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Hepatitis C Virus NS5B Protein Is a Membrane-Associated Phosphoprotein with a Predominantly Perinuclear Localization

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Hepatitis C virus NS5B protein is an RNA-dependent RNA polymerase. To investigate the properties and function of this protein, we have expressed the NS5B protein in insect and mammalian cells. NS5B was found to be present as fine speckles in the cytoplasm, particularly concentrated in the perinuclear region, suggesting its association with the nuclear membrane, the endoplasmic reticulum, or the Golgi complex. This conclusion was supported by the biochemical demonstration that NS5B was associated with the membranes in the cells. Furthermore, it was shown that NS5B protein is a phosphoprotein. These properties may be related to its function as an RNA polymerase.

INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of blood-borne, non-A, non-B hepatitis (Choo *et al.,* 1989; Kuo *et al.,* 1989), which often leads to hepatocellular carcinoma (Saito *et al.,* 1990; Shimotohno, 1993). HCV contains a single-stranded, positive-sense RNA genome of approximately 9400 nucleotides, which shares sequence homology with flaviviruses and pestiviruses (Choo *et al.,* 1989; Inchauspe *et al.,* 1991; Kato *et al.,* 1990; Miller and Purcell, 1990; Takamizawa *et al.,* 1991). The viral genome has a highly conserved 5′-untranslated region (Han *et al.,* 1991) and encodes a single polyprotein of 3010 amino acids, which is proteolytically processed into multiple viral proteins (Grakoui *et al.,* 1993c; Hijikata *et al.,* 1991; Lin *et al.,* 1994; Manabe *et al.,* 1994). These include core (C), envelope (E1, E2), proteinase, helicase, replicase, and other functionally unidentified nonstructural proteins. The viral structural proteins are cleaved by a host signal peptidase (Han *et al.,* 1991; Hijikata *et a.,* 1991; Matsuura *et al.,* 1994), and the nonstructural proteins (NS) are processed by viral proteinases (Grakoui *et al.,* 1993a,b; Hahm *et al.,* 1993; Tomei *et al.,* 1993).

Studies of the biochemical properties and functional roles of HCV gene products have been hampered by the low level of protein expression in virus-infected tissues and the lack of an efficient cell culture system *in vitro.* Most of the biochemical studies so far have been focused on the viral structural proteins and nonstructural protein NS3, which is a serine proteinase. Among the other nonstructural proteins, NS4 is a cofactor for the NS3 proteinase (Failla *et al.,* 1994; Lin and Rice, 1995), and NS5B has RNA-dependent RNA polymerase (RDRP) activity, which has been detected *in vitro* but so far lacks demonstrable specificity in its template requirement (Behrens *et al.,* 1996). Because this activity is likely important for viral replication, we are particularly interested in understanding the biochemical properties of NS5B. In this study, using both mammalian and baculovirus expression systems, we found that NS5B is localized in the cytoplasm, preferentially in the perinuclear region, and associated with the membrane. Furthermore, we found that it is a phosphoprotein. These properties may be significant in HCV RNA synthesis.

MATERIALS AND METHODS

Expression of NS5B in insect cells by recombinant baculovirus

A cDNA corresponding to the NS5B-coding region of the Korean isolate of the HCV genome (genotype Ib) (Cho *et al.,* 1993) was amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase (Boehringer Mannheim). PCR primers were designed to create a *Bam*HI site at both ends, plus an artificial initiation codon (ATG). The PCR product was digested by *Bam*HI and inserted into the *Bam*HI sites of a baculovirus transfer vector, pVL941 (Hwang *et al.,* 1992). After confirmation of the sequence by the dideoxynucleotide chain termination se-

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quencing method (Sanger *et al.,* 1977), both wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA and recombinant transfer plasmid DNA were cotransfected into *Spodoptera frugiperda* (Sf9) cells, and recombinant baculovirus was selected and amplified as described previously (Hwang *et al.,* 1992). The Sf9 insect cells were cultured in Grace's insect tissue culture medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS). The AcNPV and recombinant baculoviruses were grown and assayed in Sf9 cells as previously described (Hwang and Lai, 1993; Hwang *et al.,* 1992). For expression of NS5B protein in insect cells, monolayers of Sf9 cells were infected with recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 10 and incubated at 27°. Cell lysates prepared at various time points after infection were analyzed by electrophoresis on a 10% SDS–PAGE gel and stained with Coomassie brilliant blue.

