (si-A2#1 and -2) or with a

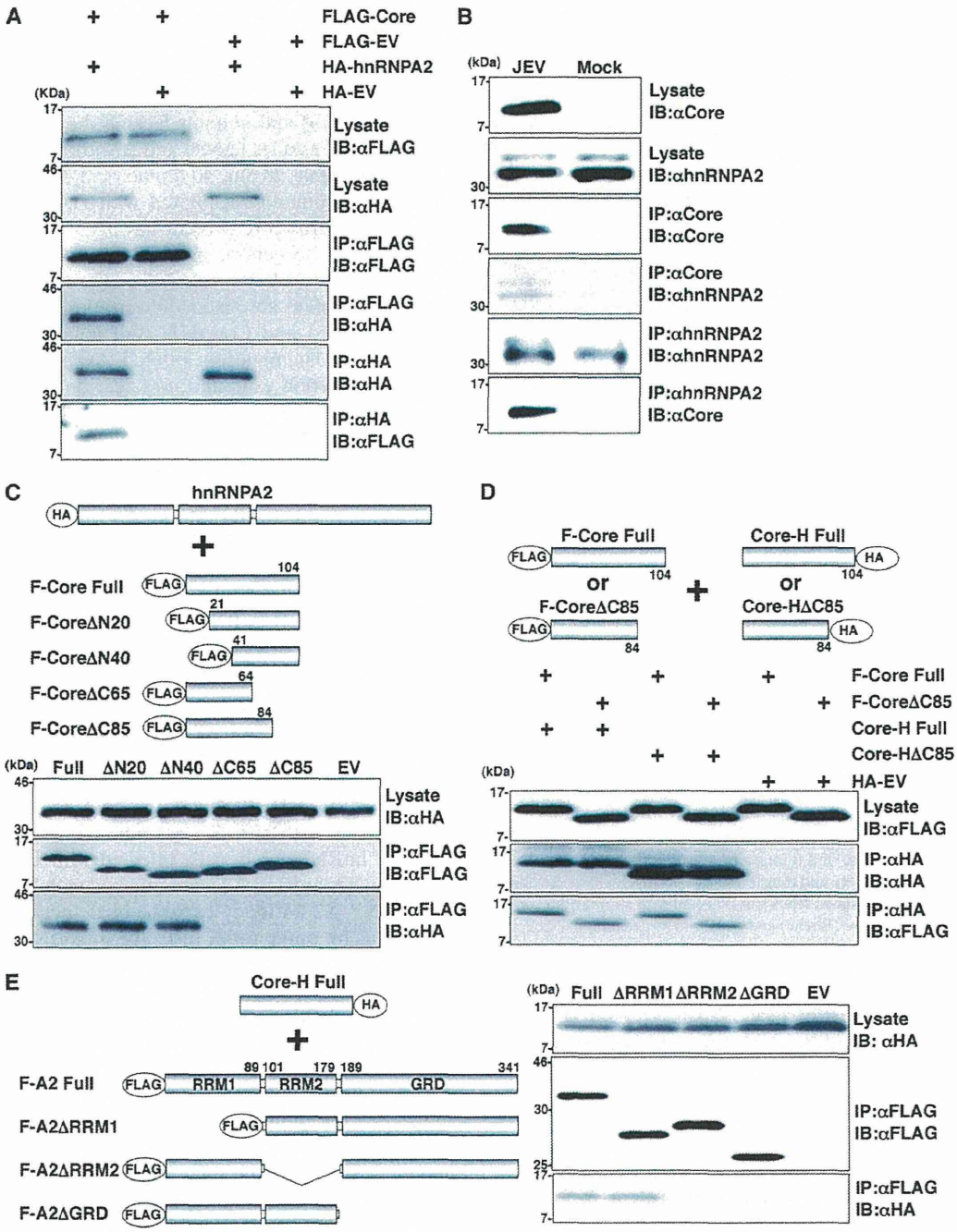


FIG. 1. Interaction of JEV core protein with hnRNP A2. (A) FLAG-Core and HA-hnRNP A2 were coexpressed in 293T cells and immunoprecipitated (IP) with mouse anti-HA MAb (HA11) or mouse anti-FLAG MAb (M2). The immunoprecipitates were subjected to immunoblotting (IB) to detect coprecipitated counterparts. As a negative control, an empty vector (EV) was used. (B) Interaction of JEV core protein with endogenous hnRNP A2 in 293T cells infected with JEV. Cells infected with JEV at an MOI of 1.0 were lysed at 24 h postinfection, and JEV core protein or hnRNP A2 was immunoprecipitated with rabbit anti-core PAb or mouse anti-hnRNP A2 MAb (DP3B3). The precipitates were analyzed by IB with appropriate antibodies. (C) Interaction of hnRNP A2 with deletion mutants of the JEV core protein. HA-hnRNP A2 and a series of deletion mutants of FLAG-Core were cotransfected in 293T cells, precipitated with mouse anti-FLAG MAb (M2), and then subjected to IB. (D) Dimerization of C-terminal deletion mutants of JEV core protein. FLAG-Core (full or ΔC85) and Core-HA (full or ΔC85) were cotransfected into 293T cells, treated with RNase A (10 μg/ml) for 30 min at 4°C, precipitated with mouse anti-HA MAb (HA11), and then subjected to IB. (E) Interaction of JEV core protein with deletion mutants of hnRNP A2. Core-HA and a series of deletion mutants of FLAG-hnRNP A2 were cotransfected into 293T cells and processed as described for panel C.

