Hepatitis delta antigens (HDAGs) are important for the replication and assembly of hepatitis delta virus (HDV). To understand the association between HDAGs and cellular proteins and the mechanism of viral multiplication, we have studied the interaction between HDAGs and nucleolin, a major nucleolar phosphoprotein. The interaction between HDAGs and nucleolin was first demonstrated by immunofluorescence staining studies. HDAGs and endogenous nucleolin were colocalized in the nucleoli of cultured cells transfected with plasmids encoding the small and large HDAG. Coimmunoprecipitation results indicated that the NH2-terminal domain of HDAG was essential for its binding to nucleolin. In vitro ligand binding assays revealed two nucleolin binding sites, NBS1 and NBS2. Each spanned amino acid residues 35–50 and 51–65, respectively, with a conserved core sequence K(K/R)XK. HDV replication was modulated by exogenous human nucleolin. In addition, a small HDAG mutant S-d6575, which possesses both NBS1 and NBS2, was capable of transactivating HDV replication, whereas the small HDAG mutant S-d5075, which retained NBS1 but not NBS2, was unable to support the replication of HDV. Thus, the nucleolin binding activity of HDAG is critical for its nucleolar targeting and is involved in the modulation of HDV replication.

The Nucleolin Binding Activity of Hepatitis Delta Antigen Is Associated with Nucleolus Targeting*

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Hepatitis delta virus (HDV) is a human pathogen associated with fulminant hepatitis and progressive chronic liver cirrhosis (1–3). HDV is considered a satellite virus of hepatitis B virus (HBV) because it requires HBV for virion production and transmission (4–7). HDV possesses a 36-nm spherical structure that contains an internal ribonucleoprotein complex with virus-specific delta antigen (HDAG) and RNA genome encapsulated by the surface antigen of HBV (HBsAg) (5, 8–12). Two forms of HDAG are found in patients with delta hepatitis, small and large HDAGs of 24 kDa (195 amino acid residues) and 27 kDa (214 amino acid residues), respectively (13–15). The large HDAG contains the entire sequence of the small HDAG with an additional 19-amino acid extension at its COOH terminus, which is the result of RNA editing occurring at the late stage of viral replication (16). Functional domains of HDAGs have been defined. A coiled-coil structure responsible for oligomerization and nuclear localization signals are within the NH2 terminus (17–20). RNA binding motifs are within the middle helix-turn-helix region (17, 21–23). An isoprenylation motif (CRPQ) constitutes the extreme COOH terminus of the large HDAG (24). Despite the sequence and structural similarities, the two HDAGs exhibit distinct biological functions in viral multiplication. The small HDAG is required for the replication of HDV RNA (25), whereas the late appearing large HDAG inhibits viral replication by a trans-dominant suppression mechanism (26). In addition, the large HDAG is crucial for virion assembly (27, 28), whereas the small HDAG is packaged into virion only in the presence of the large HDAG (29).

HDV genome is a circular single-stranded RNA of approximately 1.7 kilobases with extensive intramolecular complementarity that is organized into an unbranched rod-shaped structure (13, 14, 30). Characteristics of the rod-shaped structure and an intrinsic self-cleavage ribozyme activity (31–34) of the HDV RNA have led to the suggestion that HDV RNA undergoes replication by a double rolling circle mechanism similar to that of viroid (35). Follow-up experiments have provided further evidence to support this hypothesis (36). Studies demonstrating the sensitivity of HDV RNA replication to ρ-amanitin suggest a linkage between HDV replication and an RNA polymerase II-like activity (37, 38). Previous studies have demonstrated that the large HDAG is localized in the nucleoplasm and nucleoli of cultured cells transfected with a plasmid encoding the HDAG (39, 40). The distribution pattern was also observed with viral RNA and both the small and large HDAGs in HDV-infected hepatocytes (41–45). Nevertheless, the replication of HDV is thought to take place in the nucleoplasm (46). The significance of nucleolus localization of the HDAGs and viral RNA is not clear.

