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# Human Hepatitis Delta Antigen Is a Nuclear Phosphoprotein with RNA-Binding Activity

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**The genetic origin, structure, and biochemical properties of the delta antigen (HDAg) of a human hepatitis delta virus (HDV) were investigated. A cDNA fragment containing the open reading frame encoding the HDAg was transcribed into RNA and used for in vitro translation in rabbit reticulocyte lysates. The HDAg open reading frame was also inserted into an expression vector containing a simian virus 40 T-antigen promoter and expressed into COS 7 cells. In both systems, a protein species of 26 kilodaltons was synthesized from this open reading frame and could be specifically immunoprecipitated with antisera obtained from patients with delta hepatitis. A similar protein was also synthesized from antigenomic-sense monomeric HDV RNA in both systems, although the efficiency of translation was lower than that of the isolated open reading frame. This protein was found to be phosphorylated at the serine residues. Immunoperoxidase studies with anti-HDV sera demonstrated that the HDAg was expressed mainly in the nuclei of the transfected COS 7 cells. Moreover, the HDAg was shown to bind the genomic RNA of HDV. These studies indicate that HDAg is encoded by the antigenomic-sense RNA of HDV and is a nuclear phosphoprotein associated with an RNA-binding activity.**

Hepatitis delta virus (HDV) is a recently recognized human pathogen (27, 28). The virus is defective and may either coinfect with hepatitis B virus (HBV), resulting in fulminant hepatitis, or superinfect chronic HBV carriers resulting in severe chronic hepatitis and liver cirrhosis (1, 10, 14). HDV infection is endemic in many parts of the world. In the United States, it is prevalent among intravenous drug abusers who are HBV carriers (13, 22).

The HDV is a 36-nm particle with an envelope made up of HBV surface antigen; hence, the production of HDV virions is dependent on HBV co-infection (28). Inside the envelope is an HDV-specific protein, hepatitis delta antigen (HDAg), which is detectable only after the virion is disrupted with detergents, such as Nonidet P-40 (4, 5, 28). The HDAg is probably associated with the viral genome; however, no nucleocapsid structure has ever been observed (5, 28). This antigen is also detected in the nuclei of the infected hepatocytes (27). The HDAg in the sera and liver of infected humans, chimpanzees, or woodchucks has been shown to consist of either two protein species of between 24 and 30 kilodaltons (kDa) (2, 3, 25, 40) or a major protein of 26 kDa and multiple minor proteins (26). The relationship of these proteins observed is not clear, although immunoprecipitation with a single monoclonal antibody (25) suggests that these protein species are related in sequence. No other HDV-specific proteins have been detected.

The HDV genome is an approximately 1.7-kilobase (kb) single-stranded, circular RNA (6, 16, 23, 38). Recently, complete sequences of HDV RNA from both a chimpanzee-adapted HDV isolate and a direct human isolate were obtained (23, 38, 39). These sequences reveal that HDV RNA contains extensive sequence complementarity, a property shared with viroid and virusoid RNAs (38). Several viroid and virusoid consensus sequences which may be required for RNA replication (8) are also conserved in HDV RNA (23, 38). However, one notable difference between HDV and viroid RNAs is the presence of multiple potential open reading frames (ORFs) in HDV RNA. At least five ORFs capable of coding for more than 100 amino acids are detected. These ORFs are present in both genomic and antigenomic orientations (23, 38). In the cloned human isolate, the largest ORF in the antigenomic sense (ORF-2) (23, 38) has been implicated as the region encoding the HDAg. This ORF can code for a protein of 214 amino acids with a molecular mass of 24 kDa. Indeed, the expression of this ORF as a fusion protein in *Escherichia coli* yielded proteins immunoprecipitable with convalescent sera from patients with delta hepatitis (38). The predicted sequence of this protein contains highly charged amino acids in the N-terminal two-thirds and uncharged amino acids at the C terminus (23, 39). The abundance of highly basic amino acid residues suggests that the HDAg interacts with the HDV RNA. It has been shown that HDV RNA replication probably occurs in the nuclei of infected hepatocytes (12, 36). Moreover, Rizzetto et al. have shown that the HDAg was detected in the nuclei of the hepatocytes of delta hepatitis patients (27). Thus, the HDAg may play an important role in the replication and pathogenesis of HDV.

