Journal of Virology, July 2005, p. 8113–8120 Vol. 79, No. 13
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.13.8113–8120.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Novel Nuclear Export Signal-Interacting Protein, NESI, Critical for the Assembly of Hepatitis Delta Virus

Yun-Hsin Wang,[1] Shin C. Chang,[2] Cheng Huang,[1] Ya-Ping Li,[1]
Chia-Huei Lee,[1]† and Ming-Fu Chang[1]*

Institute of Biochemistry and Molecular Biology[1] and Institute of Microbiology,[2]
National Taiwan University College of Medicine, Taipei, Taiwan

Received 5 October 2004/Accepted 14 February 2005

The process of host factor-mediated nucleocytoplasmic transport is critical for diverse cellular events in eukaryotes and the life cycle of viruses. We have previously identified a chromosome region maintenance 1-independent nuclear export signal (NES) at the C terminus of the large form of hepatitis delta antigen (HDAg), designated NES(HDAg-L) that is required for the assembly of hepatitis delta virus (HDV) (C.-H. Lee et al., J. Biol. Chem. 276:8142–8148, 2001). To look for interacting proteins of the NES(HDAg-L), yeast two-hybrid screening was applied using the GAL4-binding domain fused to the NES(HDAg-L) as bait. Among the positive clones, one encodes a protein, designated NESI [NES(HDAg-L) interacting protein] that specifically interacted with the wild-type NES(HDAg-L) but not with the export/package-defective HDAg-L mutant, NES*(HDAg-L), in which Pro-205 has been replaced by Ala. Northern blot analysis revealed NESI as the gene product of a 1.9-kb endogenous mRNA transcript that is present predominantly in human liver tissue. NESI consists of 467 amino acid residues and bears a putative actin-binding site and a bipartite nuclear localization signal. Specific interaction between HDAg-L and NESI was further confirmed by coimmunoprecipitation and immunofluorescence staining. Overexpression of antisense NESI RNAs inhibited the expression of NESI and abolished HDAg-L-mediated nuclear export and assembly of HDV genomic RNA. These data indicate a critical role of NESI in the assembly of HDV through interaction with HDAg-L.

Hepatitis delta virus (HDV) is a satellite of hepatitis B virus (HBV) (15, 25, 29, 30) that provides surface antigens (HBsAg; small, middle, and large form) required for targeting of HDV to hepatocytes and for assembly and release of infectious HDV particles (34, 38). Inside HDV particles, HDV genomic RNA comprises a single-stranded circular RNA molecule of 1.7 kb that is associated with delta antigens (HDAgs) as ribonucleoprotein (RNP) complexes (4, 7, 17, 21, 32). Both the small form (HDAg-S) and large form HDAg (HDAg-L) are encoded by the antigenomic strand of the HDV RNA (40). The two HDAgs are identical in the N-terminal 195 amino acid residues, but there is an extra 19-amino-acid extension at the C terminus of the HDAg-L (4, 40) that is derived from posttranscriptional RNA editing (2). The HDAg-S is essential for the replication of HDV RNA (8), whereas the HDAg-L is capable of interacting via the unique C-terminal domain with the small HBsAg to form virus-like particles (3, 6). Isoprenylation of the C terminus of HDAg-L is critical for the assembly of HDV (13). Through interacting with HDAg-L, HDAg-S is copackaged into virion (6).

The life cycle of HDV also relies on host machineries. In the initial stage, the viral genome is imported into the nuclei of host cells through the RNA-binding activity and nuclear localization signal (NLS) of HDAgs. The NLS of HDAgs is recognized by the NLS receptor, importin $\alpha 2$ (10). Upon import of HDV RNP complexes into nuclei of infected cells, the viral RNA undergoes replication through a double rolling circle mechanism (1, 18, 35) mediated by HDV ribozyme and host RNA polymerase activities (24, 33). In the late stage of the HDV life cycle, the progeny HDV RNA genome is likely to form complexes with HDAgs that are then exported to the cytoplasm for further assembly with HBsAg. We have previously demonstrated that HDAg-L is a nucleocytoplasmic shuttling protein with a nuclear export signal (NES) located at the C terminus, designated NES(HDAg-L) (20). The export activity of HDAg-L was further confirmed by Lischka et al. (22). In the presence of HBsAg, a large proportion of nucleus-localized HDAg-L, but neither the HDAg-S nor the export/package-defective HDAg-L mutant HDAg(P205A), relocalized to the cytoplasm. We proposed that the NES(HDAg-L) confers the nuclear export function of HDAg-L and escorts the viral genomic RNA and HDAg-S from the nucleus to the cytoplasm for viral assembly (20). Different from the prototype leucine-rich NES, the NES(HDAg-L) is rich in proline residues and is insensitive to leptomycin B, indicating that the nuclear export of HDAg-L is mediated by a chromosome region maintenance 1 (CRM1)-independent pathway (20). To investigate the nuclear export mechanisms mediated by the NES(HDAg-L), we adopted the yeast two-hybrid screening method to search for cellular factors that interact with NES(HDAg-L). Several potential clones were obtained from a human liver cDNA expression library. One encodes a protein, designated NESI [NES(HDAg-L)-interacting protein], capable of interacting with the wild-type NES(HDAg-L) and HDAg-L but not with the export/package-defective mutants. The specific interaction between

* Corresponding author. Mailing address: Institute of Biochemistry and Molecular Biology, National Taiwan University College of Medicine, No. 1, Jen-Ai Road, First Section, Taipei 100, Taiwan. Phone: 886 2 23123456, ext. 8217. Fax: 886 2 23915295. E-mail: mfchang@ntumc.org.

† Present address: Fu Jen Catholic University School of Medicine, Taipei County, Taiwan, Republic of China.

HDAg-L and NESI was demonstrated by coimmunoprecipitation and immunofluorescence staining assays. Overexpression of the NESI antisense RNA that blocked the nuclear export of viral genomic RNA significantly inhibited the assembly of both HDAg-L and HDV RNA. The results indicate that NESI plays critical roles during the processes of HDV assembly.

MATERIALS AND METHODS

Plasmids. (i) Plasmids pECE-d-BE, pECE-d-SM, pECE-d-BE(P205A), pECE-C-ES, pSVD2, pD3, and pEGFP-N1. Plasmids pECE-d-BE and pECE-d-SM contain cDNAs encoding the HDAg-L and HDAg-S, respectively (4, 6). Plasmid pECE-d-BE(P205A) encodes the mutant HDAg-L with the amino acid residue Pro-205 replaced by Ala (20). Plasmid pECE-C-ES encodes the small forms of the HBsAg, p24 and gp27 (6). Plasmid pSVD2 contains a dimeric HDV cDNA under the control of the simian virus 40 early promoter (6). Plasmid pD3 contains a trimeric HDV cDNA flanked by T7 and SP6 promoter that allow in vitro synthesis of an antigenomic and genomic trimeric HDV RNA, respectively, pEGFP-N1 encodes green fluorescent protein (GFP) under the control of the immediate early promoter of cytomegalovirus (CMV; BD Biosciences).