Expression of NS5B in mammalian cells

To construct the mammalian expression vector, the cDNA for the NS5B-encoding region was amplified by PCR and cloned into the *Bam*HI site of the plasmid pcDNA3 (Invitrogen) under the control of both a cytomegalovirus (CMV) immediate early promoter and a T7 promoter. Construction procedures were similar to those described above for the NS5B-expressing recombinant baculovirus. The expression of the NS5B was achieved by two different methods: in the first method, the protein was expressed from the T7 promoter of the plasmid by vTF7-3 recombinant vaccinia virus, which expresses T7 RNA polymerase (Fuerst *et al.,* 1986). For this purpose, COS 7 cells (Gluzman, 1981) were grown in Dulbecco's modified Eagle media (DMEM) supplemented with 5% FCS in a 60-mm-diameter dish and infected with vTF7-3 at a multiplicity of infection of 5. At 2 hr postinfection, the infected cells were transfected with either pcDNA3 or pcDNA3-NS5B plasmid (5 μg each) by using DOTAP (Boehringer Mannheim) as described previously (Hwang and Lai, 1994). Cells were harvested after incubation for 12 hr at 37° and analyzed by immunoblotting. In the second method, NS5B was expressed from the CMV promoter of the recombinant plasmid. For this purpose, the same transfection protocol was used, but in the absence of vaccinia virus infection. Cells were used for immunofluorescence analysis at 48 hr posttransfection.

Preparation of rabbit polyclonal antibody against NS5B and human HCV patient sera

Recombinant baculovirus-infected cell lysates were separated by SDS–PAGE (Fig. 1) and the NS5B protein band was carefully excised and purified by using a Bio-Rad electroeluter (Model 422) as previously described (Hwang *et al.,* 1992). Female New Zealand White rabbits were purchased from SamYook Lab Animal Center. Each animal was immunized with 100 μg of NS5B protein in complete Freund's adjuvant by multiple subcutaneous injections. Four booster injections were performed at 2- to 3-week intervals with 70 μg of NS5B protein in incomplete Freund's adjuvant per injection. Animals were anesthetized and whole sera were collected by cardiac puncture.

The rabbit antiserum was used at 1/2000 dilution for immunofluorescence or immunoprecipitation.

Human serum used in this study was obtained from a Korean chronic HCV (genotype Ib) patient. The antibody was of IgG class. The antibody was used at 1:250 dilution for immunoblotting and immunoprecipitation.

Immunoblot analysis

Proteins were separated by electrophoresis in 10% polyacrylamide gel containing 0.5% SDS and transferred to a nitrocellulose membrane (Millipore) by electrotransfer for 1 hr at 4°. The membrane was incubated in TBS buffer (20 m*M* Tris–HCl, 500 m*M* NaCl, pH 7.5) containing 3% gelatin for 1 hr at room temperature and washed twice with TTBS buffer [0.05% Tween 20 (v/v) in TBS]. The membrane was then incubated overnight at 4° with either human HCV patient sera or rabbit polyclonal antibody in TTBS buffer containing 1% gelatin. After washing several times with TTBS, the membrane was further incubated with either alkaline phosphatase-conjugated goat anti-human IgG or goat anti-rabbit IgG (Jackson ImmunoResearch) for 1 hr at room temperature. The bound antibody was visualized by incubating membrane in the carbonate buffer (100 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, pH 9.8) containing 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (BCIP)/*p*-nitroblue tetrazolium chloride (NBT) (Bio-Rad) reagents.

Immunofluorescence

COS 7 cells grown on chamber slides (Nunc, Inc., IL) were either mock-transfected or transfected with pcDNA3-NS5B recombinant plasmid DNA as described above. At 40 hr posttransfection, slides were washed in phosphate-buffered saline (PBS) and fixed in cold acetone for 5 min. Cells were rinsed in PBS twice and incubated with either preimmune serum or rabbit anti-NS5B antibody for 1 hr at 37°. After washing in PBS, cells were further incubated at 37° for 30 min with rhodamine-conjugated goat anti-rabbit IgG (American Qualex). Cells were washed several times in PBS and then examined using a Zeiss confocal microscope.

For Sf9 cells, a monolayer of insect cells infected with the recombinant baculovirus was processed at 36 hr postinfection as described above for COS 7 cells, except that either normal human serum or HCV patient serum

was used as the primary antibody and goat anti-human IgG (American Qualex) as a secondary antibody.