To understand the biochemical characteristics and functions of the HDAg, we expressed either the region of ORF-2 or the antigenomic HDV monomer under the control of promoters of bacteriophage T7 RNA polymerase and the simian virus 40 (SV40) T antigens. A protein species of 26 kDa was obtained from both systems. This protein was specifically precipitated by hepatitis delta convalescent serum. It was also found that this protein is phosphorylated and accumulates in the nuclei. Furthermore, this protein could bind the genomic RNA of HDV. Thus, HDAg is a nuclear phosphoprotein associated with an RNA-binding activity.

## MATERIALS AND METHODS

**Cells.** The COS 7 cell line, an SV40-transformed derivative of African green monkey cell line CV-1p (9), was obtained

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from A. S. Lee of the University of Southern California. The cell line was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

**HDV cDNA clones and recombinant DNA methodology.** The human HDV cDNA clones have been previously described (23). Standard recombinant DNA techniques were used for the construction of subclones and growth and purification of plasmids. Restriction endonucleases and DNA-modifying enzymes were purchased from several commercial sources and used as recommended by the manufacturers.

**Construction of pT7 recombinant vectors.** Clone HN88 DNA containing the postulated HDAg-coding region (23) was excised from pBR322 with *Pst*I and *Bam*HI, and the resulting 1.1-kb fragment was directionally inserted into the *Bam*HI and *Pst*I sites of the transcription vector pT7-3 containing the bacteriophage T7 RNA polymerase promoter (35). This construct was designated pT7-d-BP. RNA transcribed from this construct is of the antigenomic orientation and has approximately 150 nucleotides of HDV RNA 5′ to the postulated initiator AUG of the HDAg.

To include the genetic information of the entire HDV RNA, an antigenomic HDV monomer was constructed. The 1.4-kb *Sal*I-to-*Pst*I DNA fragment was excised from the clone HN88 and ligated to the 0.3-kb *Pst*I-to-*Sal*I DNA fragment of HD489 (23). This DNA was then inserted into the *Pst*I site of pT7-3 (35). Plasmids producing HDV RNA transcripts of either genomic or antigenomic orientation were obtained and designated pT7-d-P1 and pT7-d-P3, respectively. T7 polymerase-mediated transcription from pT7-d-P3 produces a 1.75-kb antigenomic-sense RNA with approximately 750 nucleotides upstream of the AUG of the HDAg.

An additional pT7-3 plasmid containing a 3.4-kb *Sal*I-*Sal*I HDV dimer DNA was constructed and designated pT7-DD15. The construction of this dimer RNA will be described elsewhere.

**Construction of HDV eucaryotic expression vectors.** The shuttle vector pECE (21) was obtained from J.-H. Ou of the University of Southern California. This vector contains the SV40 early promoter and origin of replication, a polylinker region, and a polyadenylation signal. A 1.2-kb *Bam*HI-*Eco*RI fragment was excised from pT7-DD15 and inserted into the *Bam*HI and *Eco*RI sites of pECE. This construct was designated pECE-d-BE. To construct a plasmid containing the entire HDV genome, a 1.75-kb DNA fragment was excised from pT7-d-P1 at polylinker sites *Xba*I and *Hin*dIII and inserted into the corresponding sites in pECE. This insert includes the entire HDV genome starting and ending at the *Pst*I site (23). This construct was designated pECE-d-PP.

**In vitro transcription and translation of HDV.** The synthesis of capped RNAs from the recombinant pT7 vectors was carried out as previously described (32). The RNAs (200 ng per reaction) were translated in an mRNA-dependent rabbit reticulocyte lysate (New England Nuclear Corp.) as recommended by the manufacturer, except that potassium acetate and magnesium acetate were optimized to 100 and 1.5 mM, respectively. Translation mixtures were incubated for 60 min at 30°C with [$^{35}$S]methionine (2,400 μCi/ml; New England Nuclear) as the radiolabel.

**Anti-HDAg IgG.** The immunoglobulin G (IgG) fraction was purified from HDV-infected human sera by passage over a *Staphylococcus* protein A column. The IgG fraction purified from the sera of a non-A, non-B hepatitis patient was used as a control antibody.