(ii) Plasmids pAS2-1-NES and pAS2-1-NES*. Plasmids pAS2-1-NES and pAS2-1-NES* encode fusion proteins of the wild-type NES(HDAg-L) and mutant NES*(HDAg-L), respectively, fused to the DNA binding domain of GAL4 and were used as baits in yeast two-hybrid screening. To construct plasmid pAS2-1-NES, a DNA fragment spanning amino acid residues 198 to 210 of the HDAg-L was generated by annealing the synthetic oligonucleotides NES1 (5′-GATCATACTCTTCCCAGCCGACCCTCCCTTCTCTCCCCAGAGTTGACTGCA-3′) and NES2 (5′-GTCAACTCTGGGGAGAGAAGGGAGGGTCGGCTGGGAAGAGTAT-3′) and inserted into the BamHI and PstI sites of plasmid pAS2-1 (BD Biosciences). To construct plasmid pAS2-1-NES*, synthetic oligonucleotides NES1* (5′-GATCATACTCTTCCCAGCCGACCCTGCCTTCTCTCCCCAGAGTTGACTGCA-3′) and NES2* (5′-GTCAACTCTGGGGAGAGAAGGCAGGGTCGGCTGGGAAGAGTAT-3′) were used in the annealing reaction.

(iii) Plasmids pACT2-HA-NESI, pGEMTeasy-HA-NESI, pcDNA3.1-HA-NESI, pcDNA-NESI-V5HisTopo, and pcDNA3.1-AS-NESI. Plasmid pACT2-HA-NESI represents a cDNA plasmid isolated from clone 13-1 of the human liver cDNA library (purchased from BD Biosciences) used in yeast two-hybrid screening. The pACT2-HA-NESI encodes a fusion protein containing the activation domain of GAL4, a hemagglutinin (HA) epitope, and the full-length NESI. Plasmid pGEMTeasy-HA-NESI was generated by inserting a PCR fragment representing the HA-NESI fusion protein into the pGEMTeasy vector (Promega). The PCR fragment was amplified from pACT2-HA-NESI by using the primer set 5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′ and 5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3′. The plasmid pGEMT easy-HA-NESI possesses both T7 and SP6 promoter that allow the synthesis of sense and antisense NESI RNA in vitro by using T7 and SP6 RNA polymerase, respectively. To construct plasmids pcDNA3.1-HA-NESI and pcDNA3.1-AS-NESI, a cDNA fragment representing the HA-NESI fusion protein was excised from pGEMTeasy-HA-NESI following a treatment with AvaI and SacI restriction endonuclease, blunted, and ligated to the EcoRV site of pcDNA3.1 (Invitrogen). This resulted in an insertion of the cDNA fragment in either orientation and generated a plasmid, designated pcDNA3.1-HA-NESI, capable of producing a sense RNA of NESI and a plasmid, designated pcDNA3.1-AS-NESI, capable of producing an antisense NESI RNA from the CMV promoter in transfected cells. Plasmid pcDNA-NESI-V5HisTopo encodes a fusion protein of His-tagged NESI. To generate pcDNA-NESI-V5HisTopo, a PCR fragment representing NESI was amplified from liver cDNA by using the primer set 5′-caccATGAAAAATCAAAATGGTACATTTAAGGACTGGGC-3′ and 5′-AGCAGAATAGACAGAGGGCTCA-3′ and ligated to pcDNA3.1D/V5-His-TOPO (Invitrogen). Lowercase letters indicate extra sequences that are not derived from the coding sequences of NESI but are added to the primer for cloning purposes.

(iv) Plasmids pCMV-Tag2C-HDAgL and pCMV-Tag2C-HDAgS. Plasmids pCMV-Tag2C-HDAgL and pCMV-Tag2C-HDAgS encode fusion proteins containing a Flag epitope-tagged HDAg-L and HDAg-S, respectively. To construct plasmids pCMV-Tag2C-HDAgL and pCMV-Tag2C-HDAgS, cDNA fragments encoding HDAg-L and HDAg-S were excised from plasmids pT7-d-BP and pX9 (4, 5), respectively, following a treatment with restriction enzyme BamHI and Hind III, and cloned into the BamHI and Hind III sites of the plasmid pCMV-Tag2C (Stratagene).

Yeast two-hybrid screening. To perform two-hybrid screening in the budding yeast *Saccharomyces cerevisiae*, plasmid pAS2-1-NES representing NES(HDAg-L) fused to the DNA binding domain of GAL4 was used as bait to screen a human liver cDNA library representing fusion proteins of the activation domain of GAL4. The screening procedure to identify NES(HDAg-L)-interacting protein was performed according to the manufacturer's instruction (BD Biosciences).

Cell lines and DNA transfection. HepG2 cells (a human hepatoma cell line) and COS7 cells (a monkey kidney cell line) were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated at 37°C in 5% CO_2. DNA transfection was performed with cationic liposomes as described previously (5).

Harvest of virus-like particles. Harvest and partial purification of virus-like particles followed previously described procedures (37) with modifications. In brief, culture medium was collected 4 days posttransfection and clarified by centrifugation at 4,000 rpm in a Kubota RA-4F rotor for 5 min. The supernatant was layered over a 20% sucrose cushion (20% sucrose, 20 mM HEPES, pH 7.4, 0.1% bovine serum albumin) and centrifuged at 40,000 rpm in an SW41 rotor (Beckman) for 5 h at 4°C to pellet the virus-like particles. The virus-like particles were resuspended in phosphate-buffered saline (PBS) for further analysis.

Isolation of cellular and viral RNA. Isolation of total RNA from cultured cells and HDV RNA from virus-like particles was carried out by a single-step extraction method as described previously (9). Isolation of nuclear and cytoplasmic RNA following a partition of fractions (23) was performed by using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction.

Northern blot analysis. Northern blot analysis was performed using a digoxigenin (DIG)-labeled probe according to the procedures described by the manufacturer (Roche Biochemicals). To detect NESI transcript among various human tissues, a DIG-labeled antisense NESI RNA was transcribed in vitro from plasmid pGEMTeasy-HA-NESI in the presence of DIG-11-UTP and SP6 RNA polymerase. To detect HDV genomic RNA in transfected cells and virus-like particles, a DIG-labeled HDV trimeric antigenomic RNA was transcribed in vitro from plasmid pD3. In addition, a DIG-labeled antisense RNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared from pIBI30GAPDH (28), which contains the cDNA of rat GAPDH, to detect GAPDH transcript in various human tissues as an internal control.

