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Complex Formation of NS5B with NS3 and NS4A Proteins of Hepatitis C Virus

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At present, the mechanism of replication of the HCV genome remains unclear. Recently, NS5B and NS3 of HCV have been shown to exhibit RNA-dependent RNA polymerase and helicase activities, respectively, both of which are indispensable for virus RNA replication. In this study, we examined the complex formation of NS5B with NS3 and NS4A, a cofactor for NS3. We show here that NS5B forms a complex with NS3 through an amino-terminal portion of NS3. The NS3-NS5B complex formation took place both in the presence and absence of NS4A. We also demonstrate that NS5B form a complex with NS4A in the absence of NS3. These results suggest that NS3, NS4A and NS5B interact with each other to form a complex that functions as part of the replication machinery of HCV.

HCV is a major causative agent of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus, whose genome is single-stranded, positive-sense RNA of approximately 9.5 kb. The HCV genome encodes a polyprotein consisting of about 3,010-3,033 amino acid residues. This polyprotein is processed into mature viral proteins by cellular as well as viral proteases, which are arranged in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (1–3). The NS3 protein of HCV is predicted to possess protease (4) and helicase activities (5) and is associated with NS4A (6–9), which anchors NS3 to the endoplasmic reticulum membrane. Moreover, we found that NS3 inhibited actinomycin D-induced apoptosis in NIH3T3 cells (10). NS5B has been shown to be an RNA-dependent RNA polymerase (11).

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Abbreviations: DEN 2, dengue virus type 2; FITC, fluorescein isothiocyanate; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; NS, nonstructural protein; NS3ΔC, a carboxy-terminally truncated NS3; NS3ΔNΔC, an amino-/carboxy-terminally truncated NS3; NS3F, the full-size NS3; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate; UTR, untranslated region.

At present, replication cycle of HCV remains unknown, due to the lack of an appropriate in vitro culture system. To develop an effective antiviral drug(s) against HCV, the mechanism of HCV replication needs to be elucidated. Being a member of the *Flaviviridae* family, HCV may share a common mechanism(s) of virus replication with flaviviruses, such as dengue virus type 2 (DEN-2) and Japanese encephalitis virus (JEV). As with HCV, NS5 and NS3 of DEN-2 or JEV have been reported to have RNA-dependent RNA polymerase and helicase activities, respectively, and association between the two proteins has been demonstrated (12, 13). Thus, in DEN-2 and JEV, NS3 and NS5 are thought to be components of putative viral replicase. Moreover, NS3-NS5 complex of JEV has been shown to bind to the plus-strand 3′ untranslated region (UTR) of the viral genome (13). These findings lead us to an assumption that NS3 and NS5B of HCV might form a complex. We show here that NS5B of HCV interacts with NS3 and NS4A, a cofactor for NS3, to form NS3-NS4A-NS5B complex.

MATERIALS AND METHODS

Construction of expression plasmids. The entire NS5B region of the HCV genome was amplified by using a set of primers, NS5B-Eco-1 (sense; 5′-GAGGAATTCGCC**ATG**GGCTCGATGTCCTAC-3′; underline, *Eco*RI recognition site; boldface letters, translation initiation codon) and NS5B-Eco-2 (antisense; 5′-AGCTGAATTC**TCA**TCGGTTGGGGAGCAGGT-3′; underline, *Eco*RI recognition site; boldface letters, complementary sequence of a stop codon), from HCV MKC1a cDNA. Amplified cDNA fragment was subcloned into the unique *Eco*RI site of pBlueScript II SK- (Stratagene Cloning Systems, La Jolla, CA, USA) to generate pBSns5B. Plasmids for the expression of the full-size NS3 (NS3F), carboxy-terminally truncated NS3 (NS3ΔC), amino-/carboxy-terminally truncated NS3 (NS3ΔNΔC), and NS4A, which are designated as pBSns3/1027-1657, pBSns3/1027-1459, pBSns3/1201-1459 and pBSns4A/1658-1711, respectively, were described previously (14, 15). These plasmids were used in a vaccinia T7 hybrid expression system (14, 15). For expression of NS5B, NS3F, NS3ΔC and NS3ΔNΔC by plasmid based transient expression system in COS-7 cells, each of the HCV cDNA fragments were transferred into the unique *Eco*RI site of pSG5 (Stratagene Cloning Systems, La Jolla, CA, USA) to generate pSG5ns3/1027-1657, pSG5ns3/1027-1459, pSG5ns3/1201-1459 and pSG5ns5B.