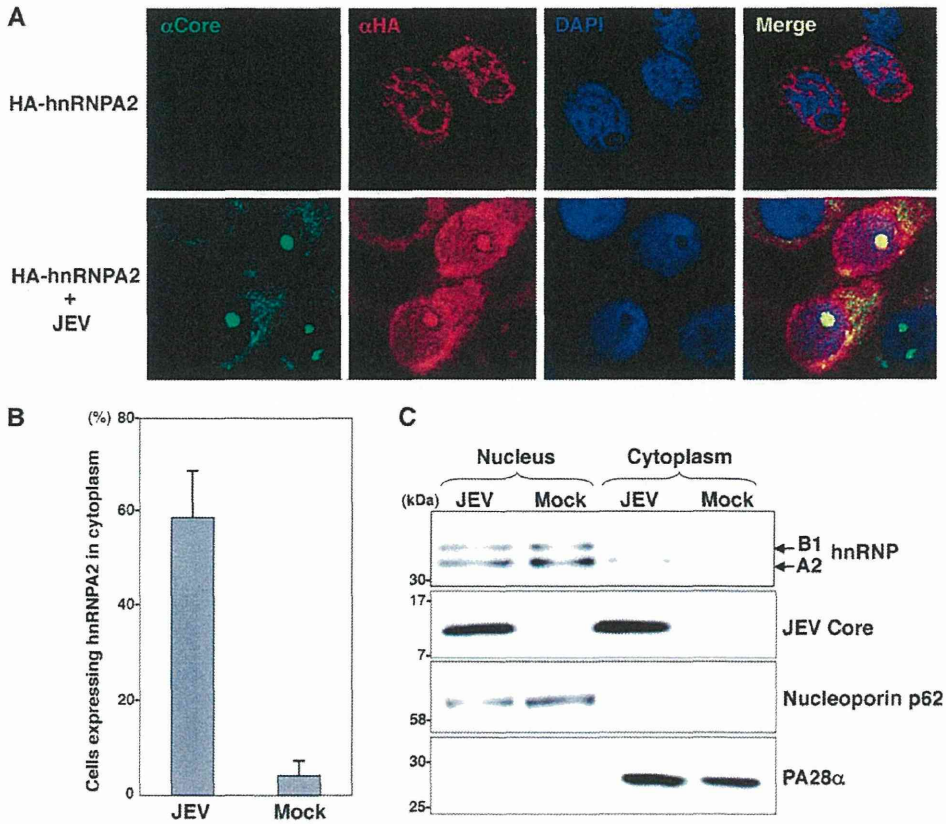


FIG. 2. Translocation of hnRNP A2 from the nucleus to the cytoplasm upon infection with JEV. (A) Vero cells transfected with a plasmid encoding HA-hnRNP A2 were infected with JEV at an MOI of 1.0 and then fixed with cold acetone at 24 h postinfection. JEV core and HA-hnRNP A2 were stained with rabbit anti-core PAb and mouse anti-HA MAb (HA11), followed by AF488-conjugated anti-rabbit IgG and AF594-conjugated anti-mouse IgG antibodies, respectively. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue). (B) Percentages of cells exhibiting translocation of HA-hnRNP A2 to the cytoplasm. Three hundred cells expressing HA-hnRNP A2 were counted in three independent experiments. Error bars indicate the standard deviations of the means. (C) Intracellular fractionation of 293T cells infected with JEV. JEV core and hnRNP A2 in the nuclear and cytoplasmic fractions were detected by immunoblotting with rabbit anti-core PAb and mouse anti-hnRNP A2 MAb (DP3B3), respectively. Nucleoporin p62 and PA28α were used as nuclear and cytoplasmic markers, respectively.

control siRNA (si-NC) at 24 h posttransfection. Transfection of the siRNAs exhibited no cytotoxicity, so total RNA was extracted from the infected cells, and the level of JEV RNA was determined by real-time PCR at 24 h postinfection (Fig. 3A). The levels of JEV RNAs in cells transfected with si-A2#1 and -2 were reduced by approximately 40% and 90%, respectively, compared with those in cells treated with si-NC. The expression of JEV NS3 protein was decreased in accord with the reduction of hnRNP A2 (Fig. 3B). Furthermore, a reduction of viral production in the culture supernatants was observed by the knockdown of hnRNP A2 (Fig. 3C). To confirm the specificity of the suppression of JEV propagation by the knockdown of hnRNP A2, a mutant HA-hnRNP A2 protein resistant to si-A2#2 by the introduction of silent mutations (siR) was introduced into cells transfected with si-A2#2 (Fig. 3D). The reduction of JEV RNA propagation by the knockdown of hnRNP A2 was partially rescued by the expression of siR. These results suggest that hnRNP A2 is required for the propagation of JEV.

Translocation of hnRNP A2 from the nucleus to the ER by expression of JEV core protein enhances viral replication. Next, to determine the biological significance of hnRNP A2 in the replication of JEV RNA, we examined the effect of knockdown of hnRNP A2 in JEV-SGR-293T cells, since the subgenomic replicon RNA of JEV replicates autonomously in 293T cells in the absence of structural proteins. Knockdown of hnRNP A2 in the JEV-SGR-293T cells had no significant effect on the replication of the subgenomic RNA and the expression of the NS3 protein (Fig. 4A), suggesting that hnRNP A2 requires JEV structural proteins, probably the core protein, to enhance viral replication. To assess this possibility, we examined the effect of the exogenous expression of the JEV core protein on the subcellular localization of hnRNP A2 and the replication of the subgenomic RNA. HA-hnRNP A2 and FLAG-Core were coexpressed in subgenomic replicon Huh7 cells (JEV-SGR-Huh7) because these replicon cells were established in our laboratory previously (18). Huh7 cells exhibit a wider cytoplasmic view than 293T cells but have smaller and fewer nucleoli than Vero cells. HA-hnRNP A2 was detected in the nuclei of the Huh7 replicon cells transfected with an empty plasmid (Fig. 4B). Although FLAG-Core was not colocalized with calregulin, GM130, and EEA1, which are markers of the ER, Golgi apparatus, and early endosome, respectively, HA-hnRNP A2 was colocalized with FLAG-Core