RT-PCR. To detect distribution of HDV genomic RNA in the nucleus and cytoplasm of transfected cells, a two-step semiquantitative reverse transcriptase PCR (RT-PCR) method was used. In brief, HDV-R (5′-GCTCTAGACATCCCCCTCTCGGTGC-3′) was used as an HDV-specific primer in the first step of cDNA synthesis of HDV genomic RNA with SuperScript III reverse transcriptase (Invitrogen) and 1 μg of the total RNA of nuclear or cytoplasmic fraction. PCR amplification was then carried out with primer set HDV-R and HDV-F (5′-GGAATTCACTGCTCGAGGATCTCTT-3′) and VioTaq (Viogene) under standard conditions (35 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s) to generate a 213-bp RT-PCR product of the HDV genomic RNA. As a control, cDNA synthesis was performed in parallel with oligo$(dT)_{12-18}$ primer followed by PCR-amplification with the primer set β-actin-F (5′-GCTCGTCGTCGGACAACGGCT-3′) and β-actin-R (5′-CAAACATGATCTGGGTCATCTTCTC-3′) to generate a 353-bp RT-PCR product of the β-actin mRNA. The RT-PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. Photographs were taken with a DC290 camera and captured through 1D Image Analysis software (Kodak).

Coimmunoprecipitation and Western blot analysis. Coimmunoprecipitation experiments were carried out as previously described (19) except that rabbit polyclonal antibodies specific for HA tag (BD Biosciences) and M2 agarose beads specifically recognizing the Flag tag (Sigma) were used. Western blot analysis was performed as previously described (19). The specific primary antibodies used include protein G-purified rabbit antiserum specific for HDAgs (5), chicken immunoglobulin Y (IgY) against HDAgs (Lin et al., unpublished data), goat polyclonal antibodies specific to HBsAg (Dako), mouse monoclonal antibody specific to histidine tag (Novagen), mouse monoclonal antibody specific to GFP (BD Biosciences), and protein G-purified antiserum specific against NESI that was raised in this study by immunizing rabbit with a peptide containing the C terminus of NESI (amino acid residues 453 to 467). To determine the package activity of HDV, virus-like particles were collected from culture media of transfected cells, and protein lysate was prepared following a resuspension of the viral pellet in 2× sample buffer (1× buffer is 12.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol, 0.25% bromophenol blue, 5% β-mercaptoethanol). The protein lysates were boiled for 3 min and subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and Western blot analysis to examine the presence of HDAg-L and HBsAg. The specific interactions between

antigens and antibodies were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Indirect double immunofluorescence staining. Permeabilization and fixation of cultured cells were carried out with Triton X-100 and paraformaldehyde, respectively, as previously described (19). After a rehydration with multiple rinses in PBS, the cells were treated with PBS containing 4% bovine serum albumin; double immunofluorescence staining of cells was carried out with protein G-purified rabbit antiserum specific for HDAgs and mouse monoclonal antibody specific to HA epitope (Roche Biochemicals) as the primary antibodies and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) and Texas red-labeled horse anti-mouse IgG (Vector) as the secondary antibodies, respectively. Immunostained cells were washed thoroughly with PBS and mounted in a buffer containing 0.1 M PBS, pH 8.0, 2% *n*-propyl gallate, and 60% glycerol. Photographs were taken using a Zeiss Axiophot microscope equipped with epifluorescence.

RESULTS

Identification of a NES(HDAg-L)-interacting protein by yeast two-hybrid screening. HDAg-L is a nucleocytoplasmic shuttling protein. The proline-rich NES(HDAg-L) mediates the relocalization of HDAg-L to the cytoplasm in the presence of HBsAg (20). The NES(HDAg-L) is a transferable nuclear export signal capable of directing a heterologous protein from the nucleus to the cytoplasm. In addition, Pro-205 is critical for the nuclear export of NES(HDAg-L) and the assembly of HDV (20). To identify cellular factors that interact with the NES(HDAg-L), yeast two-hybrid screening was performed with the NES(HDAg-L) fused to the C terminus of the DNA binding domain of GAL4 (GAL4DB) as bait. This process takes advantage of two factors: (i) the yeast two-hybrid system enables the analysis of protein-protein interactions occurring in the nucleus of transfected cells and (ii) NES(HDAg-L) is located at the C terminus of the viral protein in an exposed configuration (39). A cDNA library representing fusion proteins of the transcription activation domain of GAL4 (GAL4AD) and cDNAs derived from human liver cells was screened. Diploid colonies were grown on plates in the absence of Trp, Leu, adenine, and His, and plasmids were purified from these colonies. The plasmids represent fusion proteins of the GAL4AD and putative NES(HDAg-L)-interacting proteins and were individually cotransformed with the bait plasmid pAS2-1-NES that encodes GAL4DB-NES(HDAg-L) into yeast strain PJ69-2A for further screening. Several potential clones carry plasmids that encode proteins capable of interacting with GAL4DB-NES(HDAg-L) were obtained (data not shown). Nevertheless, among these clones, only clone 13-1 that carries plasmid pACT2-HA-NESI encoding a protein, designated NESI [NES(HDAg-L)-interacting protein], fused to the GAL4AD failed to interact with an export/package-defective NES mutant, GAL4DB-NES*(HDAg-L) in which Pro-205 has been replaced by Ala (Fig. 1). NESI encoded by the clone 13-1 is, therefore, representing an export/package activity-associated human protein. Sequence analysis and a database search indicated that *nesi* is a novel gene.

Detection of the NESI transcript in human liver tissue and isolation of the full-length cDNA of *nesi*. To understand the distribution of NESI RNA transcript in the hepatoma cell line and human tissues, Northern blot analysis was performed with total RNA isolated from HepG2 cells and various human tissues using a DIG-labeled antisense NESI RNA as the probe. As shown in Fig. 2, a NESI transcript was detected in HepG2 cells and predominantly, if not exclusively, in human liver tissue (panel B). Nevertheless, the cellular NESI transcript was approximately 1.9 kb and ran slower than NESI RNA transcribed in vitro from a cDNA plasmid (pGEMTeasy-HA-NESI) derived from the clone 13-1 (Fig. 2A). This indicated that the *nesi* cDNA present in the liver cDNA library may not be full length. To obtain the full-length cDNA of *nesi*, RT-PCR was performed using RNA isolated from human liver tissue as a template. Primers used in the RT-PCR were designed according to the results of rapid amplification of cDNA ends (data not shown). The *nesi* cDNA contains an open reading frame en-

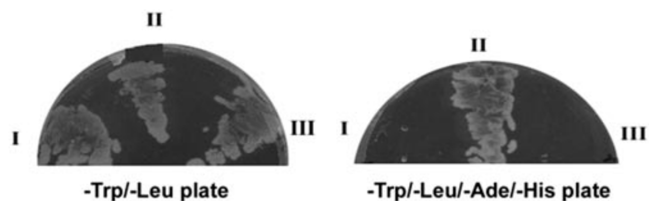

FIG. 1. Characterization of the specific interaction between GAL4DB-NES(HDAg-L) and GAL4AD-NESI. Yeast strain PJ69-2A that bears *ADE2* and *HIS3* reporters was cotransformed with plasmid pACT2-HA-NESI encoding the GAL4AD-NESI and plasmid pAS2-1 encoding the GAL4DB (I), plasmid pAS2-1-NES encoding the GAL4DB-NES(HDAg-L) (II), or plasmid pAS2-1-NES* encoding the mutant GAL4DB-NES*(HDAg-L) (III) to examine the specific interaction between NES(HDAg-L) and NESI encoded by the clone 13-1. Transformants were grown in synthetic medium containing dextrose on plates selecting for the absence of Trp and Leu (-Trp/-Leu SD) as a control (left panel). Yeast cells cotransformed with plasmid pAS2-1-NES were grown on a selection plate lacking Trp, Leu, adenine, and His (-Trp/-Leu/-Ade/-His SD) but not cells cotransformed with plasmid pAS2-1 or pAS2-1-NES* (right panel), indicating a specific interaction between NES(HDAg-L) and NESI.