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Transient expression of HCV proteins. HCV proteins were expressed by vaccinia T7 hybrid expression system in HeLa cells, as described previously (14, 15) or by plasmid based transient expression system in COS-7 cells. In brief, cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3). After 1 h, the cells were transfected with the pBlueScript based-expression plasmids using Lipofectin reagent (Life Technologies, Inc. Gaithersburg, MD, USA). After cultivation for 12-16 h, the cells were analyzed for HCV protein expression. For plasmid based transient expression, COS-7 cells were transfected with expression plasmids using calcium phosphate co-precipitation method and were examined 48 h post-transfection.

Indirect immunofluorescence analysis. HeLa cells transfected with the plasmids were fixed with methanol at −20°C for 20 min. The fixed cells were incubated with mouse monoclonal antibody against either NS3, NS4A or NS5B, washed three times with PBS, and then incubated with FITC-conjugated goat anti-mouse IgG. For double staining experiments, a patient's serum that strongly reacted to NS3 but barely to NS5B or NS4A, and anti-NS5B monoclonal antibody were used as first antibodies, and FITC-conjugated rabbit anti-human IgG and TRITC-conjugated goat anti-moue IgG, respectively, were used as second antibodies. After washing three times, the samples were observed under a fluorescent microscope.

Immunoprecipitation analysis. HeLa cells transfected with the plasmids were labeled at 12 h postinfection with 25 μCi of ^{35}S-translabel (Amersham) per ml in serum-free Dulbecco's minimal essential medium. Cell lysates were prepared in RIPA buffer consisting of 150 mM NaCl, 0.5% Triton X-100, and 10 mM Tris-HCl (pH 7.5). After being clarified by microcentrifugation, the cell lysates were incubated for 1 h at 4°C with the anti-NS3 or anti-NS5B antibody and 10 μl of protein G/protein A sepharose (Oncogene Research Products, Cambridge, MA, USA). After washing three times with RIPA buffer, the immunoprecipitates were subjected to SDS-PAGE and visualized by using BAS2000 system.

For transfected COS-7 cells, cells lysates were prepared in RIPA buffer consisting of 150 mM NaCl, 0.5% Triton X-100, and 10 mM Tris-HCl (pH 7.5). Cell lysates prepared from transfected COS-7 cells were incubated for 1 h at 4°C with the anti-NS3 polyclonal antibody and 10 μl of protein G/protein A sepharose. After being washed three times with RIPA buffer, immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting with the NS5B monoclonal antibody, using the ECL system (Amersham).

Cell fractionation and immunoblotting analysis. To analyze subcellular localization of NS5B, the nuclei were separated from the cytoplasm, as described previously (14, 15). In brief, transfected cells were washed once with PBS, collected in 0.5 ml of PBS by a scraper and divided into two microfuge tubes; one was used for cytoplasmic fractionation and the other for nuclear fractionation. Cytoplasmic fraction was obtained in 100 μl of an extraction buffer A consisting of 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl at 4°C for 30 min. To obtain nuclear fraction, cells were disrupted by a Dounce homogenizer in an ice-cold buffer B consisting of 10 mM Hepes-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, and centrifuged at 2,500 x g for 5 min. The pellet was suspended in a buffer C consisting of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100, and overlaid onto 0.5 M sucrose solution. After centrifugation at 3,000 × g for 10 min, sedimented nuclei were disrupted by ultrasonication (200 W, 150 sec) in 100 μl of buffer A. The cytoplasmic and nuclear fractions of the cells were analyzed by SDS-PAGE followed by immunoblotting with the NS5B monoclonal antibody.