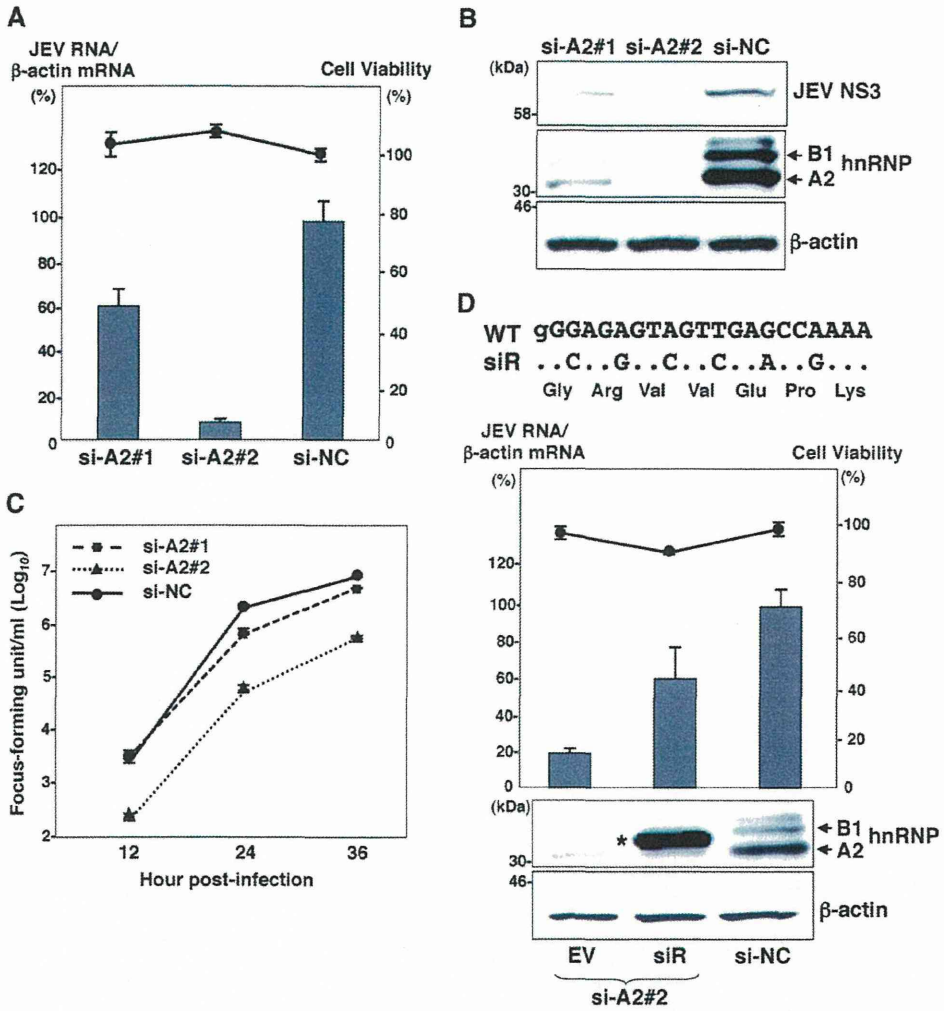


FIG. 3. Effect of hnRNP A2 knockdown on JEV propagation. (A) JEV was infected with 293T cells at an MOI of 1.0 24 h after transfection with si-A2#1, si-A2#2, or si-NC. Total cellular RNA was extracted at 24 h postinfection and subjected to RT. The level of JEV RNA (NS5) was determined by real-time PCR and calculated as a percentage of the control β-actin mRNA level (bar graph). Cell viability was determined 48 h after transfection with each siRNA and calculated as a percentage of the viability of cells treated with si-NC (line graph). The data are representative of three independent experiments. Error bars indicate the standard deviations of the means. (B) Cell lysates collected at 24 h postinfection were subjected to immunoblotting with mouse MAbs to JEV NS3, hnRNP A2 (DP3B3), and β-actin. (C) Culture supernatants were harvested at 12, 24, and 36 h postinfection, and infectious titers were determined by focus-forming assays in Vero cells. Closed squares, triangles, and circles indicate the infectious titers in the culture supernatants of cells transfected with si-A2#1, si-A2#2, and si-NC, respectively. The results shown are from three independent assays, with the error bars representing the standard deviations. (D) (Top) Nucleotide and amino acid sequences of wild-type (WT) and siRNA-resistant (siR) HA-hnRNP A2. Capital letters in the WT sequence indicate the target sequence of si-A2#2, and dots indicate the same nucleotides. (Middle) 293T cells cotransfected with si-A2#2 and siR or empty vector (EV) or transfected with si-NC were infected with JEV at an MOI of 1.0 at 24 h posttransfection. Total cellular RNA was extracted at 24 h postinfection and subjected to RT. The level of JEV RNA (NS5) was determined by real-time PCR and calculated as a percentage of the control β-actin mRNA level (bar graph). Cell viability was determined 48 h after transfection with each siRNA and calculated as a percentage of the viability of cells treated with si-NC (line graph). (Bottom) Cell lysates collected at 24 h postinfection were subjected to immunoblotting with hnRNP A2 (DP3B3) and β-actin. The exogenous mutant hnRNP A2 (siR) resistant to si-A2#2 is indicated by an asterisk. The data are representative of three independent experiments. Error bars indicate the standard deviations of the means.

and calregulin, but not with GM130 and EEA1, at 24 h post-transfection (Fig. 4B and data not shown). We have shown previously that two amino acid residues (Gly^{42} and Pro^{43}) in the JEV core protein are responsible for nuclear localization (47). To further confirm whether the cytoplasmic localization of hnRNP A2 by the expression of JEV core protein is caused by active export from the nucleus or passive retention in the cytoplasm, FLAG-CoreM, which is defective in nuclear localization, was introduced into JEV-SGR-Huh7 cells. As shown in Fig. 4B, hnRNP A2 was also detected in the ER of the replicon cells transfected with FLAG-CoreM. We further examined the interaction of hnRNP A2 with the core protein by an immunoprecipitation analysis. Coprecipitation of hnRNP A2 with core protein, but not with calregulin, was observed in the JEV-infected 293T cells (Fig. 4C). Next, to confirm the role of the core protein in the replication of JEV, FLAG-Core was