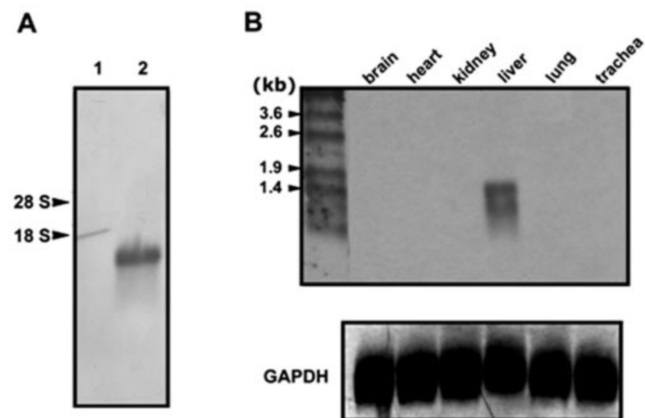

FIG. 2. The NESI transcript was detected in both HepG2 cells and the human liver tissue. Total RNA was isolated from HepG2 cells (A, lane 1) and various human tissues (BD Biosciences) as indicated (B) and subjected to Northern blot analysis with a DIG-labeled antisense NESI RNA probe. The DIG-labeled antisense NESI RNA probe was transcribed in vitro from plasmid pGEMTeasy-HA-NESI by using SP6 RNA polymerase. A NESI RNA in vitro transcribed from the plasmid pGEMTeasy-HA-NESI with T7 RNA polymerase was analyzed in parallel as a positive control (A, lane 2). Endogenous NESI transcript was detected in HepG2 cells and the human liver tissue. GAPDH RNA was analyzed to serve as an internal control. RNA size markers are shown on the left. kb, kilobases.

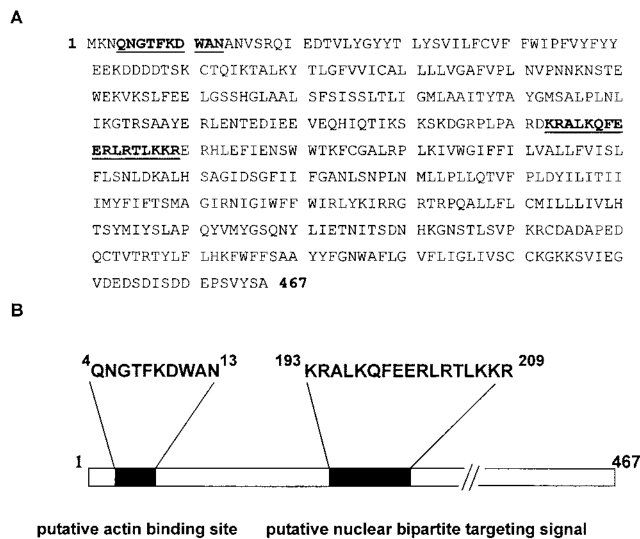

FIG. 3. Deduced amino acid sequence and schematic illustration of NESI. The deduced amino acid sequence of NESI is shown in panel A. The putative actin-binding site and nuclear bipartite targeting signal assigned by InterPro database (http://www.ebi.ac.uk/interpro) are shown in boldface and underlined; they are further illustrated in panel B. Numbers indicate the amino acid positions.

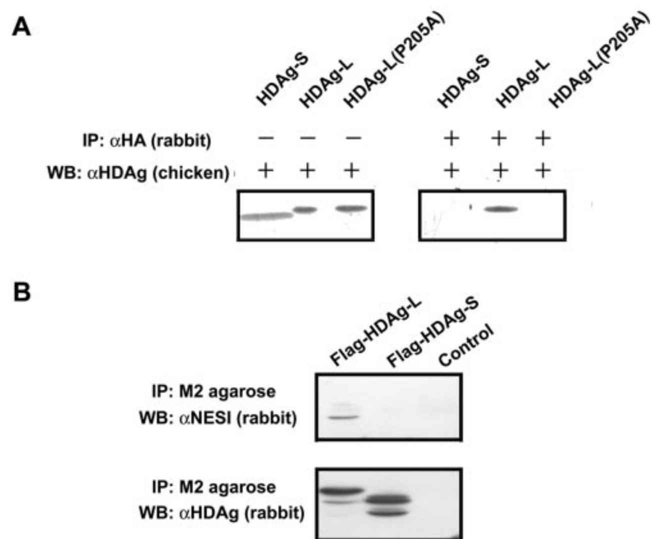

FIG. 4. NESI interacts with HDAg-L in mammalian cells. (A) Coimmunoprecipitation of HDAg-L and HA-NESI protein. HepG2 cells were cotransfected with the expression plasmid of HA-NESI fusion protein (pcDNA3.1-HA-NESI) and a plasmid representing HDAg-S (pECE-d-SM), HDAg-L (pECE-d-BE), or HDAg-L(P205A) mutant protein [pECE-d-BE(P205A)]. Cell extracts were prepared 2 days posttransfection and subjected to immunoprecipitation with rabbit polyclonal antibodies specific for HA tag, followed by Western blot analysis with chicken antibodies against HDAgs as indicated. In addition, direct Western blot analysis was performed to serve as a control of the expression levels of HDAgs. (B) Specific interaction between HDAg-L and endogenous NESI. HepG2 cells were transfected individually with the expression plasmid of Flag-HDAg-L (pCMV-Tag2C-HDAgL), Flag-HDAg-S (pCMV-Tag2C-HDAgS), and the control plasmid pCMV-Tag2C. Immunoprecipitation was performed with M2-agarose beads specific for the Flag tag, and Western blot analysis was performed with rabbit antibodies against NESI and HDAgs as indicated.

coding NESI with 467 amino acid residues (GenBank accession no. AY136817). The deduced amino acid sequence revealed that NESI possesses a putative actin-binding site (^{4}QNGTFKDWAN^{13}) and a putative nuclear bipartite targeting signal (^{193}KRALKQFEERLRTLKKR^{209}) (Fig. 3).