Membrane flotation analysis

This analysis was performed according to the previously described procedure (Matsumoto *et al.,* 1996; Sanderson *et al.,* 1994). Briefly, insect cells infected with recombinant baculoviruses were harvested at Day 3 postinfection, lysed in 0.5 ml hypotonic buffer (10 m*M* Tris–HCl, pH 7.5, 10 m*M* KCl, 5 m*M* $MgCl_2$), and further disrupted by passage through a 25-gauge hypodermic needle 10–20 times. Cell lysates were centrifuged at 1500 rpm for 10 min in a microfuge, and the supernatant was carefully collected. Approximately 0.5 ml of supernatant was dispersed into 3.0 ml of 72% sucrose in low-salt buffer (LSB, 50 m*M* Tris–HCl, pH 7.5, 25 m*M* KCl, 5 m*M* $MgCl_2$) and overlaid with 4 ml of 55% sucrose in LSB and 1.5 ml of 10% sucrose in LSB. Gradients were centrifuged in a Beckman SW41TI rotor at 4° for 14 hr at 38,000 rpm. Fractions (1 ml) were then collected from the top of the gradient. Each fraction was diluted in 4 ml of LSB and recentrifuged in a Beckman SW55TI rotor at 46,000 rpm for 90 min at 4°. The resulting pellets were analyzed by immunoblotting, using rabbit anti-NS5B antibody and ^{125}I-protein A as previously described (Hwang and Lai, 1993).

Radiolabeling and immunoprecipitation

Sf9 cells infected with recombinant baculoviruses were first incubated with methionine-free Grace's insect cell medium (GIBCO BRL) for 1 hr at 2 days postinfection and labeled with 100 μCi/ml of [^{35}S]methionine and cysteine (NEN) for 4 hr. The cells were washed twice in PBS, lysed in RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 150 m*M* NaCl, 50 m*M* Tris–HCl (pH 8.0), 0.1% SDS, 0.1 m*M* PMSF], and centrifuged at 15,000 *g* for 15 min. Aliquots of cell lysates were incubated with either control sera from a healthy donor or HCV patient sera or rabbit preimmune or anti-NS5B sera for 2 hr at 4°. The immune complex was further incubated with protein A–Sepharose for 1 hr and washed five times in RIPA buffer by centrifugation in a microfuge. The immunoprecipitates were analyzed as previously described (Hwang and Lai, 1994). For the analysis of phosphorylation, the recombinant baculovirus-infected Sf9 cells were incubated for 1 hr in phosphate-free Grace's insect cell medium at 48 hr postinfection and labeled with 300 μCi/ml of [^{32}P]orthophosphate (ICN) for 4 hr. Cells were washed in PBS, and proteins were immunoprecipitated as described above.

In vitro transcription and translation

Plasmid DNA (pcDNA3) containing the NS5B-coding region was linearized with *Eco*RI, and transcription was performed with T7 RNA polymerase for 70 min at 37°. Aliquots of the transcribed RNAs were translated in rabbit reticulocyte lysate (Promega) for 1 hr at 30°C using [^{35}S]methionine.

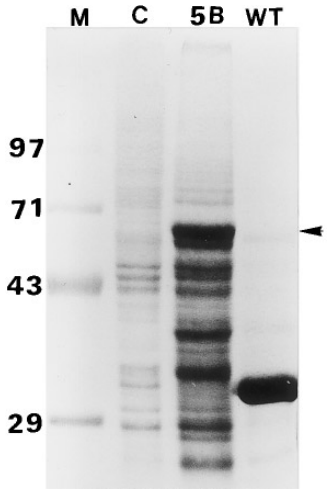

FIG. 1. Expression of NS5B in insect cells. Wild-type (WT) or recombinant baculovirus (5B)-infected Sf9 cells were harvested at 3 days postinfection. Proteins were separated by electrophoresis in a 10% polyacrylamide gel containing SDS and stained with Coomassie brilliant blue. M, molecular mass marker (in kDa). C, uninfected cells. The arrow on the right indicates the NS5B protein.