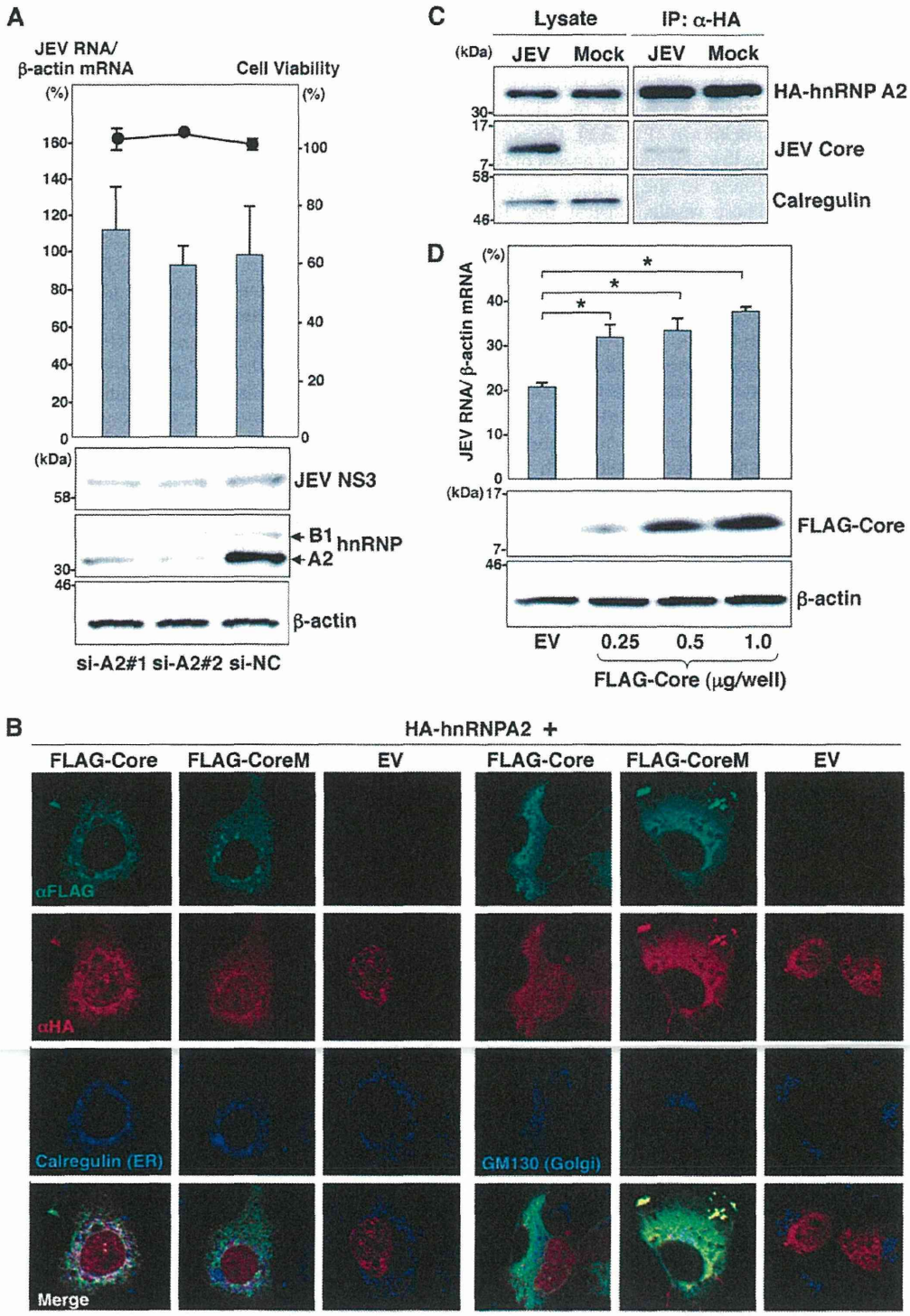


FIG. 4. Translocation of hnRNP A2 from the nucleus to the ER by expression of JEV core protein enhances viral RNA replication. (A) JEV subgenomic replicon (JEV-SGR-293T) cells were transfected with either si-A2#1, si-A2#2, or si-NC and harvested at 48 h posttransfection. (Top) The level of JEV RNA (NS5) was determined by real-time PCR and calculated as a percentage of the control β-actin mRNA level (bar graph). Cell viability was determined 48 h after transfection with each siRNA and was calculated as a percentage of the viability of cells treated with si-NC (line graph). (Bottom) Cell lysates collected at 48 h posttransfection were subjected to immunoblotting with mouse MAbs to JEV NS3, hnRNP A2 (DP3B3), and β-actin. (B) JEV subgenomic replicon (JEV-SGR-Huh7) cells were transfected with an expression plasmid for HA-hnRNP A2 together with that for FLAG-Core or FLAG-CoreM (a mutant defective in nuclear localization) or with an empty vector (EV) and then fixed with cold acetone at 24 h posttransfection. FLAG-Core was stained with either mouse anti-FLAG MAb (M2) and AF488-conjugated anti-mouse IgG or rabbit anti-FLAG PAb and AF488-conjugated anti-rabbit IgG. HA-hnRNP A2, calregulin, and GM130 were stained with rat anti-HA MAb (3F10), rabbit anti-calregulin PAb, and mouse anti-GM130 MAb, followed by AF594-conjugated anti-rat IgG, AF633-conjugated anti-rabbit IgG,