**Immunoprecipitation.** Immunoprecipitation of the HDAg was performed by the method of Kessler (15). The in vitro-translated proteins or transfected COS 7 cell extracts were incubated with 3 μl of HDV-specific or control IgG (1.5 mg/ml) for 1 to 4 h at 4°C. The antigen-antibody complexes were collected by binding to Pansorbin (Calbiochem, La Jolla, Calif.) and washed three times with NET–Nonidet P-40 buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet p-40) and eluted by boiling for 2 min in lysis buffer (0.1 M β-mercaptoethanol, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 0.08 M Tris hydrochloride [pH 6.8], 10% glycerol). The bacteria were removed by centrifugation, and the proteins were analyzed by SDS–5 to 15% polyacrylamide gel electrophoresis (PAGE) (19). Gels were fixed for 30 min in 50% methanol–7% acetic acid, dried, and exposed to Kodak X-ray film at −70°C.

**Transfection and labeling of COS 7 cells.** The DNA transfections were performed by either the calcium phosphate or the DEAE-dextran method. The procedure for the calcium phosphate method was adapated as described previously (24). Briefly, plasmid DNA (2 μg/ml), HeLa cell DNA (7 μg/ml), and calcium phosphate (125 mM final concentration) were mixed and applied to a 100-mm dish of subconfluent COS 7 cells. The treated cells were incubated at 37°C for 4 h, followed by a glycerol shock to promote uptake of exogenous DNA. Incubation was continued for 44 to 48 h. The transfected cells were then incubated for 1 h in methionine-free DMEM and labeled with [$^{35}$S]methionine (0.1 mCi/ml) for 4 h. The monolayers were then washed two times with phosphate-buffered saline, and total cell extracts were prepared by scraping the cells in 300 μl of lysis buffer (0.1 M β-mercaptoethanol, 1% [wt/vol] SDS, 0.08 M Tris hydrochloride [pH 6.8], 10% glycerol). Cellular DNA was sheared by passing the extract through a 25-gauge needle. The whole cell extract was then tested for the presence of the HDAg by immunoprecipitation.

The DEAE-dextran transfection method followed essentially the procedure described by Sussman and Milman (34), except that COS 7 cells were cultured at 37°C in the presence of 5% $CO_2$ during the course of the transfection experiment. Plasmid DNA (2 μg/ml) was resuspended in DMEM containing DEAE-dextran and added to a 100-mm dish of subconfluent COS 7 cells. Dimethyl sulfoxide shock was performed after 4 h of incubation at 37°C. Incubation was continued for 44 to 48 h after transfection. Transfected cells were metabolically labeled with either [$^{35}$S]methionine (0.1 mCi/ml) for 4 h after incubation with methionine-free medium for 1 h or [$^{32}$P]orthophosphoric acid (0.3 mCi/ml) for 4 h after incubation with phosphate-free medium for 2 h. Total cell extracts were prepared as described above and used for immunoprecipitation.

**Phosphoamino acid analysis.** The pECE-d-BE-transfected COS 7 cells were labeled with $^{32}P_i$ as described above. The HDAg was immunoprecipitated by anti-HDV IgG and purified by SDS-PAGE (5 to 15% polyacrylamide). The protein was identified by autoradiography of the wet gel, and the fragment containing HDAg was excised from the gel. The HDAg protein was electroeluted from the gel slice and recovered by precipitation with 20% trichloroacetic acid in the presence of 150 μg of bovine serum albumin as a carrier. The precipitated protein was pelleted by centrifugation in a microfuge for 20 min. The protein pellet was suspended in 0.5 ml of 1 N NaOH and reprecipitated with 0.2 ml of 50% trichloroacetic acid. The precipitate was washed once with ethanol-ether (70:30) and once with ether. The purified $^{32}$P-labeled protein was then digested with 6 N HCl at 110°C

for 1 h and dried in a lyophilizer. The sample was suspended in pH 1.9 buffer (formic acid-acetic acid-water [1:4:100]) and applied to thin-layer cellulose plates (Brinkmann Instruments, Inc.). Electrophoresis was carried out in pH 1.9 buffer at 1,000 V for 65 min (30). Phosphoamino acid standards (Sigma Chemical Co.) were stained with ninhydrin solution in *n*-butanol.

**Indirect immunoperoxidase staining.** The staining of transfected COS 7 cells with anti-HDV sera followed a modification of published procedures (11). Briefly, the COS 7 cells at 25 h posttransfection were washed with phosphate-buffered saline and then incubated with the IgG fraction of anti-HDAg serum for 30 min in a moist chamber at room temperature. After incubation, the cells were washed with phosphate-buffered saline and treated with horseradish peroxidase-conjugated rabbit antihuman IgG (DAKO) at 1:16 dilution for 30 min in a moist chamber. Subsequently the cells were treated with 3-3'-diamino-benzidine hydrochloride and hydrogen peroxide. After dehydration, the cells were examined under light microscopy.