RESULTS

NS5B interacts with NS3 through an amino-terminal portion of NS3. When NS3F and NS5B were each expressed alone, they were localized both in the cytoplasm and the nucleus (Fig. 1, a, b). When co-expressed, NS3F and NS5B were co-localized, exhibiting practically the same staining pattern, as determined by double staining analysis (Fig. 1, c, d). Also, NS3ΔC and NS5B were shown to be co-localized (Fig. 1, e, f). On the other hand, NS3ΔNΔC exhibited a staining pattern different from that of NS3F and NS3ΔC, and was not co-localized with NS5B (Fig. 1, g, h). To confirm the possible interaction between NS3 and NS5B, immunoprecipitation analysis was performed. NS3F and NS5B were co-precipitated from co-transfected cell lysates by either anti-NS3 or anti-NS5B monoclonal antibody (Fig. 2A). Likewise, NS3ΔC was shown to be co-localized with NS5B (Fig. 2B). On the other hand, NS3ΔNΔC and NS5B were not co-precipitated by the same monoclonal antibodies, although they were each precipitated by the corresponding antibodies (Fig. 2C).

To rule out the possibility that the NS3-NS5B interaction was mediated by vaccina viral proteins, we used a plasmid-based transient expression system in COS-7 cells that does not utilize recombinant vaccinia virus. NS5B was again shown to be co-precipitated with NS3F and NS3ΔC, but not with NS3ΔNΔC (Fig. 3). Taken together, these results strongly suggest that NS5B interacts with NS3 through an amino-terminal portion of NS3.

NS4A, a cofactor for NS3, does not interfere with complex formation between NS3 and NS5B. Since NS3 has been shown to form a stable complex with NS4A through an amino-terminal portion of NS3, we investigated the effect of NS4A on NS3-NS5B complex formation. Double staining analysis revealed that, even when NS4A was additionally expressed, NS5B was co-localized with NS3 (Fig. 4, a, b). It should also be noted that the subcellular localization pattern of NS3F and NS5B is indistinguishable from that of NS4A expressed alone (Fig. 4, c). These results clearly demonstrated that NS5B interacts with NS3 even in the presence of NS4A, suggesting the possibility that NS3, NS4A and NS5B form a complex. To confirm the association among them, immunoprecipitation analysis was performed. Anti-NS3 monoclonal antibody co-precipitated NS5B and NS4A in addition to NS3F (Fig. 5). Similarly, anti-NS4A monoclonal antibody co-precipitated NS3F and NS5B, and anti-NS5B monoclonal antibody co-precipitated NS3F and NS4A, in addition to their corresponding antigens.

NS5B interacts with NS4A in the absence of NS3. A question was then raised as to whether or not NS5B interacts with NS4A in the absence of NS3. Immunofluorescence analysis of cells co-transfected with NS4A and NS5B revealed co-localization of the two proteins in the cells (data not shown). Immunoprecipitation analysis revealed that anti-NS4A and anti-NS5B monoclonal antibodies co-precipitated NS5B