NESI interacts with HDAg-L in mammalian cells. To examine whether NESI is capable of binding HDAg-L in mammalian cells, a coimmunoprecipitation experiment was performed following cotransfection of HepG2 cells with an expression plasmid of HA-NESI fusion protein (pcDNA3.1-HA-NESI) and a plasmid representing HDAg-L (pECE-d-BE), HDAg-S (pECE-d-SM), or the HDAg-L(P205A) mutant protein [pECE-d-BE(P205A)]. Cell lysates from the transfected cells were immunoprecipitated with rabbit anti-HA antibody, and the immunoprecipitates were analyzed by Western blot analysis with chicken antibodies against HDAgs. As shown in Fig. 4A, only the export/package-competent wild-type HDAg-L was coprecipitated with HA-NESI. Neither the export/package-deficient HDAg-L(P205A) mutant nor the HDAg-S that lacks the export/package signal could be precipitated. The specific interaction between HDAg-L and NESI was further confirmed by transfecting HepG2 cells with plasmid pCMV-Tag2C-HDAgL encoding a Flag-tagged HDAg-L or plasmid pCMV-Tag2C-HDAgS encoding a Flag-tagged HDAg-S. Two days posttransfection, cell lysates were harvested and subjected to immunoprecipitation with M2 agarose beads specific for the Flag tag, followed by Western blot analysis using rabbit antibodies raised against a peptide sequence of NESI. A signal representing the endogenous NESI was detected only in the presence of HDAg-L but not in the presence of HDAg-S (Fig. 4B). Taken together, the results confirm the data of the yeast two-hybrid assay and indicate a specific interaction between HDAg-L and NESI through NES(HDAg-L).

Colocalization of NESI and HDAg-L. Previous studies have demonstrated that HDAg-L localizes in the nuclei of transfected cells and infected hepatocytes, either appearing as homogeneous nucleoplasm or as nucleolus distribution (4, 20). To investigate the functional significance of the interaction between NESI and HDAg-L, the subcellular localization of transiently expressed HA-NESI and HDAg-L were examined by indirect double immunofluorescence staining 2 days posttransfection. As shown in Fig. 5, HA-tagged NESI and HDAg-L were colocalized in the nucleus, suggesting an interaction of the nucleus-localized NESI with HDAg-L.

Effects of antisense NESI RNAs on the nuclear export of HDV genomic RNA and the assembly of HDAg-L-mediated genomic RNA into virus-like particles. To form infected HDV particles, both HDAgs have to be imported into the nucleus to form RNP with the nuclear viral RNA and then exported out of the nucleus to assemble with HBsAgs in the cytoplasm. The nuclear export activity is indispensable for HDAg-L to form virion and virus-like particles with the small form of HBsAg (20). HDAg-S that lacks nuclear export activity is assembled into virus-like particles via its binding activity to the viral RNA and the oligomerization activity of HDAgs (6). In earlier studies, we have shown that NESI specifically interacted with NES(HDAg-L) in a yeast two-hybrid system and with HDAg-L

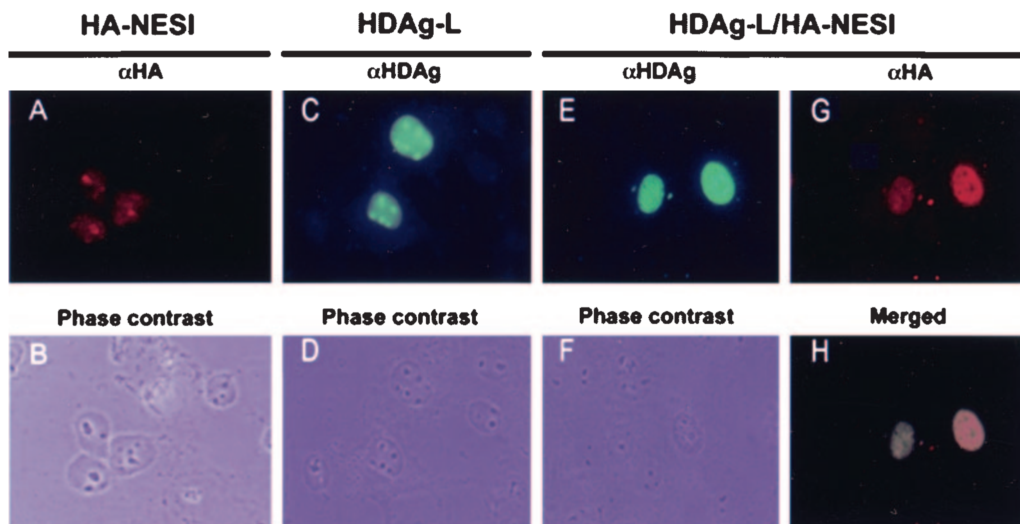

FIG. 5. Colocalization of HDAg-L and HA-NESI in the nucleus of transfected COS7 cells. COS7 cells were transfected with plasmid pcDNA3.1-HA-NESI encoding an HA-tagged NESI (A and B) or plasmid pECE-d-BE encoding the HDAg-L (C and D) or were cotransfected with both plasmids (E to H). Two days posttransfection, cells were fixed and subjected to indirect double immunofluorescence staining by using a mouse monoclonal antibody to the HA tag (A and G) and protein G-purified rabbit antiserum to HDAg (C and E) as primary antibodies. Panels B, D, and F are phase-contrast micrographs representing cells of the same fields as panels A, C, and E, respectively. HA-tagged NESI was visualized with Texas-red optics (A and G), and HDAg-L was visualized with fluorescein optics (C and E). A superimposed image of panels E and G is shown in panel H. Photographs were taken by epifluorescence microscopy.

in mammalian cells (Fig. 1 and 4). When indirect double immunofluorescence staining was performed, we found that NESI and HDAg-L were predominantly colocalized in the nucleus of transfected cells (Fig. 5). We therefore postulated that an antisense NESI RNA may affect the expression of endogenous NESI and block the efficiency of the HDAg-L-mediated nuclear export and package of HDV genomic RNA. To test this hypothesis, the effect of antisense RNA on the expression of NESI was first examined. Various amounts of the effector plasmid pcDNA3.1-AS-NESI encoding an antisense NESI RNA were cotransfected with plasmid pcDNA-NESI-V5HisTopo encoding a His-tagged NESI fusion protein. Two days posttransfection, protein lysates were prepared for use in Western blot analysis. As shown in Fig. 6, expression of the

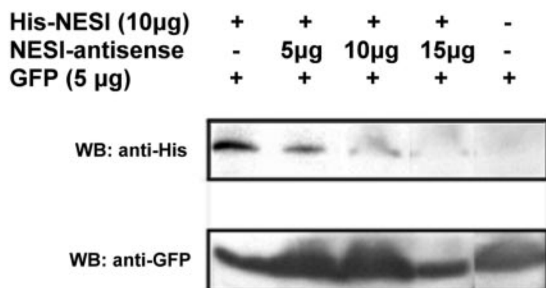

FIG. 6. Overexpression of an antisense NESI RNA blocked the expression of an exogenous His-tagged NESI in mammalian cells. COS7 cells were cotransfected with plasmid pEGFP-N1 encoding GFP, plasmid pcDNA-NESI-V5HisTopo encoding a His-tagged NESI, and various amounts of the effector plasmid pcDNA3.1-AS-NESI that expresses an antisense RNA of NESI as indicated. Two days posttransfection, protein lysates were prepared from the transfected cells and subjected to Western blot analysis using antibodies as indicated.