Determination of the functional motifs that contribute to the nucleolar distribution of HDAGs and identification of HDAG-associated proteins are both necessary for understanding how HDAGs are involved in HDV multiplication. Brazas and Ganem (47) have recently identified a cellular homolog of HDAG, termed delta-interacting protein A (DIPA), and demonstrated that overexpression of DIPA specifically attenuated HDV RNA replication. In this study, we have demonstrated that HDAGs colocalized and associated with nucleolin (formerly termed C23), a major nucleolar phosphoprotein of 100 kDa. We also defined two nucleolin binding sites within the NH2-terminal domain of HDAG. The binding site that encompasses amino acid residues 51–65 is required for nucleolus targeting. Furthermore, overexpression of exogenous nucleolin in both Huh-7 and BHK-21 cells modulated HDV RNA replication. It seems to
be correlated among the nucleocidin binding activity, nucleolar distribution, and transactivating activity of the small HDAg.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids pECE-d-BE, pECE-d-5M, and pSVD2—Plasmids pECE-d-BE and pECE-d-5M encode the wild type large and small HDAg, respectively, as described previously (22, 39). Plasmid pSVD2 contains a dimeric HDV cDNA under the control of the simian virus 40 early promoter as described previously (20).

Plasmid pECE-aCAT—Plasmid pECE-aCAT encodes a fusion protein with the NH2-terminal 31 amino acid residues of chloramphenicol acetyltransferase linked to amino acid residues 10–214 of the large HDAg as described previously (20).

Plasmids pECEL-d10/55, pECEL-d25/88, pECEL-d40/163, pECEL-d164/195, pECEL-d35/75, pECEL-d50/75, and pECEL-d65/75—These plasmids encode large HDAg mutants with internal deletions of amino acid residues 10–55, 35–88, 89–163, 164–195, 35–75, 50–75, and 65–75, respectively, as described previously (20). Structures representing individual HDAg mutants and the positions of amino acid residues flanking each deletion are summarized in Figs. 4A and 5A.

Plasmid pCMV-Nu—Plasmid pCMV-Nu contains a full-length cDNA of human nucleolin under the control of the cytomegalovirus immediate-early gene promoter and linked to the simian virus 40 splicing and poly(A) signals, as described previously (48).

Plasmid pSVD2m—For construction of plasmid pSVD2m, the SacII–SacII monomeric HDV cDNA fragment from plasmid pSVD2 (20) was treated with T4 DNA polymerase and subcloned into a modified pSV2d from which the SacII–SacII DNA fragment had been deleted and in which the remaining DNA had been blunted by T4 DNA polymerase. The resultant plasmid, pSVD2m, contains a dimeric HDV cDNA that encodes a frameshifting HDAg mutant with the first 10 amino acid residues of the wild type HDAg followed by 51 unrelated amino acid residues. Specific mutations in plasmid pSVD2m were confirmed by DNA sequencing, using the dyeoxy chain termination method (49).

Plasmid pDS-d50/75 and pDS-d65/75—Strategies used for construction of pDS-d50/75 and pDS-d65/75 were similar to those used for the construction of pECEL-d50/75 and pECEL-d65/75, respectively, except that the parental plasmid used was pECE-d-5M instead of pECE-d-BE. Plasmid pDS-d50/75 and pDS-d65/75 encode small HDAg mutants with internal deletions of amino acid residues 50–75 and 65–75, respectively.

Cell Lines and DNA Transfection

COS7 cells (a monkey kidney cell line) and Huh-7 cells (a human hepatoma cell line) were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum plus 100 units of penicillin and 100 μg of streptomycin/ml. BHK-21 cells (a baby hamster kidney cell line) were cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics as described above. The BHK-21 cell line transfected with pCMV-Nu has been used as the system to study the effect of nucleolin on the regulation of acute phase response genes (48). DNA transfection was performed with cationic liposomes as described previously (17).