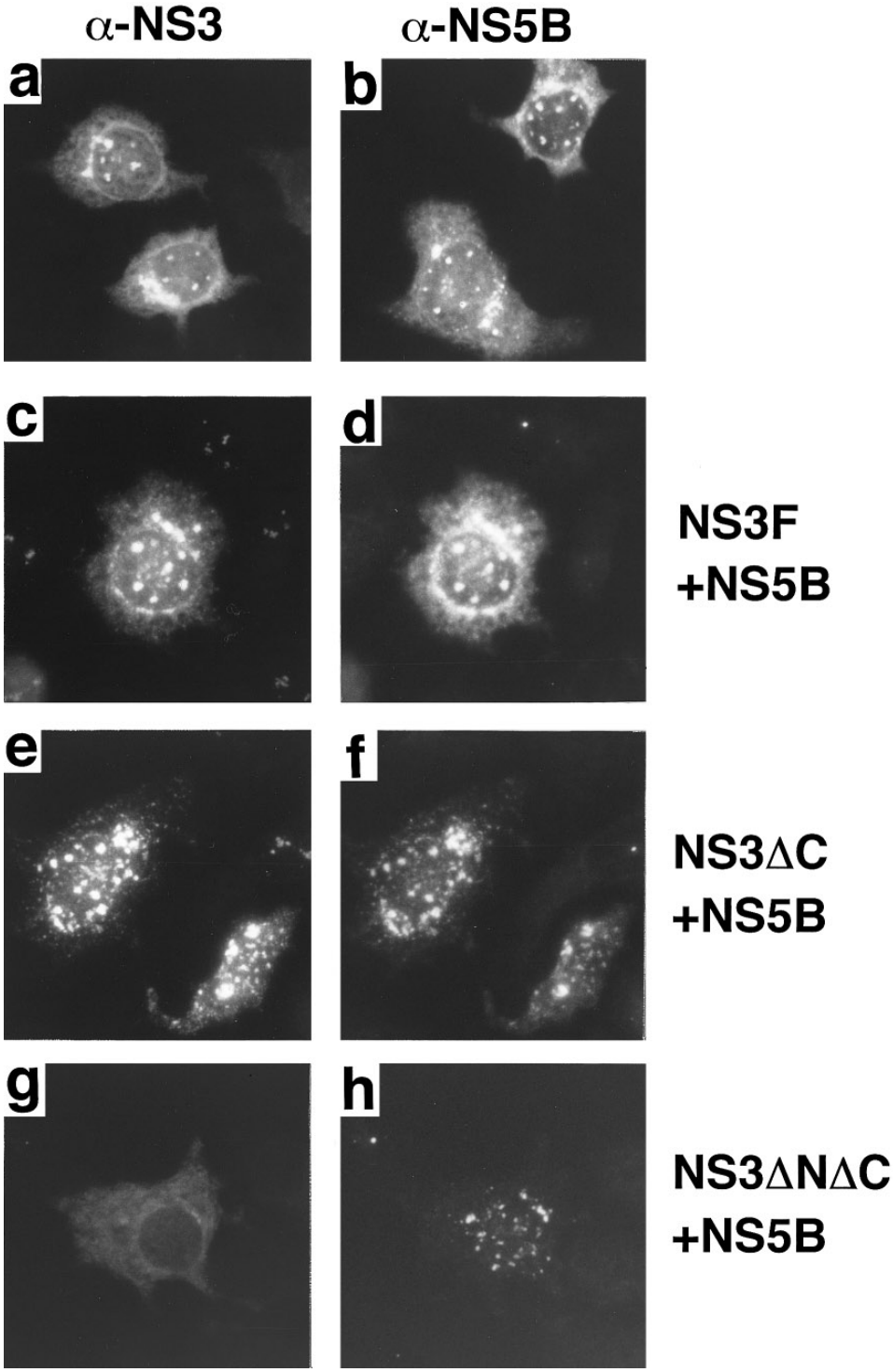

FIG. 1. Subcellular co-localization of NS3 and NS5B in HeLa cells. Cells transfected with pBSns3/1027-1657 (a) and pBSns5B (b) were stained with anti-NS3 and anti-NS5B monoclonal antibodies, respectively. In double staining analysis, cells co-transfected with pBSns3/1027-1657 and pBSns5B (c, d), cells co-transfected with pBSns3/1027-1459 and pBSns5B (e, f) or cells co-transfected with pBSns3/1201-1459 and pBSns5B (g, h) were stained simultaneously with anti-NS3 human serum (c, e, g) and anti-NS5B monoclonal antibody (d, f, h).

and NS4A, respectively, in addition to their corresponding antigens (Fig. 6). Thus, NS5B was shown to interact with NS4A even in the absence of NS3. Taken together, these results strongly suggest that NS3, NS4A and NS5B interact with each other to form a complex.