antisense NESI RNA efficiently blocked the expression of NESI in a dose-dependent manner. The antisense NESI RNA was then applied to examine the role of NESI on the package of HDV. The effector plasmid expressing antisense NESI RNA was cotransfected with plasmids encoding the HDAg-L, the small form of HBsAg, and a dimeric form of HDV genomic RNA in the HDV package system. Four days posttransfection, viral proteins and RNA were isolated from virus-like particles present in the culture medium. Western and Northern blot analyses were performed in parallel with the total protein and RNA that were isolated from the transfected cells. The results demonstrated that the level of HDV genomic RNA present in the NESI antisense RNA-expressing cells was equivalent to that of the control cells. Strikingly, in the presence of NESI antisense RNA, packaging of the viral genomic RNA into virus-like particles was completely abolished (Fig. 7A). The Western blot analysis shown in Fig. 7B demonstrated that in the presence of NESI antisense RNA, HDAg-L was produced in the transfected cells at a level similar to that of the control cells. The detection of HBsAg in the culture medium indicated that the assembly of HBsAg and the release of virus-like particles into culture medium were not blocked by the NESI antisense RNA. Nevertheless, in the presence of NESI antisense RNA, package efficiency of the HDAg-L into virus-like particles was decreased to less than 40% (Fig. 7B). The reason that the packaging of HDV genomic RNA was completely abolished but the packaging of HDAg-L was abolished to a lesser extent may be partially explained by the observation that an NLS-deleted HDAg-L mutant could bypass the nucleus localization and assemble directly with the small form of HBsAg in the cytoplasm (6). The fact that NESI antisense RNA blocks the release of virus-like particles containing HDV genomic RNA may reflect a block on the nuclear export of the

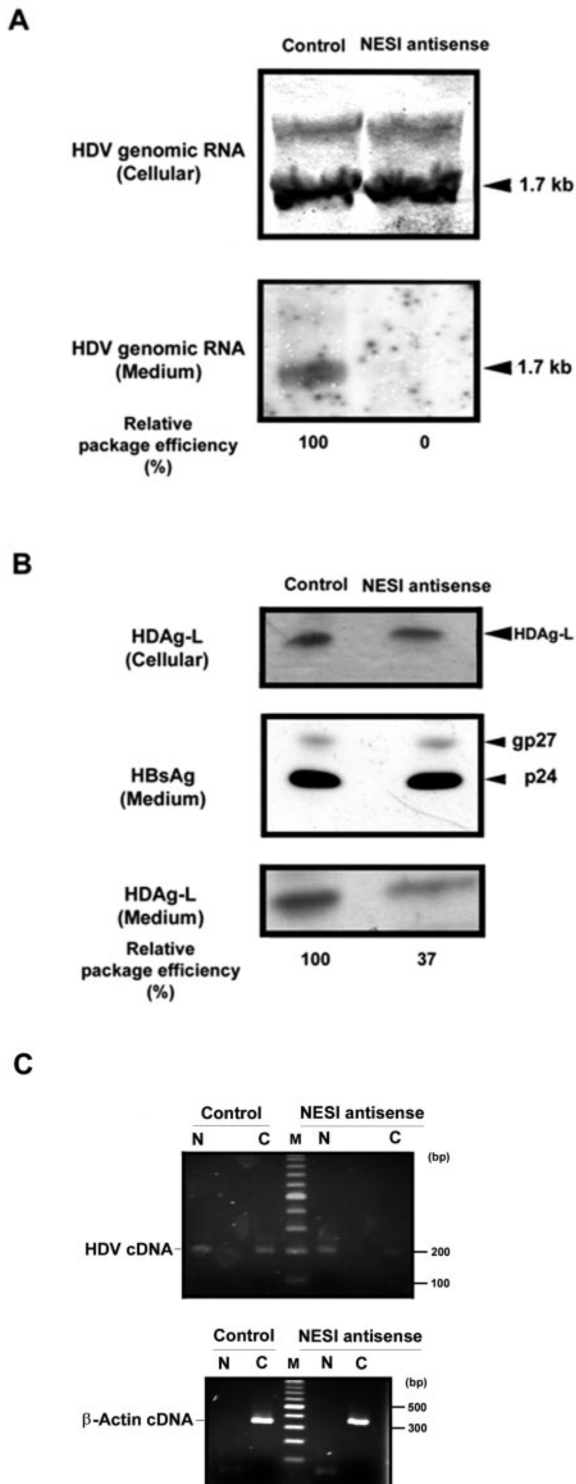

FIG. 7. Overexpression of the antisense NESI RNA blocked the nuclear export of HDV genomic RNA and assembly of the viral RNA into virus-like particles mediated by HDAg-L. HepG2 cells were co-transfected with plasmid pSVD2 expressing a dimeric HDV RNA and plasmids encoding the small form of HBsAg (pECE-C-ES) and HDAg-L (pECE-d-BE) in the presence of the control plasmid pcDNA3.1 (Control) or the effector plasmid pcDNA3.1-AS-NESI (NESI antisense) that expresses an antisense RNA of NESI. Four days posttransfection, both RNA and protein lysates were prepared from the transfected cells and from the viral pellets collected from the culture media. A DIG-labeled trimeric HDV antigenomic RNA transcribed in vitro from plasmid pD3 was used as a probe to perform Northern blot analysis (A). Protein G-purified rabbit antiserum specific for HDAgs and goat polyclonal antibodies specific to HBsAg were used to perform Western blot analysis as indicated (B). Following a partition, RNAs were isolated from both nuclear and cytoplasmic fractions of the transfected HepG2 cells and subjected to RT-PCR analysis to detect HDV genomic RNA (C, upper panel) and β-actin mRNA (C, lower panel). M, 100-bp DNA ladder. N, nuclear fraction; C, cytoplasmic fraction.

viral RNA. To further investigate whether antisense NESI RNA prevents HDV RNAs from leaving the nucleus through interfering with the nuclear export of HDAg-L, total RNAs from partitioned lysates of transfected cells were isolated to examine the distribution of HDV genomic RNA by RT-PCR analysis. As shown in Fig. 7C, antisense NESI RNA treatment significantly diminished the HDV genomic RNA in the cytoplasm compared to the control group (upper panel). The efficient separation of the nuclear and cytoplasmic fractions was evident by the results that the distribution of cellular β-actin mRNA control was mainly in the cytoplasmic fractions. The distribution of β-actin mRNA was not affected by the antisense NESI RNA (Fig. 7C, lower panel). Collectively, these results demonstrated that NESI is involved in the HDAg-L-mediated assembly of HDV genomic RNA.