Antibodies

A rabbit antiserum against HDAg was prepared as described previously (9) and purified further over a protein G affinity column (Pierce). The mouse monoclonal antibody (mAb) to nucleolin was a gift from S.-C. Lee (National Taiwan University, College of Medicine) (48, 50). The mouse mAb to the splicing factor SC35 was a gift from C.-H. Wu (National Taiwan University, College of Medicine). The mouse mAb to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Biodine. Rabbit IgG was purchased from Sigma, and mouse IgG was a gift from Z.-F. Chang (National Taiwan University, College of Medicine).

Indirect Double Immunofluorescence Staining

Indirect double immunofluorescence staining was carried out as follows. Cells were grown on coverslips and allowed to adhere overnight before DNA transfection. Two days post-transfection, culture medium was removed, and the cells were washed twice in phosphate-buffered saline (PBS, pH 7.4) followed by a fixation at room temperature for 12 min with PBS containing 4% paraformaldehyde. The fixative was removed by three washes with PBS, and the cells were permeabilized by exposure to 100% methanol for 2 min at room temperature. After a rehydration with multiple rinses in PBS, the cells were treated with PBS containing 4% bovine serum albumin and proceeded to double immunofluorescence staining, using protein G-purified rabbit anti-serum specific for HDAg (9) and mouse mAb to nucleolin (48, 50) as the primary antibodies and fluorescein isothiocyanate-labeled goat antirabbit IgG (Jackson) and Texas Red-labeled goat anti-mouse IgG (Vector) as the secondary antibodies, respectively. The secondary antibodies were subjected to acid hydrolysis in vacuo (6 N HCl, at 110 °C for 72 h) and analyzed by high performance liquid chromatography. The synthetic peptides were coupled to rabbit IgG through the cysteine residues by using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Sigma) as the coupling reagent (53). In brief, for each peptide, 0.7 mg of MBS in dimethylformamide was added to 4 mg of rabbit IgG in 0.25 ml of 10 mM PBS, pH 8.0, 2% n-propyl gallate, and 60% glycerol. Photographs were taken using a Leica epifluorescence microscope.

Comunmunoprecipitation

2 days post-transfection, cells were washed twice with PBS and lysed in RIPA buffer containing 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl, pH 7.2, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were clarified by centrifugation at 30,000 × g for 15 min, and supernatants were collected. To perform communoprecipitation experiments, protein G-purified rabbit anti-serum specific for HDAg or mouse mAb to nucleolin was added to the supernatants and incubated for 1 h at 4 °C followed by an incubation with protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) overnight at 4 °C. The immunoprecipitates were washed three times by vigorous mixing with RIPA buffer and then resuspended in sample buffer (70 mM Tris-HCl, pH 6.8, 11.2% glycerol, 3% SDS, 0.01% bromaehloro blue, 5% β-mercaptoethanol), heated, and pelleted. The resultant supernatants were subjected to Western blot analysis.

Western Blot Analysis

To carry out Western blot analysis, immunoprecipitates or cell lysates prepared from transfected cells were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred onto an Immobilon-P membrane (Millipore) as described previously (22). The membrane was incubated with antibodies to HDAg, nucleolin, splicing factor SC35, or GAPDH, and specific interactions between antigens and antibodies were detected by incubation with 3,5-diaminobenzidine tetrahydrochloride chemiluminescence system.

Isolation of Total Cellular RNA and Northern Blot Analysis

Total cellular RNA was isolated from transfected cells 6 days post-transfection by a single-step extraction method as described previously (51). Northern blot analysis was performed as described (22) using a 32P-labeled genomic strand of HDV RNA as the probe. The 28 S rRNA detected by hybridization with a 32P-labeled cDNA fragment of 28 S rRNA was used as an internal control.