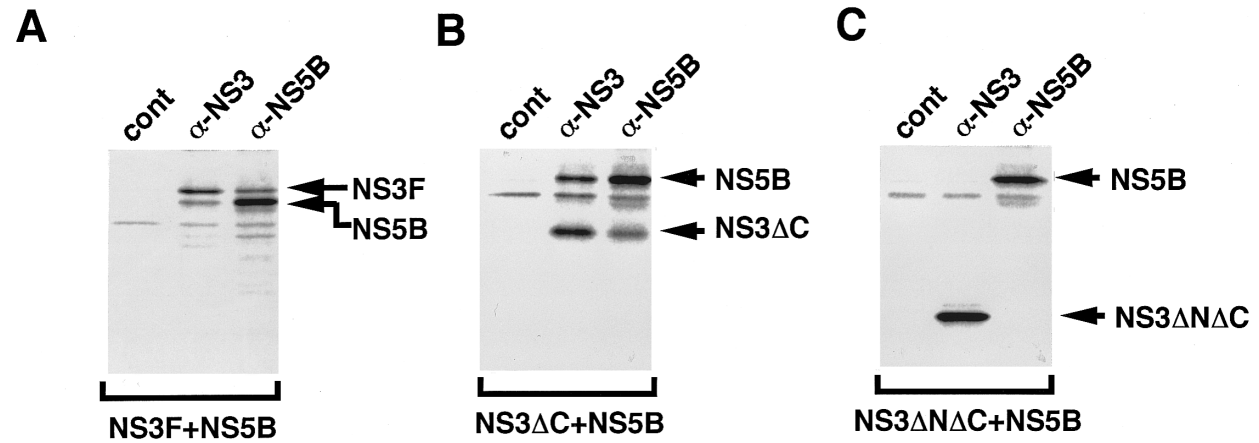

FIG. 2. Complex formation between NS3 and NS5B in HeLa cells. Lysates of cells co-transfected with pBSns3/1027-1657 and pBSns5B (A), pBSns3/1027-1459 and pBSns5B (B) or pBSns3/1201-1459 and pBSns5B (C) were immunoprecipitated with either normal mouse IgG as a control (cont), anti-NS3 or anti-NS5B monoclonal antibody, and analyzed by SDS-PAGE.

NS5B is present in both cytoplasmic and nuclear fractions. When expressed alone, NS5B appeared to be localized both in the cytoplasm and the nucleus (Fig. 1b). To confirm the nuclear localization of NS5B, cell fractionation analysis was performed. NS5B was detected both in the cytoplasmic and nuclear fractions of the cells (Fig. 7), the results being consistent with the immunofluorescence observations (Fig. 1b).

DISCUSSION

Both NS3 and NS5B of HCV have been considered to be important for replication of viral RNA, with the former functioning as RNA helicase and the latter RNA-dependent RNA polymerase. In this study, we examined the interaction between NS3 and NS5B. Immunofluorescence and immunoprecipitation analyses demonstrated that NS5B interacts with NS3 through an amino-terminal portion of NS3 in vaccinia T7 hybrid expression system (Figs. 1 and 2). Practically the same results were obtained by using another plasmid-based transient expression system in COS-7 cells that does not utilize vaccinia virus (Fig. 3). It is not unreasonable to assume that the interaction between NS3 and NS5B takes place also in HCV-infected cells. As is the case with DEN-2 or JEV, NS3-NS5B complex is likely to be part of replication machinery of HCV. In the case of DEN-2, NS5 has been shown to be a phosphoprotein and unphosphorylated form of NS5 interacts with NS3 (12). Recently, NS5B of HCV has been shown to be phosphorylated (16). Thus, status of phosphorylation of NS5B might influence the complex formation between NS3 and NS5B. Moreover, immunofluorescence and cell fractionation analyses demonstrated that NS5B is present in the nucleus (Figs. 1b and 7). In the case of DEN-2, Kapoor et al. (12) has shown that NS5B is present in the nucleus. Thus, these findings imply that replication machinery of HCV resembles that of DEN-2.

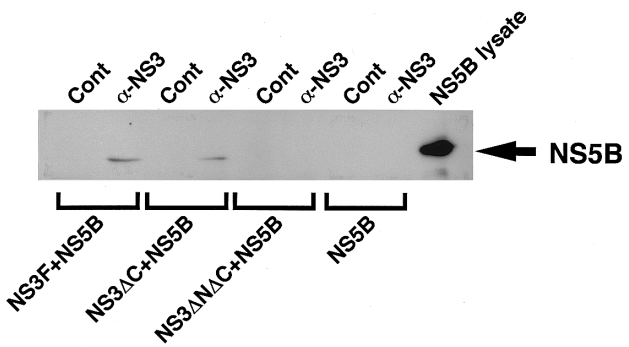

FIG. 3. Complex formation between NS3 and NS5B in COS-7 cells. Lysates of cells co-transfected with pSG5ns3/1027-1657 and pSG5ns5B (lane 1), pSG5ns3/1027-1459 and pSG5ns5B (lane 2), pSG5ns3/1201-1459 and pSG5ns5B (lane 3) and pSG5 and pSG5ns5B (lane 4) were immunoprecipitated with either normal mouse IgG as a control (Cont) or anti-NS3 polyclonal antibody, and analyzed by immunoblotting with anti-NS5B monoclonal antibody.