**RNA-binding assay.** The RNA-binding assay was performed by the procedure of Robbins et al. (29). Briefly, whole cell extracts were prepared from COS 7 cells transfected with either pECE-d-BE or pECE at 48 h posttransfection and immunoprecipitated with anti-HDV IgG as described above. The immunoprecipitated proteins were separated by SDS-PAGE (5 to 15% polyacrylamide) and then electrotransferred to a nitrocellulose filter membrane at 200 mA for 16 h at 4°C (37). The nitrocellulose filter was incubated in standard binding buffer (SBB; 10 mM Tris hydrochloride [pH 7.0], 1 mM disodium EDTA, 50 mM NaCl, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone) for 1 h at room temperature followed by a 1-h incubation at room temperature in SBB containing a $^{32}$P-labeled RNA transcribed in vitro from plasmid pT7-d-P1. This RNA represents the full-length genomic RNA of HDV. After binding, the blot was washed four times in SBB and air dried, and the bound RNA was visualized by autoradiography.

## RESULTS

**In vitro translation of HDAg.** To determine the genetic origin and biochemical properties of HDAg, we first studied the translational capacity of the ORFs of HDV cDNA clones. Since ORF-2 was predicted to be the region encoding the HDAg (23), the cDNA fragment corresponding to this ORF was inserted downstream of the T7 promoter in a pT7 vector (Fig. 1a). Transcription by T7 RNA polymerase of this plasmid, pT7-d-BP, resulted in the synthesis of a 1.1-kb RNA starting at approximately 150 nucleotides upstream of the postulated HDAg initiator AUG. To assess the translational capacity of the total antigenomic-sense HDV RNA, a monomer HDV cDNA clone was also inserted into the same vector. Transcription from this construct, pT7-d-P3, resulted in the synthesis of a 1.75-kb RNA starting 750 nucleotides upstream of the HDAg initiator AUG and represented HDV antigenomic-sense monomeric RNA. These RNAs were then translated in a rabbit reticulocyte lysate system.

Translation of both RNAs resulted in the synthesis of a single major protein of approximately 26 kDa (Fig. 2, lanes 2 and 3). The pT7-d-BP RNA, which contains only the ORF-2, synthesized a much larger amount of p26 than the pT7-d-P3 RNA, which contains the entire anti-genomic monomer HDV RNA, although equal amounts of RNA were used for translation. A minor smaller protein (18 kDa) was also occasionally synthesized by both RNAs (Fig. 2). No additional translation products were detected from the monomer pT7-d-P3 RNA, although several additional ORFs were present.

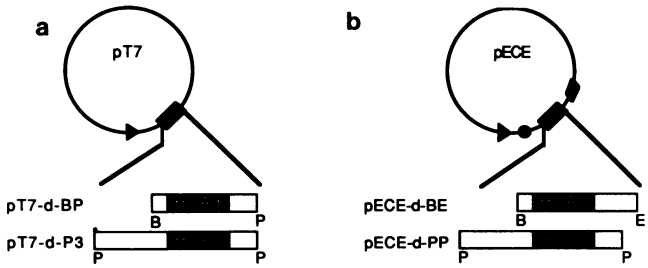


FIG. 1. Schematic diagrams of the pT7 and pECE recombinant plasmids used to express the HDAg in vitro and in vivo. (a) A 1.1-kb *Bam*HI-*Pst*I fragment of HDV cDNA clone HN88 (23) was inserted into the polylinker region ( ▒ ) of the pT7-3 vector downstream of the T7 RNA polymerase promoter (▶). This construct was designated pT7-d-BP. Alternatively, a 1.7-kb monomer HDV cDNA clone from the *Pst*I-to-*Pst*I sites was inserted into pT7-3 (pT7-d-P3). These two plasmids produce HDV-specific RNAs of antigenomic sense. (b) cDNA clones representing the HDAg ORF (*Bam*HI to *Eco*RI) or the HDV monomer (*Pst*I to *Pst*I) were inserted into the polylinker site ( ▒ ) of the expression vector pECE. This vector contains the SV40 early promoter (▶) upstream of the insert and a polyadenylation signal ( ◥ ) following the insert. In addition, there is an SV40 origin of replication (●) so that high copy numbers of this plasmid can be generated when it is transfected into COS 7 cells.