RESULTS AND DISCUSSION

Expression and characterization of NS5B in insect and mammalian cells

To study the biochemical properties of NS5B, we first constructed a recombinant baculovirus containing the HCV NS5B-coding sequence under the control of the polyhedrin promoter and used it to infect Sf9 cells; protein expression was analyzed 3 days postinfection. The results showed that a major protein species of approximately 64 kDa, which is equivalent to the predicted molecular weight of NS5B, was detected in the recombinant virus-infected cells, but not in uninfected cells or in wild-type baculovirus-infected cells (Fig. 1). The nature of this protein was confirmed by immunoblotting using sera from human HCV patients. As shown in Fig. 2A, the patient's serum detected the 64-kDa protein and two other smaller proteins of 62 and 35 kDa, which are likely the degradation products of NS5B. None of these proteins reacted with sera from normal blood donors (data not shown). No proteins were detected in the wild-type baculovirus-infected or uninfected cells (Fig. 2A). The 64-kDa protein was then used to prepare a rabbit polyclonal antibody (see Materials and Methods). The specificity of this rabbit antibody was determined by immunoprecipitation of the *in vitro* translation product of the NS5B ORF in rabbit reticulocyte lysates, which showed that this anti-

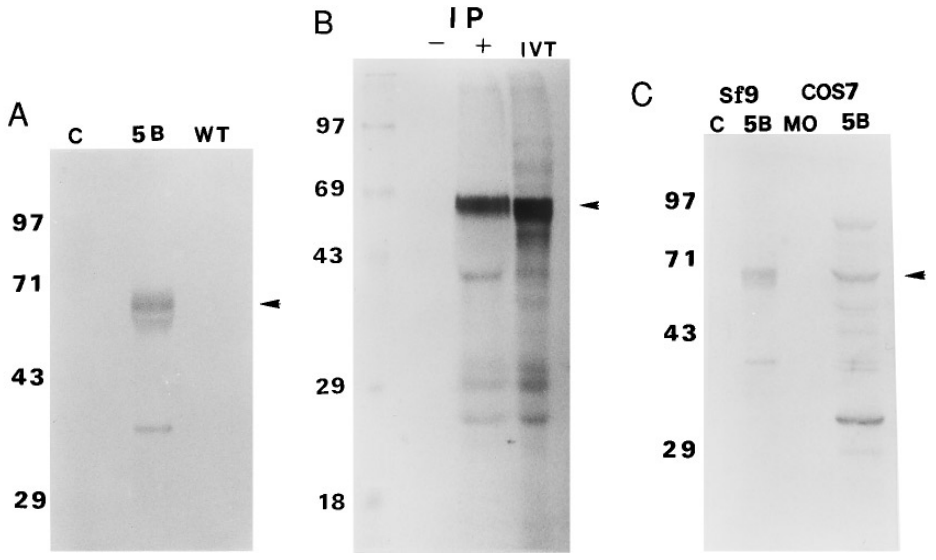

FIG. 2. (A) Characterization of NS5B by immunoblot analysis. Recombinant baculovirus-infected Sf9 cell lysates were separated as shown in Fig. 1 and electrotransferred to nitrocellulose membrane. The NS5B protein was detected by immunoblotting with an HCV patient serum. (B) Immunoprecipitation of the *in vitro*-translated NS5B. NS5B was translated *in vitro* in rabbit reticulocyte lysate and precipitated with either rabbit preimmune serum (−) or an antibody raised against the 64-kDa protein from the recombinant baculovirus-infected insect cells (+). IVT, *in vitro*-translated products without immunoprecipitation. (C) Comparison of NS5B in insect and mammalian cells. COS 7 cells were infected with recombinant vaccinia virus expressing T7 polymerase (vTF7-3) (Fuerst *et al.,* 1986) and transfected with plasmid DNA expressing NS5B. Sf9 cells were infected with the recombinant baculovirus (5B). The proteins were separated by SDS–PAGE and detected by immunoblotting with HCV patient serum. C, uninfected insect cell lysate. MO, mock-transfected COS 7 cells. The arrow indicates the NS5B.

body precipitated the 64-kDa protein and also several smaller proteins that may represent premature termination or degradation products (lane 2, Fig. 2B). The preimmune serum did not precipitate any protein (lane 1, Fig. 2B). The NS5B protein expressed in insect cells was then compared with that expressed in mammalian cells using an expression vector under a T7 promoter in the presence of a recombinant vaccinia virus encoding T7 RNA polymerase (see Materials and Methods) (Fig. 2C). The sizes of the proteins expressed in the two different cells were indistinguishable, suggesting that they have very similar posttranslational modifications, if any (Fig. 2C). However, in COS 7 cells, several additional proteins were detected; the nature of these proteins is not clear. No proteins were detected in the uninfected insect cells or untransfected COS 7 cells.