Since it has been shown that NS3 tightly interacts with NS4A (6-9), we investigated the effect of NS4A on NS3-NS5B complex formation. Our results clearly demonstrated that, even when NS4A was expressed additionally, NS5B interacted with NS3 (Figs. 3 and 4). It should be noted that subcellular localization of NS3-NS5B complex was indistinguishable from that of NS4A expressed alone (Fig. 3c), the latter having been reported to be localized in the endoplasmic reticulum membrane (6). Moreover, NS5B was shown to interact with NS4A in the absence of NS3 (Fig. 5). Thus, NS3, NS4A and NS5B are likely to interact with each other to form a complex on the endoplasmic reticulum membrane. To our knowledge, this is the first report demonstrating that NS5B interacts with NS3 and NS4A.

The HCV genome is assumed to replicate on the en-

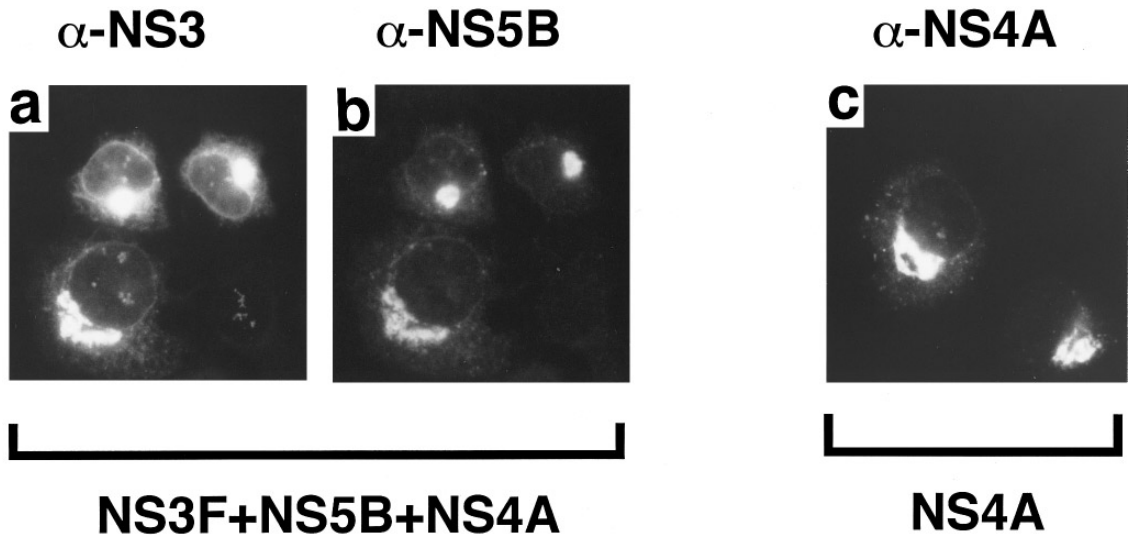

FIG. 4. Subcellular co-localization of NS3 and NS5B in the presence of NS4A. Cells co-transfected with pBSns3/1027-1657, pBSns4A/1658-1711 and pBSns5B were double-stained with anti-NS3 human serum (a) and anti-NS5B monoclonal antibody (b). Cells transfected with pBSns4A/1658-1711 alone were stained with anti-NS4A monoclonal antibody (c).

doplasmic reticulum membrane (16). Based on our present results and those reported previously (1), it is likely that NS4A anchors NS3 and NS5B to the endoplasmic reticulum membrane. Therefore, NS4A can be considered to play an important role in supporting the replication of HCV RNA, functioning as a co-factor for NS5B. Indeed, NS5B co-expressed with NS4A from an HCV polyprotein precursor is more active in an RNA polymerase activity than that expressed in the absence of NS4A (11).

The detailed structure of the 3′ UTR of the HCV genome, including the 3′X sequence, was determined (17, 18) and certain cellular proteins were shown to bind specifically to this region (19). As is the case with JEV (13), NS3-NS5B complex of HCV would also bind