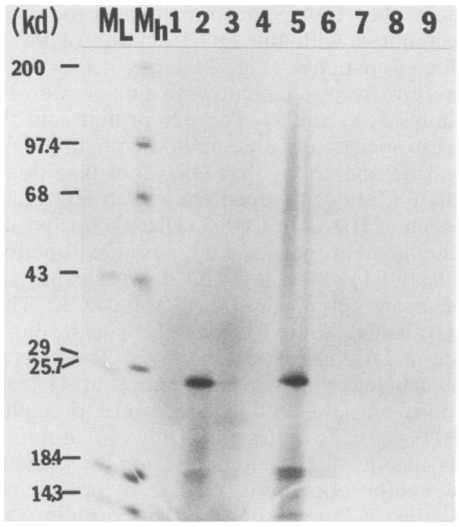


FIG. 2. In vitro translation products of RNAs transcribed from recombinant plasmids containing HDV sequences. Capped RNAs were synthesized by T7 RNA polymerase from linearized plasmids pT7-d-BE and pT7-d-P3. The RNA was translated in rabbit reticulocyte lysates in the presence of [$^{35}$S]methionine. Translation products were analyzed by electrophoresis on an SDS–5 to 15% polyacrylamide gradient gel and visualized by autoradiography. Lanes contained translation products of no added RNA (lane 1), pT7-d-BP RNA (lane 2), and pT7-d-P3 RNA (lane 3); after immunoprecipitation with anti-HDV IgG with no added RNA (lane 4), pT7-d-BP RNA (lane 5), and pT7-d-P3 (lane 6); and after immunoprecipitation with anti-NANB IgG with no added RNA (lane 7), pT7-d-BP RNA (lane 8), and pT7-d-P3 RNA (lane 9). Low- and high-molecular-weight markers (Bethesda Research Laboratories, Inc.) were run in parallel (lanes $M_L$ and $M_h$, respectively).

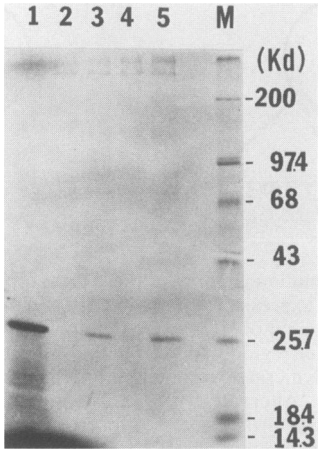


FIG. 3. Expression of the HDAg in COS 7 cells. COS 7 cells were transfected with either expression vector pECE or recombinant plasmid pECE-d-BE containing the HDAg-coding region by either calcium phosphate or DEAE-dextran methods. Transfected cells were metabolically labeled with [$^{35}$S]methionine (0.1 mCi/ml) for 4 h. Cellular lysates were immunoprecipitated with anti-HDV IgG and then analyzed by SDS-PAGE on 5 to 15% polyacrylamide linear gradient gels and by autoradiography. Products were immunoprecipitated from COS 7 cells transfected by calcium phosphate method with pECE vector (lane 2) or pECE-d-BP (lane 3) or by the DEAE-dextran method with pECE vector (lane 4) or pECE-d-BP (lane 5). Lane 1 is an in vitro translation product of the HDAg as shown in Fig. 2. Molecular weight markers are presented in lane M.