Particular Purification of Nucleolin

Nucleolin was partially purified from cultured cells as described previously (32). In brief, COS7 cells grown to confluence were harvested and homogenized with a tight fitting Dounce homogenizer by 50 strokes in homogenization buffer containing 300 mM KCl, 1% leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and the homogenate was layered over a 5–30% (w/v) stepwise sucrose gradient prepared in AM rotor at 14,000 rpm for 5 min (4 °C), and the resulting supernatant was layered over a 5–30% (w/v) stepwise sucrose gradient prepared in 4 °C. The immunoprecipitates were washed three times by vigorous mixing with RIPA buffer and then resuspended in sample buffer (70 mM Tris-HCl, pH 6.8, 11.2% glycerol, 3% SDS, 0.01% bromphenol blue, 5% β-mercaptoethanol), heated, and pelleted. The resultant supernatants were subjected to Western blot analysis.

Synthesis and IgG Coupling of HDAg Peptides

Pepitides HDAg(35–50) and HDAg(51–65) were synthesized by peptide synthesizer (Applied Biosystems Inc. model 431). HDAg(35–50) and HDAg(51–65) are: NH2–Arg–Lys–Leu–Lys–Lys–Ile–Lys–Leu–Glu–Glu–Asp–Asp–Pro–Trp–Cys–COOH and NH2–Gly–Asn–Ile–Lys–Gly–Ile–Gly–Lys–Asp–Gly–Glu–Cys–COOH, respectively. To confirm the amino acid composition, each synthetic peptide was subjected to acid hydrolysis in vacuo (6 N HCl, at 110 °C for 72 h) and analyzed by high performance liquid chromatography. The synthetic peptides were coupled to rabbit IgG through the cysteine residues by using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Sigma) as the coupling reagent (53). In brief, for each peptide, 0.7 mg of MBS in dimethylformamide was added to 4 mg of rabbit IgG in 0.25 ml of 10 mM PBS, pH 8.0, 2% n-propyl gallate, and 60% glycerol. Photographs were taken using a Leica epifluorescence microscope.
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7.2, and stirred for 30 min at room temperature. The MBS-activated rabbit IgG MBS-activated rabbit IgG, was passed through a Sephadex G-25 column (Pharmacia), equilibrated, and eluted with 50 mM PBS, pH 6.0, to remove free MBS. The coupling reaction was carried out at room temperature for 3 h with the MBS-activated rabbit IgG and 5 mg of the synthetic peptide in 10 mM PBS, pH 7–7.5. Uncoupled peptide was removed from the conjugated product by passing through a Sephadex G-25 column.

In Vitro Ligand Binding Assay

IgG-conjugated HDAg peptides, IgG-HDAg(35–50) and IgG-HDAg(61–65), were used as the ligands to perform binding assays with nucleolin. For each binding reaction, the ligand was used at a final concentration of 30 mg/ml, and nucleolin was prepared by stepwise sucrose gradient from approximately 10^7 COS7 cells. 5 mg of protein A-Sepharose CL-4B (Pharmacia) was added to each of the binding reaction mixtures and allowed to be mixed completely. After rotating overnight at 4 °C, the precipitates were washed extensively with RIPA buffer and resolved on a 10% SDS-polyacrylamide gel. To examine the reaction mixtures and whether there is interaction between nucleolin and HDV, we used protein G-purified antiserum specific for HDAg (aHDAg) and mAb against nucleolin (aNu) to stain COS7 cells that transiently expressed the large and small forms of HDAg. Nucleolin is a major nucleolus phosphoprotein with RNA helix activity and the ability to shuttle between the cytoplasm and the nucleus (52–59). The staining patterns of HDAg were similar to the staining pattern of nucleolin (Fig. 1), indicating that both the large and small HDAGs are highly enriched in the nucleoli of COS7 cells 2 days post-transfection. Interestingly, in a Huh-7 cell line that stably expresses small HDAG, we observed a redistribution of the HDAG from the nucleolus to the nucleoplasm 3 days following transfection of a dimeric HDV RNA (data not shown). Colocalization of HDAGs and nucleolin suggests the possibility that there is an interaction between them and the possibility that nucleolin is involved in the replication of HDV.