DISCUSSION

By performing yeast two-hybrid analysis, we have identified NESI as a cellular factor that interacts with the wild-type nuclear export signal of HDAg-L, NES(HDAg-L), but not with the package/export-defective mutant NES*(HDAg-L). The specific interaction between HDAg-L and NESI was demonstrated by coimmunoprecipitation and an immunofluorescence staining assay. Overexpression of antisense NESI RNAs blocked the nuclear export and assembly of HDV RNA mediated by HDAg-L.

The nuclear export of viral RNP is a critical step in the life cycle of viruses that replicate in the nuclei of host cells. Most viral products that enter and exit the nucleus take advantage of the host nuclear import and export machineries. Human immunodeficiency virus (HIV) Rev protein that mediated the CRM1-dependent nuclear export of the partially spliced HIV-1 mRNA species and unspliced genomic RNA is the best-characterized example. The Rev protein is required for the viral RNA expression and virion assembly (12). Recently, a variety of alternative nuclear export signals independent of the CRM1 export receptor have been identified. These include the bidirectional signals found in the mRNA-regulating proteins, such as the KNS (for K nuclear shuttling) signal of hnRNP K (27) and the M9 signals of hnRNP A1 (26). Subcellular distribution of the TATA-binding protein-associated factor TAF_{II} 105 (31) was also regulated in a CRM1-independent manner. In a recent study, a CRM1-independent NES of human cytomegalovirus was identified within the C-terminal domain of pUL69 protein (22). The NES(pUL69) shares amino acid sequence homologous to the NES(HDAg-L), including a conserved proline residue at a comparable position to the Pro-205 of the NES(HADg-L) sequence. The conserved proline residue is essential for the NES function of both HDAg-L and

pUL69. Interestingly, the nuclear export of NES(pUL69) could not be competed away by the NES(HDAg-L) (22), indicating that the export pathway of HDAg-L and pUL69 involve distinct export receptors.

HDAg-L is localized mainly in the nucleolus of transfected cells. It targets to the nucleolus via interaction with nucleolin and B23 that shuttle between the nucleolus and nucleoplasm (16, 19). Nucleoplasm distribution is observed only in a minor population of the HDAg-L-expressing cells and is only detected at a later time point (60 h) posttransfection (20). Nevertheless, in the presence of HDV RNA, a portion of the HDAg-L relocalized to the nucleoplasm at an earlier time point in the form of viral RNP complexes (11). Formation of HDV RNP complexes in the nucleus is required for viral replication at the early stage of infection and for virion assembly, presumably after HDAg-L is synthesized in the late stage of the viral life cycle. HDV RNP complex has been described as a nucleocytoplasmic shuttling unit with a steady-state nuclear distribution (36). In addition, a minor population of the HDV RNP complex has been detected in the cytoplasm of transfected cells in the absence of HBsAg (36). In this study, coimmunoprecipitation and colocalization experiments indicate a specific interaction between HDAg-L and the newly identified NESI (Fig. 4 and 5). Taken together, these data suggest that the interaction between HDAg-L and NESI renders the NESI-HDAg-L complex in a conformation accessible to the protein complexes required for nuclear export and/or assembly of HDV RNP into virus-like particles. Indeed, we demonstrated that overexpression of an antisense NESI RNA abolished HDAg-L-mediated assembly of HDV genomic RNA into virus-like particles by inhibiting the HDAg-L-mediated nuclear export of HDV genomic RNA into the cytoplasm (Fig. 7). The observation that a trace amount of HDV genomic RNA was detected in the cytoplasm of transfected cells in the presence of antisense NESI RNA is similar to the result of a recent study in which HDV genomic RNA was detected in the cytoplasm in the absence of HDAg-L (23). These results suggest that, without forming a complex with HDAg-L, the cytoplasm-localized free form HDV RNA is not competent for viral assembly. It is likely that HDAgs are imported into the nucleus where HDV RNP complexes form, then via an interaction between the NES(HDAg-L) and the nuclear export-associated factor NESI, the RNP complexes export to the cytoplasm and form RNA-containing virus-like particles with HBsAg in the endoplasmic reticulum and Golgi apparatus. When the expression of NESI is blocked by the antisense NESI RNA, the process of forming a competent export/assembly complex of HDAg-L is inhibited and the assembly of HDV RNA is abolished.

The interaction between NESI and HDAg-L is highly associated with the export/assembly activity of HDAg-L. NESI bound to the export/assembly-competent HDAg-L but not to the export/assembly-defective HDAgs, HDAg-S, and HDAg-L(P205A) (Fig. 4). Profile analysis of NESI revealed a putative bipartite NLS spanning amino acid residues 193 to 209 and a putative actin-binding site at the N terminus (Fig. 3). Interestingly, a recent study demonstrated a critical role of nuclear actin in the nuclear export of HIV Rev protein and protein kinase inhibitor (PKI) (14). Whether actin is involved in the nuclear export of HDAg-L remains to be elucidated. Collectively, we propose that NESI plays an important role in facilitating the formation of a functionally competent export/package complex of HDAg-L. The interaction between NESI and NES(HDAg-L) may facilitate the export of HDV RNP from nucleus to cytoplasm and the assembly of viral genomic RNA mediated by HDAg-L. Further studies on the molecular mechanisms of NESI involved in the nucleocytoplasmic shuttling of HDAg-L and the assembly of HDV RNP would provide further information in understanding the functional roles of the novel NESI in eukaryotic cells.

ACKNOWLEDGMENTS

We are grateful to S.-S. Lin and W.-N. Wen for providing chicken antibodies specific to HDAgs. We also thank J.-Y. Lin, Y.-H. Wu, I.-C. Yu, Y.-H. Chen, H.-M. Shih, and J.-Y. Shew for helpful discussions and technical assistance.

This work was supported in part by research grants NSC91-2320-B-002-073, NSC92-2320-B-002-194, and NSC93-2320-B-002-123 from the National Science Council of the Republic of China.