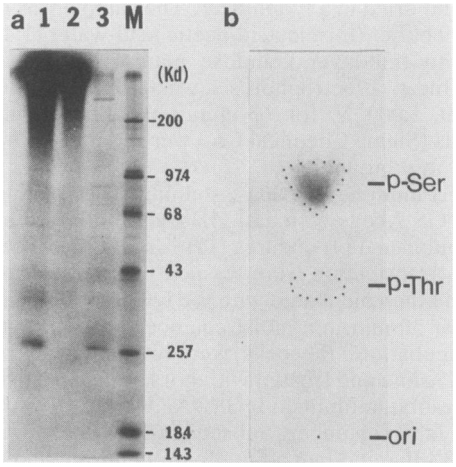


FIG. 4. Phosphorylation of the HDAg. (a) COS 7 cells were transfected with either expression vector pECE or recombinant DNA plasmid pECE-d-BE containing the HDAg-coding region by the DEAE-dextran method. Transfected cells were metabolically labeled with [$^{32}$P]orthophosphoric acid (0.3 mCi/ml) for 4 h. Cellular lysates were prepared, immunoprecipitated with anti-HDV IgG, and analyzed by SDS-PAGE (5 to 15% polyacrylamide) as described in Fig. 3. Phosphorylated products were immunoprecipitated from COS 7 cells transfected with pECE-d-BE (lane 1) or pECE vector (lane 2). An [$^{35}$S]methionine-labeled immunoprecipitate as shown in Fig. 3 was included as a control (lane 3). Molecular weight markers are presented in lane M. (b) Phosphoamino acid analysis of $^{32}$P-labeled HDAg. The $^{32}$P-labeled pp26 was electroeluted from the gel as described in the Materials and Methods. After acid hydrolysis, phosphoamino acids were separated by high-voltage electrophoresis at pH 1.9 on thin-layer cellulose plates and autoradiographed. The regions circled with dashed lines indicate either authentic phosphoserine (p-Ser) or phosphothreonine (p-Thr) identified by ninhydrin. The origin (ori) where the sample was applied is also marked.

To demonstrate that the translation products obtained were indeed the HDAg, the translation products were immunoprecipitated with the IgG fractions of the sera from HDV-infected patients (Fig. 2, lanes 4, 5, and 6) and a control serum from a patient with non-A, non-B hepatitis (Fig. 2, lanes 7, 8, and 9). The p26 protein and the smaller protein were specifically precipitated with the HDV antisera. This result demonstrates that ORF-2 of this cloned human HDV isolate (23) does indeed encode an HDAg.

**Expression of HDAg in COS 7 cells.** To further understand the biochemical properties and possible functions of the HDAg, the ORF-2 was inserted downstream of the SV40 T-antigen promoter in shuttle vector pECE. The plasmid was then transfected into COS 7 cells by using either calcium phosphate or DEAE-dextran methods. The cells were metabolically labeled with [$^{35}$S]methionine at 44 to 48 h after transfection, and the cell lysates were precipitated with human HDV-specific antiserum. Only one protein of 26 kDa (p26) was specifically precipitated from the cells transfected with the vector containing ORF-2 by either of the two methods (Fig. 3, lanes 3 and 5). This protein was indistinguishable in size from that made from this ORF in the rabbit reticulocyte lysate (Fig. 3, lane 1). No HDV-specific protein was detected in cells transfected with the vector alone (Fig. 3, lanes 2 and 4). This result further confirms that the ORF-2 codes for only one HDV-specific protein of 26 kDa. Thus, the two HDAg protein species detected in HDV-infected serum or liver (2, 3, 25, 40) may not be the direct translation products from the same ORF. The 18-kDa protein sometimes seen in the in vitro translation reaction (Fig. 2) was not detected in the transfected cells. This protein may be a p26 degradation product or the result of premature termination of translation in vitro.

**Phosphorylation of HDAg.** The predicted molecular mass of the protein encoded by ORF-2 is 24 kDa, whereas the protein made by this ORF in rabbit reticulocyte lysates or in COS 7 cells was 26 kDa. The difference between the predicted and apparent $M_r$ of the HDAg on SDS-PAGE may be due to posttranslational modification of the HDAg. We therefore examined whether the HDAg was phosphorylated, since many nucleic acid-binding proteins are phosphorylated (31, 33). Phosphorylation generally occurs at serine, threonine, and tyrosine residues. The sequence of the human HDAg predicts 14 serine residues, 3 threonine residues, and no tyrosine residues (23). To determine whether the serine or threonine residues were phosphorylated, one-dimensional thin-layer electrophoresis was performed. The COS 7 cells transfected with pECE-d-BE were metabolically labeled with $^{32}P_i$ and immunoprecipitated with anti-HDV antiserum. The data showed that the immunoprecipitated $^{32}$P-labeled protein ran as a single band with an $M_r$ identical to that of $^{35}$S-labeled p26 of HDAg (Fig. 4a). This finding suggested that the HDAg is a phosphoprotein. To rule out the possibility that $^{32}$P-labeled HDAg was due to nonspecific association of RNA or phospholipid, we performed phosphoamino acid analysis of the p26. The $^{32}$P-labeled HDAg (p26) was eluted from SDS-PAGE, hydrolyzed with 6 M HCl, and then separated by electrophoresis on thin-layer cellulose at pH 1.9. The hydrolyzed $^{32}$P-labeled amino acids from p26 comigrated with authentic phosphoserine but not with phosphothreonine (Fig. 4b), indicating that the HDAg is phosphorylated on the serine residues but not on the threonine residues. This result established that the HDAg is indeed a phosphoprotein. Thus, we termed this protein pp26.