HDAgs Are Associated with Nucleolin in Cultured Cells—Earlier studies have demonstrated that large HDAG possesses two staining patterns in the nuclei of transfected cells; one is exhibited as homogeneous nucleolus staining, and the other appears as a granule-like distribution in the nucleoplasm and nucleolus (39). To investigate the functional significance of the nucleolar localization of HDAG further, we used protein G-purified antiserum specific for HDAg (aHDAg) and mAb against nucleolin (aNu) to stain COS7 cells 24 and 27 kDa, representing the small and large HDAGs, respectively, were detected (Fig. 2A). In contrast, if cell lysates were first precipitated with aHDAg and mAb against nucleolin, (aNu) to stain COS7 cells and the possibility that nucleolin is involved in the regulation of early stage viral replication. To this end, cotransfection was performed in Huh-7 and BHK-21 cells with a dimeric HDV cDNA (pSV2D) capable of producing small HDAG and a plasmid encoding human nucleolin (pCMV-Nu). Total cellular RNA was harvested 6 days post-transfection and subjected to Northern blot analysis with a genomic strand of HDV RNA as a probe. Results in Fig. 3 show that with increasing amounts of nucleolin-encoding plasmid in the Huh-7 (Fig. 3A) and BHK-21 transfecents (Fig. 3B), the amounts of HDV antigenic RNA increased. These results suggest that nucleolin modulates HDV replication.

Assessment of the Nucleolin Binding Activity of Truncated HDAG Mutants—To identify functional domains of HDAG responsible for nucleolin binding, a series of deletion constructs encoding HDAG mutants was transfected into COS7 cells. Deletion constructs used in this study were derived from the wild type large HDAG (HDAG-L) because both large and small forms of HDAG interacted with nucleolin, and the large HDAG was expressed at a higher level in transfected COS7 cells (Fig. 2A). Cell lysates were subjected to immunoprecipitation with aNu and resolved on a 12.5% SDS-polyacrylamide gel followed by Western blot analysis with aHDAg. Results demonstrate that comparable amounts of HDAG-L and its mutants, L-sCAT, L-d89/163, and L-d164/195 were coprecipitated with nucleolin despite the lower expression level of L-d89/163 (Fig. 4). In

![Fig. 1. Colocalization of HDAGs and the endogenous nucleolin in transfected COS7 cells as detected by double immunofluorescence staining.](http://www.jbc.org/)

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**FIG. 1.** Colocalization of HDAGs and the endogenous nucleolin in transfected COS7 cells as detected by double immunofluorescence staining. A and B, COS7 cells were transfected with plasmid pECE-d-BE encoding the large HDAG (panels A–C) or pECE-d-SM encoding the small HDAG (panels D–F). 2 days post-transfection, cells were fixed and proceeded to indirect double immunofluorescence staining using protein G-purified rabbit antiserum to HDAG (aHDAG; panels A and B) and mouse mAb to nucleolin (aNucleolin; panels C and F) as the primary antibodies. Panels A and D are phase-contrast micrographs representing cells of the same fields as panels B and C, and panels E and F, respectively. The nucleolar distribution of the HDAGs was visualized with fluorescein optics (panels B and E), and nucleolin was visualized with Texas Red optics (panels C and F). The bar in panel F represents 10 μm in length.
contrast, large HDAg mutants, L-d10/55 and L-d35/88, failed to coprecipitate with nucleolin, although their expression levels were higher than that of the L-d89/163 HDAg mutant (Fig. 4). These results suggest that the NH$_2$-terminal domain of the HDAg is involved in nucleolin binding. HDAg mutants with overlapping deletions in the NH$_2$-terminal domain were analyzed further. As shown in Fig. 5, it appears that deletion mutant L-d65/75 retained nucleolin binding ability, and L-d35/75 and L-d50/75 were defective. Taken together, a putative nucleolin binding domain of the HDAg was identified to be between amino acid residues 35 and 65.