Subcellular localization of the NS5B protein

To determine the subcellular localization of NS5B, an indirect immunofluorescence staining of NS5B was first performed in COS 7 cells transfected with the NS5B-expressing plasmid (Fig. 3). For this purpose, the NS5B protein was expressed from the CMV promoter in the absence of recombinant vaccinia virus infection. The NS5B was detected by using rabbit polyclonal antibody made against the baculovirus-expressed NS5B. The results showed that NS5B was present as fine speckles exclusively in the cytoplasm. Most interestingly, these speckles were concentrated primarily in the perinuclear regions, particularly associated with the nuclear membrane (Fig. 3D). This staining was not detected when the preimmune rabbit serum was used (Fig. 3C). Nor did the rabbit serum against NS5B detect any staining in the mock-transfected cells (data not shown). A similar result was obtained when insect cells infected with the NS5B-expressing baculovirus were examined (data not shown). In this case, the NS5B was detected by using sera from HCV patients to avoid possible cross-reaction of the antibody with other insect cellular proteins. These results indicate that NS5B is localized predominantly in the perinuclear region, suggestive of its association with the nuclear membrane and the endoplasmic reticulum or Golgi complex.

Membrane association of the NS5B protein

The perinuclear localization of the NS5B protein in both mammalian and insect cells suggests that it is associated with the intracellular membranes. To investigate this possibility, we separated the recombinant baculovirus-infected Sf9 cell lysates into membrane and cytosol fractions, using the membrane flotation method as previously described (Matsumoto *et al.,* 1996; Sanderson *et al.,* 1994). In this method, the mem-