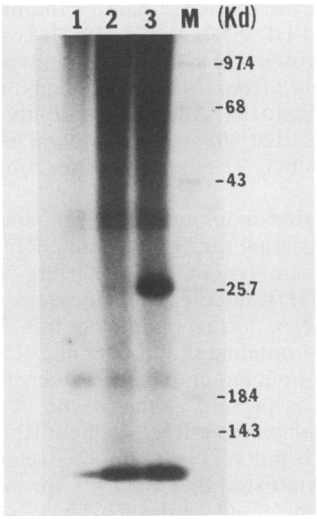


FIG. 5. Immunoprecipitation of HDAg from COS 7 cells transfected with HDV antigenomic monomer DNA. COS 7 cells were transfected with plasmid DNA by the DEAE-dextran method and labeled with $^{32}P_i$ from 44 to 48 h posttransfection as described in the Materials and Methods. Extracts were immunoprecipitated with anti-HDV IgG and analyzed by SDS-PAGE (5 to 15% polyacrylamide gradient). Proteins were immunoprecipitated by HDV antisera from COS 7 cells transfected with pECE (lane 1), pECE-d-PP (lane 2), or pECE-d-BE (lane 3). The molecular sizes of the $^{14}C$-labeled marker proteins (lane M) are given on the right.

We also examined whether the vector containing the entire monomer HDV, which expresses an antigenomic-sense RNA starting at 750 nucleotides upstream of the ORF-2, could synthesize pp26 or other HDV-specific proteins. A small amount of pp26 was synthesized from the COS 7 cells transfected with this vector (Fig. 5). The amount of protein was significantly lower than that made from the ORF-2 alone. No other proteins were detected.

**Nuclear localization of the HDAg pp26.** Rizzetto et al. first demonstrated that HDAg was localized in the nuclei of HDV-infected hepatocytes (27). The HDV-specific RNA replication also appeared to occur in the nuclei (12, 36). Thus, HDAg could be important for the replication of HDV RNA. The nuclear localization of HDAg could be a result of its association with the helper virus HBV DNA or protein or HDV RNA, inasmuch as HDAg is predicted to be highly basic (23). To rule out these possibilities, we examined the intracellular localization of HDAg in COS 7 cells transfected with the ORF-2 expressing plasmid pECE-d-BE. The cells were examined 25 h after transfection by indirect immunoperoxidase staining with the IgG fraction of anti-HDAg sera and horseradish peroxidase-conjugated rabbit anti-human antibodies. The results showed that HDAg was present exclusively in the nucleus. The nuclear staining was mainly homogeneous within the nuclei (Fig. 6a). However, in some cells, the immunoperoxidase staining was localized in certain granules within the nuclei (Fig. 6b). The significance of the latter pattern of staining is not clear. In contrast, the cells transfected with the pECE vector alone did not show any immunoperoxidase staining (Fig. 6c). These results indicate that full-length genomic HDV RNA is not required for HDAg transport to the nucleus and that the HDAg itself is responsible for its localization in the nucleus. Thus, the HDAg is a nuclear phosphoprotein.

**RNA-binding activity of the HDAg.** Since the HDAg is a highly basic protein as predicted from its amino acid composition (23), and is an internal viral protein (4, 5, 28), it might be associated with HDV genomic RNA in the virion or other cellular nucleic acids in the infected cells. To examine whether HDAg might contain an RNA-binding activity, we transferred the immunoprecipitated HDAg from SDS-PAGE to a nitrocellulose filter and then incubated the filter with $^{32}P$-labeled HDV genomic RNA. This RNA bound preferentially to the HDAg, (Fig. 7a), whereas only a small amount of RNA bound to the heavy chain of immunoglobulin. The total amount of the immunoglobulin was in overwhelming excess over the HDAg in this gel, as judged by the Coomassie blue staining where immunoglobulin heavy chain was visualized