Synthetic Peptide Containing Amino Acid Residues 35–50 or 51–65 of the HDAg Is Capable of Binding to Nucleolin in Vitro—Sequence comparison with SV40 large T antigen and histone 2B (60) revealed that HDAgs contain two potential nucleolin binding sites with a conserved core sequence K(K/R)XK within the domain of amino acid residues 35–65 (Fig. 6A). Notably, both of the putative nucleolin binding sites are conserved among HDV isolates (17). An in vitro ligand binding assay was carried out using IgG-conjugated synthetic HDAg peptides IgG-HDAg(35–50) and IgG-HDAg(51–65) as the ligands. Each ligand was incubated with partially purified nucleolin in the presence of protein A-Sepharose beads. Products containing ligand-protein A complexes were analyzed by Western blotting with Nu. The data in Fig. 6B show that both IgG-HDAg(35–50) and IgG-HDAg(51–65) conjugates bound specifically to nucleolin. Nucleolin did not complex with free rabbit IgG. These results reveal that in vitro there are two nucleolin binding sites in HDAg, one located at amino acid residues 35–50 (designated NBS1) and the other 51–65 (designated NBS2).

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**FIG. 2. Interactions between HDAgs and nucleolin.** Cell extracts were prepared from COS7 cells transfected with plasmid pECE-d-SM (S in panels A and B), pECE-d-BE (L in panels A and B, and all lanes in panels C and D), and pECE vector (V in panels A and B) 2 days posttransfection and subjected to immunoprecipitation (IP) followed by Western blot analysis (Western), using HDAg and Nu as indicated. IgG(R) and IgG(M) represent rabbit and mouse IgG, respectively, and were used as negative controls. The presence of small and large HDAg, nucleolin, SC35, and GAPDH in the cell extracts was demonstrated by direct Western blotting as controls. Positions of the major form SC35 and GAPDH are indicated by * and **, respectively. H and L represent the mouse heavy chain and light chain of IgG, respectively. The molecular size markers are indicated. Molecular sizes of the small HDAg, large HDAg, nucleolin, SC35, and GAPDH are 24, 27, 100, 36, and 36 kDa, respectively.

**FIG. 3. Effect of nucleolin on the replication of HDV RNA.** Total cellular RNA was isolated from Huh-7 (panel A) and BHK-21 cells (panel B) transfected with a dimeric HDV cDNA (pSVD2) and a plasmid encoding human nucleolin (pCMV-Nu) and subjected to Northern blot analysis. Amounts of the effector plasmid pCMV-Nu (as indicated by Nu) used in the cotransfection experiments are indicated on the top of each lane. Vector plasmid pGEM3Z-CMV (as indicated by V) was used to give equal amount of DNA in each transfection experiment. The probe used was the genomic strand of HDV RNA as described under “Experimental Procedures.” AG represents the antigenic HDV RNA, and 28 S represents the 28 S rRNA as an internal control. The amounts of antigenic HDV RNA accumulated in the cells were quantitated and normalized against the 28 S RNA. Quantitation was performed with densitometer (Alpha Innotech IS1000, Digital Imaging System), and the results are indicated as relative activity (%).