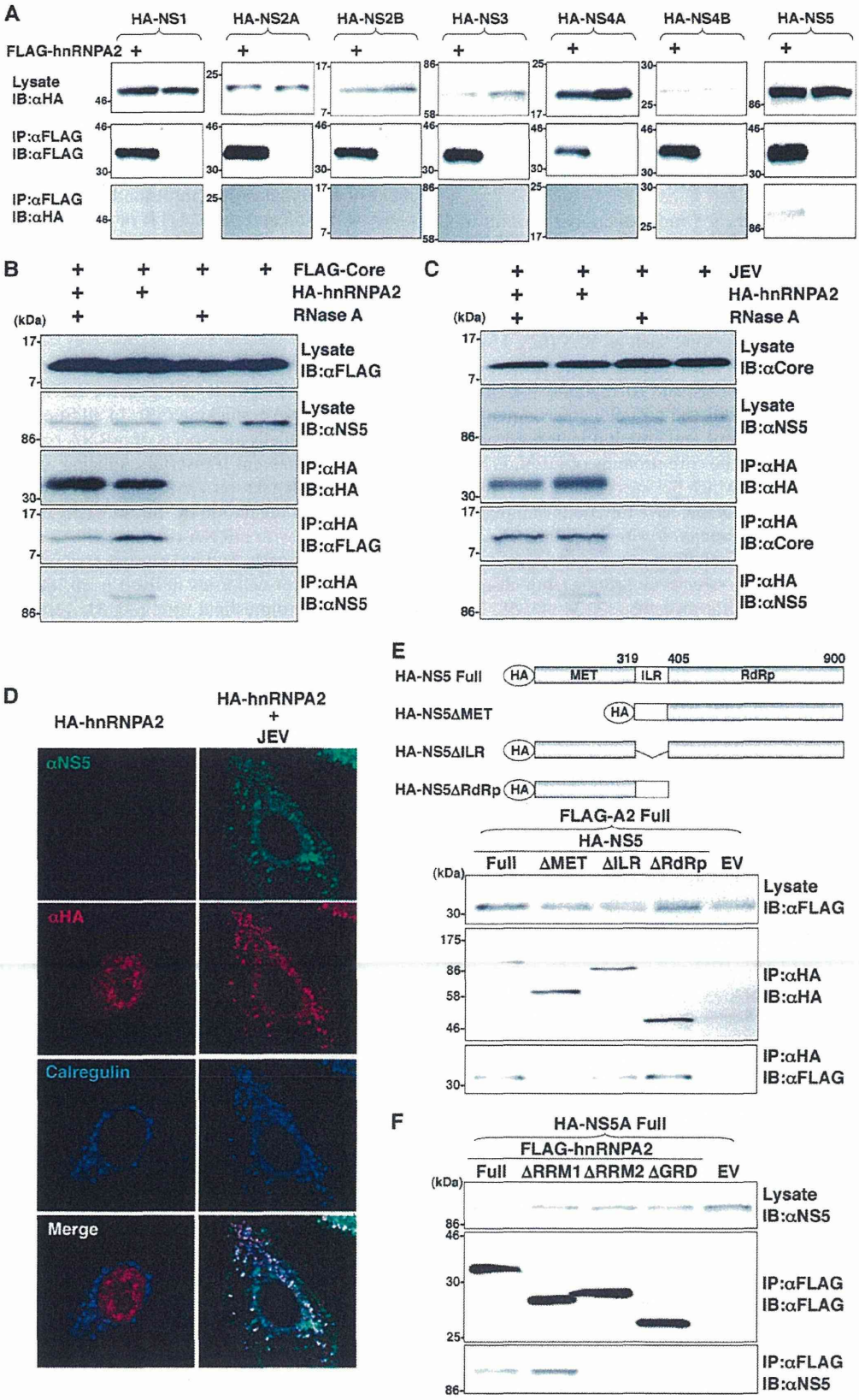
expressed in JEV-SGR-293T cells. As shown in Fig. 4D, the levels of JEV RNA were increased up to <2-fold, in accord with the expression levels of the core protein. The effect of JEV core expression on RNA replication in the subgenomic replicon cells was weak compared with that in the JEV-infected cells with hnRNP A2 knockdown (Fig. 3A); the weak effect might be attributable to the low efficiency of access of the exogenously expressed core protein to the replication complexes in the replicon cells. These results suggest that the passive ER retention of hnRNP A2 by interaction with the JEV core protein enhances viral replication.

hnRNP A2 interacts with JEV NS5 through interaction with viral RNA, in contrast to protein-protein interaction with JEV core protein. The viral RNA replication of flaviviruses takes place in a replication complex consisting of NS proteins and host proteins in the ER (45). To determine the interaction of hnRNP A2 with JEV NS proteins, FLAG-hnRNP A2 was coexpressed with each of the HA-tagged JEV NS proteins in JEV-SGR-293T cells and immunoprecipitated with anti-FLAG antibody. Among the JEV NS proteins we examined, only NS5 was coimmunoprecipitated with hnRNP A2 (Fig. 5A). More importantly, the interaction between hnRNP A2 and NS5 was observed in JEV-SGR-293T cells but not in 293T cells expressing both of the proteins (data not shown). Like the core protein, both NS5 and hnRNP A2 are RNA-binding proteins (23, 59, 74), and thus it might be feasible to speculate that viral RNA mediated the interplay between these proteins. To determine the role of viral RNA in these interactions, HA-hnRNP A2 and FLAG-Core were coexpressed in JEV-SGR-293T cells, and the cell lysates were treated with RNase A before immunoprecipitation (Fig. 5B). Coprecipitation of NS5 with hnRNP A2 in the replicon cells was abolished by the treatment with RNase A, while that of JEV core protein with hnRNP A2 was rather resistant to the treatment. The RNA-mediated interaction between NS5 and hnRNP A2 was also observed in the JEV-infected cells, but not that between the core protein and hnRNP A2 (Fig. 5C). These results indicate that hnRNP A2 interacts with JEV NS5 through the interaction with viral RNA, in contrast to protein-protein interaction with the JEV core protein. Next, to determine the subcellular localization of NS5 and hnRNP A2, Vero cells expressing HA-hnRNP A2 were infected with JEV and examined by confocal microscopy at 24 h postinfection. Both hnRNP A2 and NS5 were colocalized in the ER of cells infected with JEV (Fig. 5D). These results suggest that hnRNP A2 interacts with JEV core protein and NS5A around the ER (Fig. 4B) and in the ER, respectively. To determine the region in NS5 responsible for the interaction with hnRNP A2, three HA-NS5 mutants, lacking the MTase region (ΔMET), the internal linker region (ΔILR), and the RdRp region (ΔRdRp), were coexpressed with FLAG-hnRNP A2 in JEV-SGR-293T cells. FLAG-hnRNP A2 was immunoprecipitated with each of the NS5 constructs except for the mutant lacking the MTase region (Fig. 5E). On the other hand, NS5 was coprecipitated with full-length hnRNP A2 (FLAG-A2 Full) and with a mutant lacking the RRM1 domain (FLAG-A2 ΔRRM1) but not with mutants lacking either RRM2 or GRD (FLAG-A2 ΔRRM2 or FLAG-A2 ΔGRD) (Fig. 5F). These results indicate that RRM2 and GRD in hnRNP A2 participate in the interaction with the MTase region in NS5 as well as with the C-terminal region of the JEV core protein, as described above (Fig. 1E).