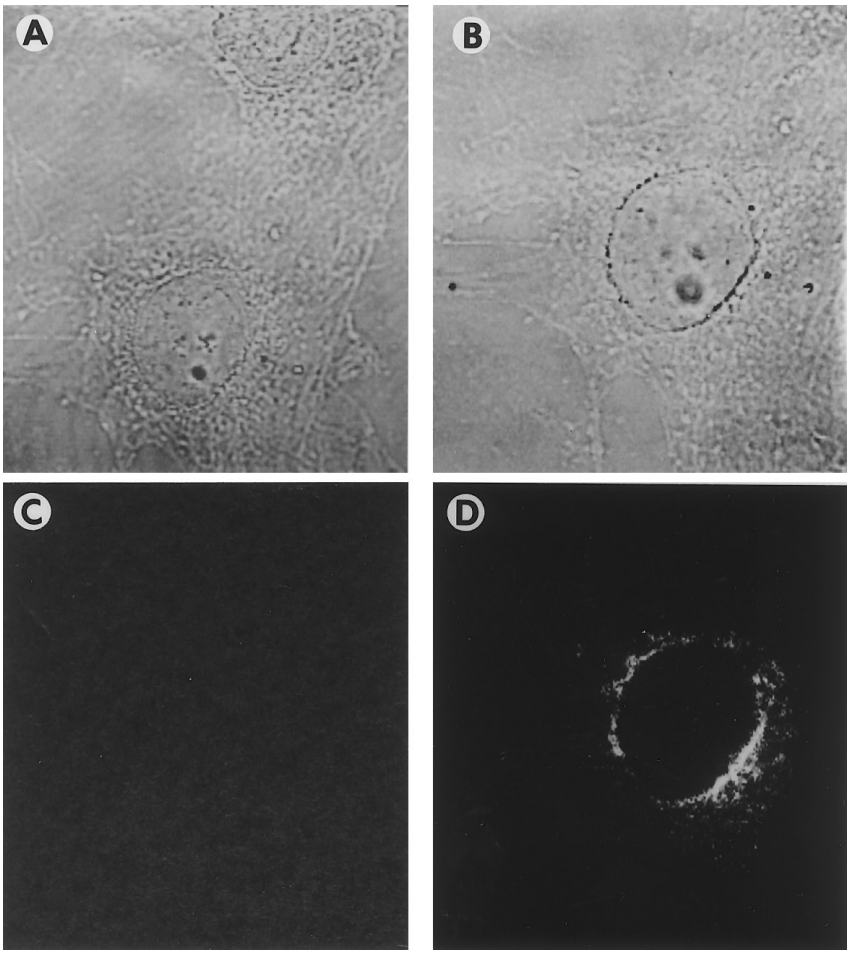

FIG. 3. Subcellular localization of the NS5B. COS 7 cells transfected with plasmid encoding NS5B were fixed and incubated with either rabbit anti-NS5B sera (B and D) or rabbit preimmune sera (A and C) as primary antibodies and rhodamine-conjugated goat anti-rabbit IgG as secondary antibodies. Stained cells were examined using a Zeiss confocal microscope. (A and B) Phase-contrast images. (C and D) Immunofluorescence staining.

brane-containing materials float to the top of the sucrose gradient, while the cytosol fractions remain at the bottom (see Materials and Methods) (Enami and Enami, 1996; Sanderson *et al.,* 1994). The integral membrane proteins, such as tumor necrosis factor receptor, were found to be localized exclusively in the membrane fraction (data not shown). The presence of the NS5B protein in each fraction was determined by immunoblotting using HCV patients' sera. As shown in Fig. 4, NS5B protein was found to be localized in both membrane (fractions 1–3) and cytosol fractions (fractions 7–9). Several NS5B species with slightly different electrophoretic mobility were detected by HCV patients' sera. It is noteworthy that, in the membrane fraction (lanes 1–3), the larger form of the NS5B was more abundant than the small form, whereas the reverse was true for the cytosol fractions (lanes 7–9), suggesting that the large form was enriched in the membrane fractions. The significance of these different forms of NS5B is not yet clear. These results indicate that some of the NS5B is associated with the cellular membrane, at least in insect cells.

Phosphorylation of the NS5B

We next examined the possible phosphorylation of NS5B. We used the baculovirus-expressed NS5B to facilitate detection of protein phosphorylation, since NS5B was expressed by the recombinant baculovirus at a much higher level than by the mammalian expression vector, and insect cells undergo most of the posttranslational modifications equivalent to those in mammalian cells (Miller, 1988). The recombinant baculovirus-infected cells were labeled with either [^{32}P]orthophosphate or [^{35}S]methionine and cysteine for 3 hr at 48 hr postinfection. Metabolically labeled NS5B proteins were immunoprecipitated with either a rabbit polyclonal antibody or HCV patient serum. Figure 5 shows that the NS5B protein

was labeled by [^{32}P]orthophosphate. No difference in electrophoretic mobility between ^{35}S- and the ^{32}P-labeled NS5B was observed. However, the [^{35}S]methionine-labeled NS5B appeared to be more diffuse than the ^{32}P-labeled protein (Fig. 5B), and in some gels, ^{35}S-labeled NS5B separated into two bands (Fig. 5A), similar to the results obtained by immunoblotting (Fig. 2). In either case, only the more slowly migrating form was phosphorylated. No proteins were detected by immunoprecipitation with normal human sera (Fig. 5A) or preimmune rabbit sera (Fig. 5B). In addition to the 64-kDa protein, several other [^{35}S]methionine- and ^{32}P-labeled proteins were coprecipitated, particularly by the rabbit sera. Whether these proteins represent the degradation products of NS5B and/or cellular proteins associated with NS5B is not known.

This study thus revealed several new properties of NS5B, which has previously been demonstrated to have an RNA-dependent RNA polymerase activity (Behrens *et al.,* 1996). So far, the possible template specificity of NS5B for HCV RNA has not been demonstrated. Conceivably, the polymerase activity and template specificity of NS5B may be regulated by posttranslational modifications, such as phosphorylation, of the protein and its interaction with other viral or cellular proteins. In this study, we detected a relatively low level of phosphorylation of NS5B. Considering that there is a total of 117 serine, threonine, and tyrosine residues in the protein, the phosphorylation level detected was low. This result suggests that only a few amino acid residues are phosphorylated and only a portion of the total protein is phosphorylated. This conclusion is consistent with the finding that the ^{32}P-labeled NS5B species corresponded only with the more slowly migrating species of the [^{35}S]-methionine-labeled protein (Fig. 5). Because of the low level of phosphorylation, we have so far not been able to determine the phosphoamino acid residues. Nevertheless, the level of phosphorylation of the NS5B protein may be regulated by the physiological conditions of the infected cells, and phosphorylation of a key amino acid residue may play a significant role in regulating its activities. Although phosphorylation of NS5B has not been demonstrated so far in mammalian cells because of the low level of radiolabeling, the finding that the NS5B in insect and mammalian cells had indistinguishable sizes suggests that most of the posttranslational modifications of the proteins, including phosphorylation, are likely similar between the two cell types.