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HDV Replication—To correlate nucleolin binding activity with subcellular distribution of the HDAg, mutant forms of the large HDAg L-d65/75 and L-d50/75 were expressed in COS7 cells, and cells were examined further by immunofluorescence staining. As shown in Fig. 7, the L-d50/75 HDAg mutant, which retains NBS1 but lacks NBS2, was distributed evenly in the nucleoplasm, a pattern different from that of nucleolin in the nucleoli (Fig. 7, E and F). The L-d65/75 HDAg mutant, which possesses both nucleolin binding sites, retained the ability to colocalize with nucleolin in the nucleoli, although it also was distributed throughout the cytoplasm (Fig. 7, B and C). Consistent with the coimmunoprecipitation results shown in Fig. 5, amino acid residues 35–50 alone are insufficient for the interaction between HDAg and nucleolin and are insufficient for the nucleolar distribution of HDAg. NBS2 is required for nucleolar targeting. Furthermore, the nucleoplasmic distribution pattern of L-d50/75 and the nucleolar distribution pattern of L-d65/75 were also observed with the small HDAg mutants S-d50/75 and S-d65/75, respectively (data not shown). To understand whether the nucleolar localization is important for small HDAg to support HDV replication, a cotransfection experiment was performed in Huh-7 cells with a replication-defective dimeric HDV cDNA mutant (pSVD2m) incapable of producing small HDAg and a plasmid encoding the wild type or a mutant form of small HDAg. Total cellular RNA was harvested 6 days posttransfection and subjected to Northern blot analysis with a genomic strand of HDV RNA as a probe. Results in Fig. 8 show that the small HDAg mutant S-d65/75, which possesses both NBS1 and NBS2, was capable of transactivating HDV replication, whereas the small HDAg mutant S-d50/75, which retained NBS1 but not NBS2, failed to support the replication of HDV. Thus, the nucleolin binding activity of the small HDAg is involved in the modulation of HDV replication. These results further suggest that the nucleolar localization of small HDAg is important for viral replication.
**DISCUSSION**

We have shown in this study that both large and small forms of HDAg not only colocalize but also interact specifically with endogenous nucleolin in the nucleoli of COS7 cells transfected with an HDAg-encoding plasmid (Figs. 1 and 2). The nucleolin binding site at amino acid residues 51–65 (NBS2) contributes to nucleolar localization of HDAg (Figs. 5 and 7) and HDV replicating HDV RNA replication. Taking these together, we put forth a working hypothesis for the HDV replication cycle: HDV replication starts when viral RNA and HDAgs are complexed and transported from cytoplasm to the nucleolus, followed by a redistribution into nucleoplasm. Nuclear transport may not necessarily involve the nucleolin, but it may facilitate HDV replication by association with the small HDAg and function as a nucleolar localization signal-binding protein (B23), a putative nucleolar localization signal-binding protein (39). We and other groups have demonstrated previously that the middle region of HDAg contributes to its binding activity to HDV RNA. The RNA binding activity is critical for small HDAg to transactivate the viral replication (9, 22, 23). Alternatively, HDAg may bind to nucleolin that, in turn, interacts with nucleoplasmin (B23), a putative nucleolar localization signal-binding protein (61). Whether the RNA binding domain of HDAg interacts with ribosomal RNA needs to be examined further.

In recent years, work on HDV replication has provided evidence for a double rolling-circle model (31–34, 36). However, two important issues concerning HDV replication remain unsolved: 1) How does HDV RNA with an extensive intramolecular base pairing structure serve as a template to synthesize the antigenomic RNA in the first-run replication? and 2) How does the antigenomic RNA form a pseudoknot structure as required for ribozyme activity? A helicase should be involved in the process of HDV replication to unwind the RNA duplex and possibly to form the pseudoknot structure. Human nucleolin known as DNA helicase IV can unwind RNA-RNA duplexes as well as DNA-DNA and DNA-RNA duplexes (59). We have demonstrated in this study that HDAg has nucleolin binding activity. From that we suggest that nucleolin plays a role in modulating HDV RNA replication. Taking these together, we put forth a working hypothesis for the HDV replication cycle: HDV replication starts when viral RNA and HDAGs are complexed and transported from cytoplasm to the nucleus, followed by a redistribution into nucleoplasm. Nuclear transport may not necessarily involve the nucleolin, but it may facilitate HDV replication by association with the small HDAg and function as a helicase to unwind the partially double-stranded HDV RNA. Late in the cycle of HDV replication, oligomerization of large and small HDAGs facilitates virion assembly. In the meantime,
viral replication attenuated. Nucleolin may also be involved in the regulation of late stage viral replication by interacting with large HDAg. This is supported by our previous results that large HDAg mutant L-450/75 lacking nucleolin binding activity failed to repress HDV replication (20). However, how binding of nucleolin to large HDAg is involved in repression of viral replication still remains a question to be addressed.

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