hnRNP A2 interacts with the 5′ UTR of the negative-sense JEV RNA and facilitates viral RNA synthesis. Next, to determine the interaction between hnRNP A2 and JEV RNA, 293T cells expressing HA-hnRNP A2 were inoculated with JEV, and the cell lysates were immunoprecipitated with an anti-HA antibody at 24 h postinfection. RNAs extracted from the precipitates were subjected to RT-PCR to detect JEV RNA. JEV NS1 RNA was detected in the precipitates obtained by anti-HA antibody for cells expressing HA-hnRNP A2 (Fig. 6A), suggesting that hnRNP A2 associates with JEV RNA. Several hnRNPs have been shown to interact with the UTR of the viral RNA of positive-strand RNA viruses, such as poliovirus and enterovirus 71 (5, 35). To determine the region in the UTRs of JEV responsible for the interaction with hnRNP A2, biotin-labeled 5′ and 3′ UTRs of the positive- and negative-sense JEV RNAs were synthesized *in vitro* (Fig. 6B), and a pulldown assay was carried out using streptavidin beads to capture the biotinylated viral RNA associated with HA-hnRNP A2 in 293T cells. HA-hnRNP A2 was pulled down with the 5′ UTR of the negative-strand viral RNA but not with other viral RNAs. To further confirm the interaction between hnRNP A2 and the viral RNA, GST-hnRNP A2 prepared in *E. coli* was incubated with the biotinylated 5′ UTR of the negative-strand JEV RNA or 3′ UTR of the positive-strand JEV RNA, and the viral RNA interacting with GST-hnRNP A2 was detected by Northern blot analyses using streptavidin. The 5′ UTR of the negative-strand JEV RNA was detected in the complex (Fig. 6C). These results indicate that hnRNP A2 interacts directly with the 5′ UTR of the negative-strand JEV RNA.

As described for Fig. 3, knockdown of hnRNP A2 suppresses JEV propagation. To further examine the roles of hnRNP A2 in viral RNA replication in more detail, syntheses of the positive- and negative-strand viral RNAs were determined for cells transfected with si-A2#2 targeted to hnRNP A2 and inoculated with JEV at 24 h posttransfection. Total RNAs extracted from the infected cells at various time points were reverse transcribed by using strand-specific primers for either the 5′ UTR of the negative-strand JEV RNA or the 3′ UTR of the positive-strand JEV RNA, with oligo(dT) primers used for

and AF633-conjugated anti-mouse IgG antibodies, respectively. (C) HA-hnRNP A2 was expressed in 293T cells, which were infected with JEV at an MOI of 1.0 and subjected to immunoprecipitation with mouse anti-HA MAb (HA11) at 24 h postinfection. The immunoprecipitates were subjected to immunoblotting using rat anti-HA MAb (3F10), rabbit anti-core PAb, or rabbit anti-calregulin PAb. (D) JEV-SGR-293T cells were transfected with empty vector (EV) or a plasmid encoding FLAG-Core, and the level of JEV RNA (NS5) was determined by real-time PCR at 48 h posttransfection and calculated as a percentage of the control β-actin mRNA level. The data are representative of three independent experiments. Error bars indicate the standard deviations of the means. The significance of differences between the means was determined by Student's *t* test.



β-actin mRNA as an internal control, and JEV RNA and β-actin mRNA levels were determined by real-time PCR. Syntheses of both the positive- and negative-strand viral RNAs were delayed from 12 h postinfection in the hnRNP A2 knockdown cells (Fig. 6D). These results suggest that hnRNP A2 facilitates viral RNA synthesis through interaction with core, NS5, and the 5′ UTR of negative-strand viral RNA.

DISCUSSION

The flavivirus core protein is a multifunctional protein involved in viral replication and pathogenesis. The core protein binds to the viral RNA and forms a nucleocapsid in the cytoplasm as a structural protein (23). Furthermore, some portion of the core protein of flaviviruses localizes in the nucleus and associates with various host factors, such as B23 (62), Hsp70 (54), Daxx (50), and Jab1 (53). The DEN core protein has been shown to interact with hnRNP K and to regulate C/EBP-β-mediated transcription to modify the host cell environment by regulating the expression of pro- and antiviral factors for viral propagation (7). In addition, the core proteins of DEN, WNV, and hepatitis C virus (HCV), which belongs to the genus *Hepacivirus* within the family *Flaviviridae*, have functions of inducing or inhibiting the apoptosis associated with host factors, suggesting that the core protein of flaviviruses participates not only in viral assembly (as a structural protein) but also in pathogenesis (as a nonstructural protein) (13, 34, 43, 58, 70). The flavivirus core protein is not essential for RNA replication, since NS proteins alone are sufficient for efficient replication of the subgenomic viral RNA (24), while the core protein has been suggested to augment viral RNA replication (48).

In this study, we demonstrated that the JEV core protein specifically interacts with hnRNP A2 and participates in viral replication. Many RNA-binding proteins, including members of the hnRNP complex, have been shown to participate in the life cycles of several positive-strand RNA viruses, such as poliovirus (5), enterovirus 71 (35, 36), Sindbis virus (36), HCV (25), and DEN (7, 20, 51), through an interaction with viral RNA. The RdRp of the positive-strand RNA viruses transcribes the viral RNA into a complementary negative-strand RNA and generates double-stranded RNA, which serves as a replicative intermediate for production of a large excess of positive-strand genomic RNA (32). In the case of poliovirus, hnRNP C1/C2 has been shown to be involved in the initiation of viral RNA synthesis through the interaction with viral replication polypeptides and the 3′ UTR of the negative-strand RNA (5). In this study, we have shown that the JEV core protein interacts with hnRNP A2 and participates in RNA replication through recruitment of hnRNP A2 to the ER; however, enhancement of JEV replication could not be explained by the interaction of the core protein with hnRNP A2. Therefore, we further examined the factors involved in the enhancement of JEV replication and found that hnRNP A2 also associates with NS5 and the 5′ UTR of negative-strand viral RNA. These findings suggest that hnRNP A2 participates in positive-strand RNA synthesis through the interaction with viral proteins and RNA. Further studies are needed to clarify the molecular mechanisms by which hnRNP A2 promotes viral RNA replication.