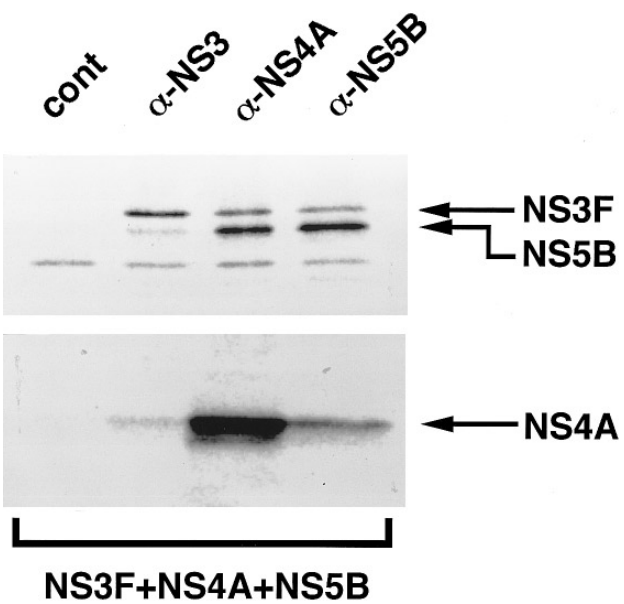

FIG. 5. Complex formation among NS3, NS4A and NS5B in HeLa cells. Lysates of cells co-transfected with pBSns3/1027-1657, pBSns4A/1658-1711 and pBSns5B were immunoprecipitated with either normal mouse IgG (cont), anti-NS3 or anti-NS5B monoclonal antibody, and analyzed by SDS-PAGE.

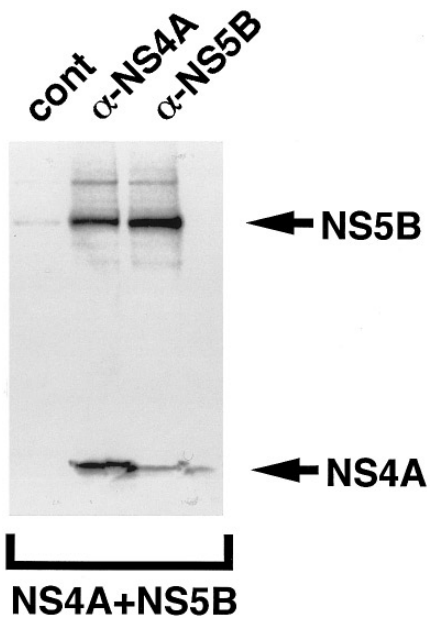

FIG. 6. Complex formation between NS4A and NS5B in HeLa cells. Lysates of cells co-transfected with pBSns4A/1658-1711 and pBSns5B were immunoprecipitated with either normal mouse IgG (cont), anti-NS4A or anti-NS5B monoclonal antibody, and analyzed by SDS-PAGE.

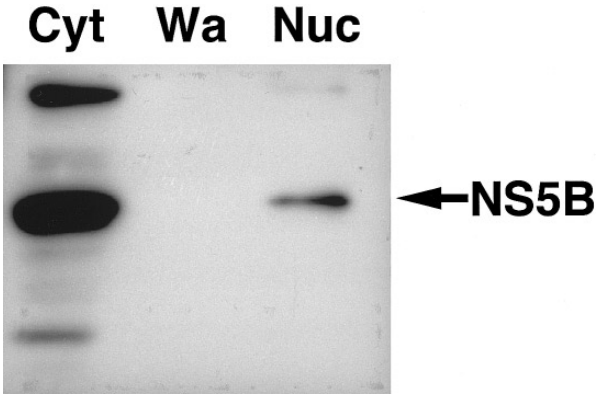

FIG. 7. Subcellular localization of NS5B in HeLa cells. Cells transfected with pBSns5B was fractionated into subcellular components. C, cytoplasmic fraction; Wa, third washings of nuclei; N, nuclear fraction.

to the plus-strand 3′ UTR of the HCV genome, functioning as part of the replication machinery. It should be emphasized, however, that the replication machinery may consist of not only NS3-NS5B but also other NS proteins. We have noticed that NS5B interacts with NS5A, though not so strongly as with NS3 or NS4A, but does not interact with NS4B in the absence of other NS proteins of HCV (data not shown). The interaction between NS5A and NS5B was reported also by other researchers (20). Moreover, it has recently been reported that NS3, NS4A and NS4B form a complex (21) and that NS4A interacts with NS5A in COS cells (22). Taken together, these results suggest that NS3, NS4A, NS4B, NS5A and NS5B interact with each other, either directly or indirectly, to form a replication complex. Further analysis is now in progress to look into more detailed interaction among those HCV proteins and, possibly, certain cellular proteins. These findings will give us a clue to elucidate the mechanism of replication of HCV.

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