REFERENCES

1. **Branch, A. D., and H. D. Robertson.** 1984. A replication cycle for viroids and other small infectious RNA's. Science **223:**450–455.
2. **Casey, J. L., and J. L. Gerin.** 1995. Hepatitis D virus RNA editing: specific modification of adenosine in the antigenomic RNA. J. Virol. **69:**7593–7600.
3. **Chang, F. L., P. J. Chen, S. J. Tu, C. J. Wang, and D. S. Chen.** 1991. The large form of hepatitis delta antigen is crucial for assembly of hepatitis delta virus. Proc. Natl. Acad. Sci. USA **88:**8490–8494.
4. **Chang, M. F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. C. Lai.** 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. J. Virol. **62:**2403–2410.
5. **Chang, M.-F., C.-Y. Sun, C.-J. Chen, and S. C. Chang.** 1993. Functional motifs of delta antigen essential for RNA binding and replication of hepatitis delta virus.J. Virol. **67:**2529–2536.
6. **Chang, M. F., C. J. Chen, and S. C. Chang.** 1994. Mutation analysis of delta antigen: effect on assembly and replication of hepatitis delta virus. J. Virol. **68:**646–653.
7. **Chang, M. F., C. H. Chen, S. L. Lin, C. J. Chen, and S. C. Chang.** 1995. Functional domains of delta antigens and viral RNA required for RNA packaging of hepatitis delta virus. J. Virol. **69:**2508–2514.
8. **Chao, M., S. Y. Hsieh, and J. Taylor.** 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. J. Virol. **64:**5066–5069.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Chou, H. C., T. Y. Hsieh, G. T. Sheu, and M. M. C. Lai.** 1998. Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. J. Virol. **72:**3684–3690.
11. **Dingle, K., V. Bichko, H. Zuccola, J. Hogle, and J. Taylor.** 1998. Initiation of hepatitis delta virus genome replication. J. Virol. **72:**4783–4788.
12. **Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Luhrmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475–483.
13. **Glenn, J. S., J. A. Watson, C. M. Havel., and J. M. White.** 1992. Identification of a prenylation site in the delta virus large antigen. Science **256:**1331–1333.
14. **Hoffmann, W., B. Reichart, A. Ewald, E. Muller, I. Schmitt, R. H. Stauber, F. Lottspeich, B. M. Jockusch, U. Scheer, J. Hauber, and M. C. Dabauvalle.** 2001. Cofactor requirements for nuclear export of Rev response element (RRE)- and constitutive transport element (CTE)-containing retroviral RNAs: an unexpected role for actin. J. Cell Biol. **152:**895–910.
15. **Hoofnagle, J. H.** 1989. Type D (delta) hepatitis. JAMA **216:**1321–1325.
16. **Huang, W. H., B. Y. M. Yung, W. J. Syu, and Y. H. Wu-Lee.** 2001. The nucleolar phosphoprotein B23 interacts with hepatitis delta antigens and modulates the hepatitis delta virus RNA replication. J. Biol. Chem. **276:** 25166–25175.
17. **Kos, A., R. Dijkema, A. C. Arnberg, P. H. van der Meide, and H. Schellekens.** 1986. The hepatitis delta (δ) virus possesses a circular RNA. Nature **323:** 558–560.
18. **Kuo, M. Y., L. Sharmeen, G. G. Dinter, and J. Taylor.** 1988. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. J. Virol. **62:**4439–4444.
19. **Lee, C. H., S. C. Chang, C. J. Chen, and M. F. Chang.** 1998. The nucleolin binding activity of hepatitis delta antigen is associated with nucleolus targeting. J. Biol. Chem. **273:**7650–7656.
20. **Lee, C. H., S. C. Chang, C. H. Wu, and M. F. Chang.** 2001. A novel chromosome region maintenance 1-independent nuclear export signal of the

large form of hepatitis delta antigen that is required for the viral assembly. J. Biol. Chem. **276:**8142–8148.
21. **Lin, J. H., M. F. Chang, S. C. Baker, S. Govindarajan, and M. M. Lai.** 1990. Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. J. Virol. **64:**4051–4058.
22. **Lischka, P., O. Rosorius, E. Trommer, and T. Stamminger.** 2001. A novel transferable nuclear export signal mediates CRM1-independent nucleocytoplasmic shuttling of the human cytomegalovirus transactivator protein pUL69. EMBO J. **20:**7271–7283.
23. **Macnaughton, T. B., and M. M. Lai.** 2002. Genomic but not antigenomic hepatitis delta virus RNA is preferentially exported from the nucleus immediately after synthesis and processing. J. Virol. **76:**3928–3935.
24. **Macnaughton, T. B., Y. J. Wang, and M. M. C. Lai.** 1993. Replication of hepatitis delta virus RNA: effect of mutations of the autocatalytic cleavage sites. J. Virol. **67:**2228–2234.
25. **Makino, S., M. F. Chang, C. K. Shieh, T. Kamahora, D. M. Vannier, S. Govindarajan, and M. M. C. Lai.** 1987. Molecular cloning and sequencing of a human hepatitis delta virus RNA. Nature **329:**343–346.
26. **Michael, W. M., M. Choi, and G. Dreyfuss.** 1995. A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell **83:**415–422.
27. **Michael, W. M., P. S. Eder, and G. Dreyfuss.** 1997. The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. **16:**3587–3598.
28. **Piechaczyk, M., J. M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. El-Sabouty, P. Fort, and P. Jeanteur.** 1984. Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. Nucleic Acids Res. **12:**6951–6963.
29. **Ponzetto, A., P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin.** 1984. Transmission of the hepatitis B virus-associated delta agent to the eastern woodchuck. Proc. Natl. Acad. Sci. USA **81:**2208–2212.
30. **Ponzetto, A., F. Negro, H. Popper, F. Bonino, R. Engle, M. Rizzetto, R. H Purcell, and J. L. Gerin.** 1988. Serial passage of hepatitis delta virus in chronic hepatitis B virus carrier chimpanzees. Hepatology **8:**1655–1661.
31. **Rashevsky-Finkel, A., A. Silkov, and R. Dikstein.** 2001. A composite nuclear export signal in the TBP-associated factor TAFII105. J. Biol. Chem. **276:** 44963–44969.
32. **Ryu, W. S., H. J. Netter, M. Bayer, and J. Taylor.** 1993. Ribonucleoprotein complexes of hepatitis delta virus. J. Virol. **67:**3281–3287.
33. **Sharmeen, L., M. Y. P. Kuo, G. Dinter-Gottlieb, and J. Taylor.** 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. J. Virol. **62:**2674–2679.
34. **Sureau, C., B. Guerra, and R. E. Lanford.** 1993. Role of the large hepatitis B virus envelope protein in infectivity of the hepatitis delta virion. J. Virol. **67:**366–372.
35. **Symons, R. H.** 1989. Self-cleavage of RNA in the replication of small pathogens of plants and animals. Trends Biochem. Sci. **14:**445–450.
36. **Tavanez, J. P., C. Cunha, M. C. A. Silva, E. David, J. Monjardino, and M. Carmo-Fonseca.** 2002. Hepatitis delta virus ribonucleoproteins shuttle between the nucleus and the cytoplasm. RNA **8:**637–646.
37. **Tsai, C. W., S. C. Chang, and M. F. Chang.** 1999. A 12-amino acid stretch in the hypervariable region of the spike protein S1 subunit is critical for cell fusion activity of mouse hepatitis virus. J. Biol. Chem. **274:**26085–26090.
38. **Wang, C. J., P. J. Chen, J. C. Wu, D. Patel, and D. S. Chen.** 1991. Small-form hepatitis B surface antigen is sufficient to help in the assembly of hepatitis delta virus-like particles. J. Virol. **65:**6630–6636.
39. **Wang, J. G., R. W. Jansen, E. A. Brown, and S. M. Lemon.** 1990. Immunogenic domains of hepatitis delta virus antigens: peptide mapping of epitopes recognized by human and woodchuck antibodies. J. Virol. **64:**1108–1116.
40. **Weiner, A. J., Q. L. Choo, K. S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton.** 1988. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24 delta and p27 delta. J. Virol. **62:**594–599.