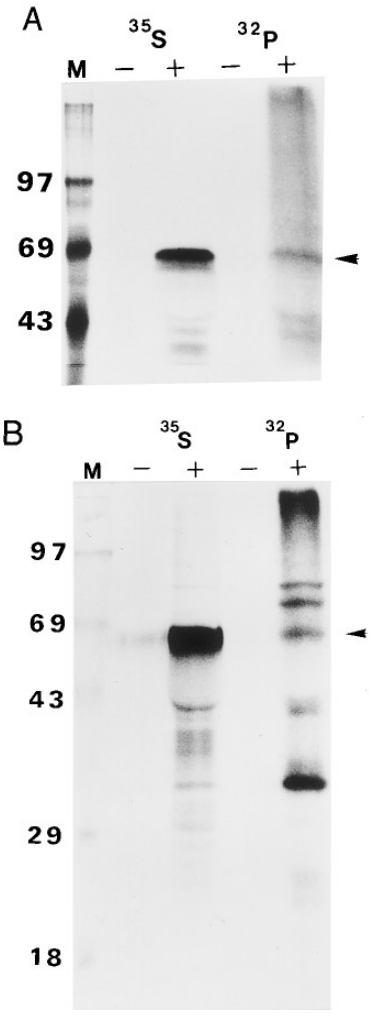

FIG. 5. *In vivo* phosphorylation of NS5B proteins. Insect cells were infected with the recombinant baculoviruses and labeled metabolically with either [^{35}S]methionine and cysteine or [^{32}P]orthophosphate for 4 hr at 2 days postinfection. The NS5B proteins were immunoprecipitated with (A) HCV patient serum (+) or normal human serum (−) or (B) rabbit anti-NS5B antibody (+) or preimmune serum (−). The immunoprecipitates were analyzed by SDS–PAGE and autoradiographed. M, molecular mass marker.

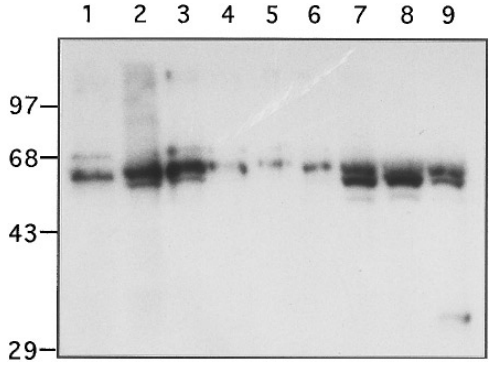

FIG. 4. Membrane flotation analysis of NS5B expressed in insect cells. Sf9 cells infected with recombinant baculovirus expressing NS5B were harvested at 3 days postinfection. Cell lysates were prepared as described previously (Matsumoto *et al.,* 1996) and subjected to fractionation by equilibrium sucrose gradient centrifugation. One-milliliter fractions were collected from the top of the gradient. Proteins in each fraction were analyzed by immunoblotting using HCV patient serum. Fractions 1–3, membrane fractions. Fractions 7–9, cytosol fractions.

It is interesting that NS5B is localized primarily in the perinuclear region and associated with cellular membranes. These findings suggest that HCV RNA replication may take place in the membrane fraction in the infected cells. This is consistent with the findings that RNA synthesis of RNA viruses usually takes place in the membrane complex (Butterworth *et al.,* 1976; Dennis and Brian, 1982; Lazarus and Barzilai, 1974). The finding that the different forms of the NS5B detected in the insect cells appear to differ in their relative distribution in the membrane and cytosol fractions (Fig. 4) may suggest that certain modifications, such as phosphorylation, of NS5B facilitate its membrane association. Although the precise relationship between these different forms of the protein is not yet known, it is interesting to note that it was the more slowly migrating form which was phosphorylated and was also more enriched in the membrane fractions (Fig. 5). It is not known whether NS5B from HCV of a different genotype displays different biochemical properties. The posttranslational modifications and membrane association of NS5B may play a significant role in the function of HCV polymerase.

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