hnRNP A2 is the most abundant of the hnRNP family proteins and is the first *trans*-acting factor described to be involved in neural mRNA trafficking (59). In addition, hnRNP A2 participates in virtually all aspects of mRNA processing, including packaging of nascent transcripts, splicing of pre-mRNAs, and translational regulation (16), and plays crucial roles in post-transcriptional regulation by shuttling between the nucleus and the cytoplasm with mRNA (8). hnRNP A2 also participates in telomere biogenesis, and its overexpression has been described for many cancer cell lines derived from the breast, pancreas, liver, and gastrointestinal tract (30, 31, 72, 75). In virus infections, hnRNP A2 has been shown to enhance mouse hepatitis virus RNA synthesis (60) and to regulate the trafficking of genomic RNA of human immunodeficiency virus (33). Although in this study we have demonstrated that hnRNP A2 interacts with the 5′ UTR of negative-strand JEV RNA, it has been shown previously that hnRNP A2 interacts with the 3′ UTR of positive-strand DEN RNA (55). This discrepancy might be attributable to differences in sequence and/or structure of the UTRs between JEV and DEN, as reported previously (21). Localization of hnRNP A2 was changed from the nucleus to the cytoplasm upon infection with JEV, as seen in many other virus infections (5, 25, 35). In addition, expression of the JEV core protein alone induced retention of hnRNP A2 in the ER and facilitated RNA replication in the replicon cells, suggesting that interaction of the core protein with hnRNP A2 is important for RNA replication of JEV.

FIG. 5. hnRNP A2 forms a complex with JEV core protein and NS5 via JEV RNA. (A) FLAG-hnRNP A2 was coexpressed with HA-JEV NS proteins in JEV subgenomic replicon (JEV-SGR-293T) cells and immunoprecipitated (IP) with mouse anti-FLAG MAb (M2). The immunoprecipitates were subjected to immunoblotting (IB) using rabbit anti-FLAG PAb. (B) FLAG-Core and HA-hnRNP A2 were coexpressed in JEV-SGR-293T cells, and cell lysates were treated with or without RNase A (10 μg/ml) for 30 min at 4°C and immunoprecipitated with mouse anti-HA MAb (HA11). The immunoprecipitates were subjected to IB with rabbit anti-FLAG PAb, mouse anti-NS5 MAb, or rat anti-HA MAb (3F10). (C) HA-hnRNP A2 was expressed in 293T cells, which were infected with JEV at an MOI of 1.0 and subjected to IP with mouse anti-HA MAb (HA11) at 24 h postinfection. The cell lysates were pretreated with or without RNase A (10 μg/ml) for 30 min at 4°C. The immunoprecipitates were subjected to IB using rabbit anti-core PAb, mouse anti-NS5 MAb, or rat anti-HA MAb (3F10). (D) Vero cells infected with JEV at an MOI of 1.0 and fixed with cold acetone at 24 h postinfection. JEV NS5, HA-hnRNP A2, and calregulin, which is an ER marker, were stained with mouse anti-NS5 MAb, rat anti-HA MAb (3F10), and anti-calregulin rabbit PAb (H-170), followed by AF488-conjugated anti-mouse IgG, AF594-conjugated anti-rat IgG, and AF633-conjugated anti-rabbit IgG, respectively. (E) Interaction of hnRNP A2 with deletion mutants of JEV NS5. FLAG-hnRNP A2 and a series of deletion mutants of HA-NS5 were cotransfected into JEV-SGR-293T cells. Deletion mutants of HA-NS5 in cell lysates were immunoprecipitated with mouse anti-HA MAb (HA11), and the immunoprecipitates were subjected to IB with mouse anti-FLAG MAb (M2) or rat anti-HA MAb (3F10). (F) Interaction of JEV NS5 with deletion mutants of hnRNP A2. A series of deletion mutants of FLAG-hnRNP A2 were expressed in JEV-SGR-293T cells and were immunoprecipitated with mouse anti-FLAG MAb (M2). The immunoprecipitates were subjected to IB with rabbit anti-FLAG PAb or mouse anti-NS5 MAb.

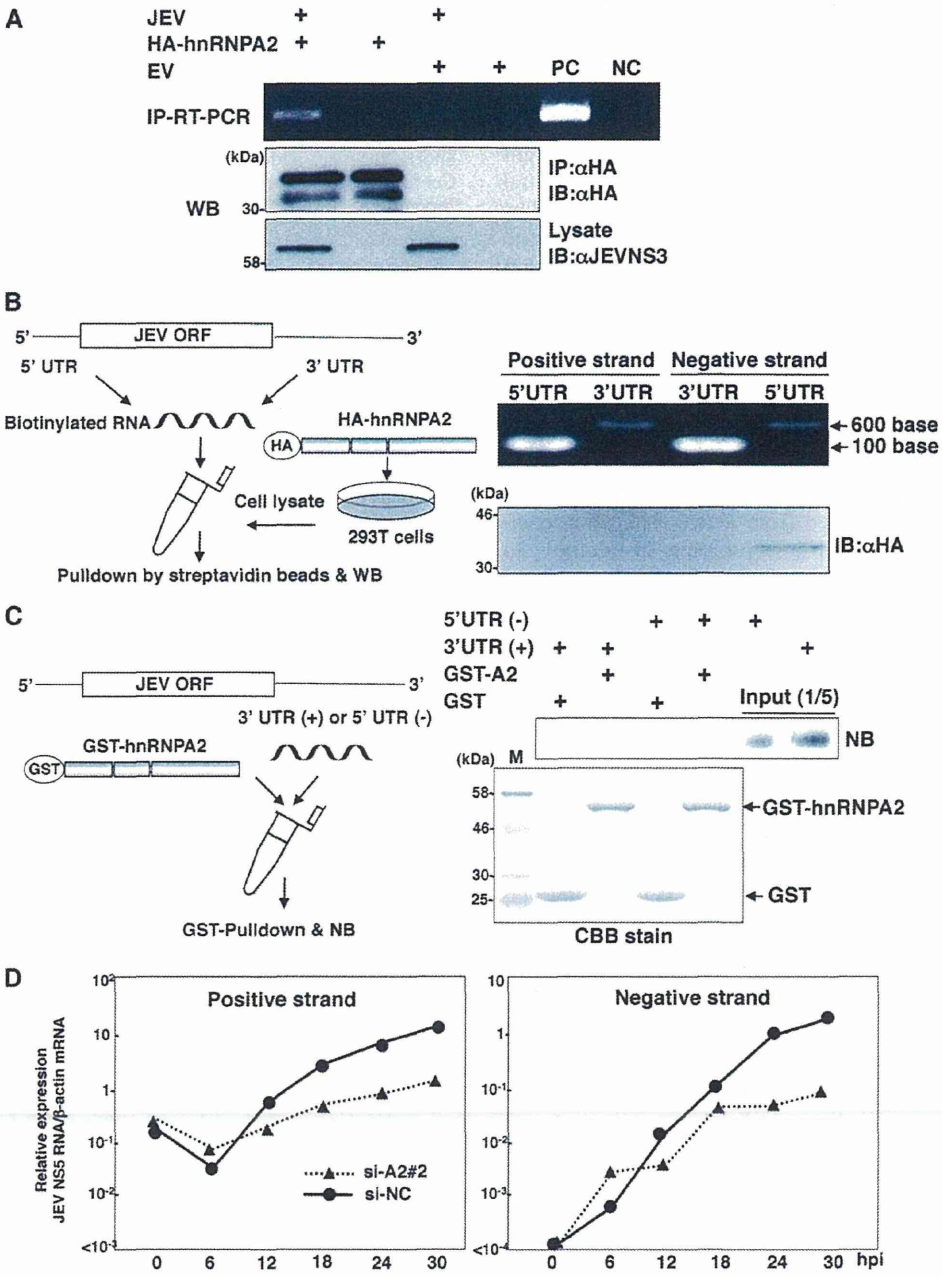


FIG. 6. hnRNP A2 interacts with the 5′ UTR of the negative-strand JEV RNA and facilitates viral RNA synthesis. (A) A plasmid encoding HA-hnRNP A2 was transfected into 293T cells, which were infected with JEV at an MOI of 1.0 at 24 h posttransfection. Cell lysates harvested at 24 h postinfection were immunoprecipitated with mouse anti-HA MAb (HA11) and subjected to IB with rat anti-HA MAb (3F10). RNAs extracted from the immunoprecipitates were subjected to RT. JEV RNA was detected by PCR using primers targeting the NS1 region. (B) Cell lysates from 293T cells transfected with a plasmid encoding HA-hnRNP A2, prepared at 24 h posttransfection, were incubated with the biotin-labeled 5′ or 3′ UTR of the positive- or negative-sense JEV RNA for 15 min at 30°C. After pulldown by streptavidin, protein complexes were subjected to IB with mouse anti-HA MAb (HA11). (C) GST-fused hnRNP A2 prepared in bacteria was incubated with the biotin-labeled 3′ UTR of positive-sense JEV RNA or 5′ UTR of negative-sense JEV RNA for 15 min at 30°C. RNAs extracted from the precipitates obtained by GST pulldown were subjected to Northern blotting (NB) with streptavidin. CBB, Coomassie brilliant blue. (D) Total RNA extracted from 293T cells transfected with si-A2#2 or si-NC and infected with JEV at an MOI of 10 at 24 h posttransfection was subjected to RT using strand-specific primers and an oligo(dT) primer. The levels of positive- and negative-strand JEV RNAs (NS5) were determined by real-time PCR and calculated as percentages of the control β-actin mRNA level. Closed triangles and circles indicate the relative RNA levels in cells transfected with si-A2#2 and si-NC, respectively. The data are representative of three independent experiments.

Although replication and packaging of the viral genome remain obscure steps in the life cycle of flaviviruses, coupling between RNA replication and particle assembly has been suggested to occur in several positive-strand RNA viruses (22, 52). Replication of flaviviruses takes place in virus-induced intracellular membrane structures on the ER known as replication complexes, which contain NS proteins, viral RNA, and host factors essential for replication, and is suggested to circumvent the activation of the host immune response triggered by viral RNA (45). The invagination of the ER membrane induced by the expression of NS proteins is connected to the cytoplasm through pores, which allows entry of nucleotides and other factors required for RNA replication and for exit of the newly synthesized RNA to the sites for translation and particle assembly (10, 44, 65). The majority of the viral RNA species in the replication complex exist as double-stranded replicative intermediates, and newly transcribed genomic RNA is exported efficiently from the replication complex to the assembly sites (10). In addition, to minimize the production of defective viral RNA transcribed from the error-prone viral RdRp (22), viral RNA must be synthesized from active replication complexes consisting of viral and host proteins; in addition, to circumvent the induction of innate immunity, the viral genome should be packaged immediately into viral particles. Therefore, structural and nonstructural viral proteins participate coordinately in viral RNA replication and particle formation, as reported for the participation of the NS3 protein of YFV in viral assembly (56). Although the biological significance of the interaction between the JEV core protein and hnRNP A2 for viral replication is unclear, it might be feasible to speculate that the core protein recruits hnRNP A2 to the replication complex to promote viral RNA replication.

The property of hnRNP family proteins shuttling between the cytoplasm and the nucleus has been suggested to participate in the maintenance of cellular homeostasis in response to stress stimuli such as heat shock (57), amino acid starvation (41), mitochondrial dysfunction (11), and nucleolar stresses (73). Infection of positive-strand RNA viruses modulates the host environment for efficient viral propagation through the remodeling of host proteins. For instance, the core protein of WNV suppresses the expression of the ER stress-protective protein OASIS and inhibits the antiviral response through induction of ER stress (2, 63), while poliovirus infection increases nuclear envelope permeability and replaces nuclear proteins required for efficient viral replication in the cytoplasm (3). In this study, hnRNP A2 was localized in both the nucleus and the cytoplasm upon infection with JEV or expression of the JEV core protein, suggesting that the JEV core protein plays an important role in the replication of JEV RNA through a modification of the host cellular environment.

Viruses are obligatory intracellular parasites, and therefore they are completely dependent on infected cells to supply energy, chemicals, and much of the machinery required for their replication. In the present study, we identified hnRNP A2 as one of the host factors participating in JEV propagation. The ER retention of hnRNP A2 through an interaction with the core protein leads to the interaction of hnRNP A2 with NS5 and the negative-strand viral RNA, resulting in the promotion of JEV RNA replication. This may help to open up a new area of inquiry into virus-cell interactions and could lead to an improved understanding of the mechanism of flavivirus RNA replication.

ACKNOWLEDGMENTS

We thank H. Murase and M. Tomiyama for their secretarial assistance. We also thank E. Konishi for providing a plasmid.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare, the Ministry of Education, Culture, Sports, Science, and Technology, and the Osaka University Global Center of Excellence Program. H. Katoh is a research fellow of the Japanese Society for the Promotion of Science.

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