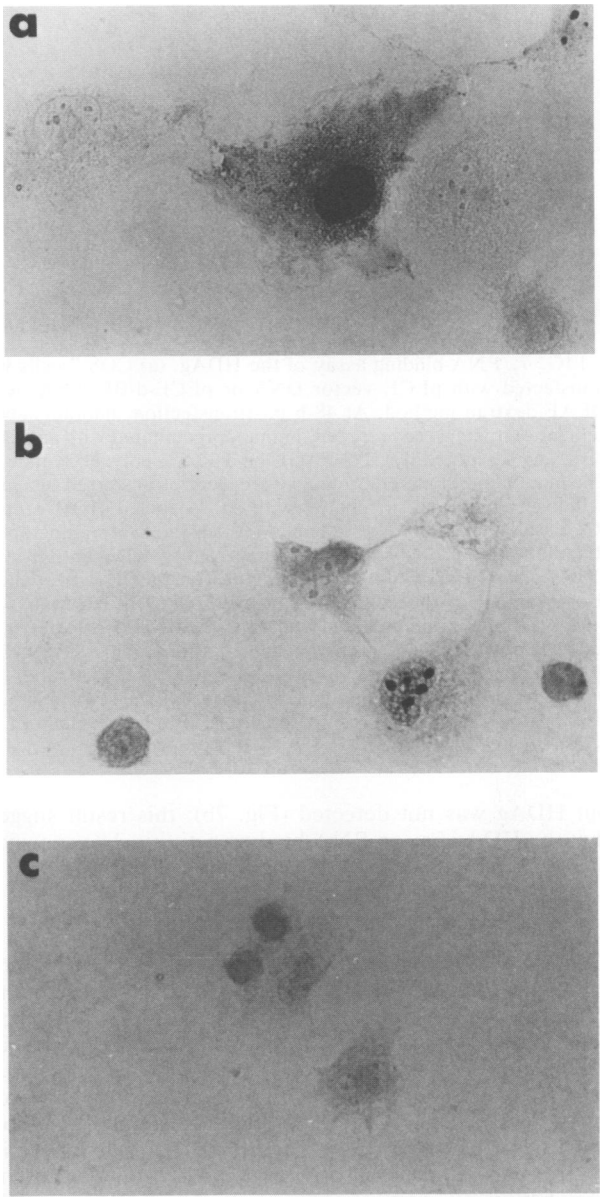


FIG. 6. Immunoperoxidase staining of HDV-transfected COS 7 cells. COS 7 cells were transfected with pECE vector alone or pECE-d-BE DNA by the DEAE-dextran method as described in the Materials and Methods. At 25 h postransfection, cells were fixed and processed for detection of HDAg by indirect immunoperoxidase staining. (a and b) Two types of nuclear staining in pECE-d-BE-transfected COS 7 cells. (c) COS 7 cells transfected with pECE vector alone.

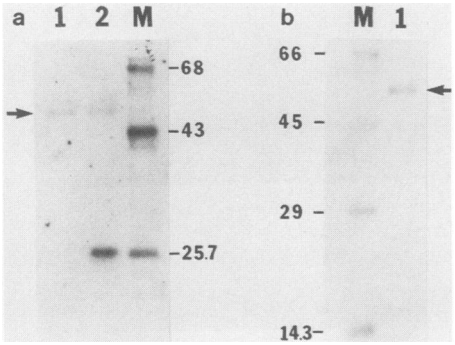


FIG. 7. RNA-binding assay of the HDAg. (a) COS 7 cells were transfected with pECE vector DNA or pECE-d-BE DNA by the DEAE-dextran method. At 48 h posttransfection, nonradiolabeled cellular extracts were prepared, immunoprecipitated with anti-HDV sera, and separated by SDS-PAGE on a 12.5% polyacrylamide gel. The immunoprecipitated proteins were electrotransferred to a nitrocellulose membrane, incubated with a $^{32}$P-labeled HDV genomic RNA as described in Materials and Methods, and autoradiographed. RNA bound to proteins immunoprecipitated by HDV-antisera from COS 7 cells transfected with pECE (lane 1) or pECE-d-BE (lane 2). (b) A sample of the cellular lysate from pECE-d-BE-transfected cells was immunoprecipitated and separated by SDS-PAGE as in (a). The polyacrylamide gel was stained with Coomassie blue and dried. Lane 1 represents proteins that are visualized after this procedure. Molecular weight markers (lanes M) were run in parallel, and the sizes are expressed in kilodaltons. The heavy chain of IgG is marked with an arrow.

but HDAg was not detected (Fig. 7b); this result suggests that the HDAg has an RNA-binding activity. The specificity of RNA binding is currently under investigation.

## DISCUSSION

In this study we have examined the in vitro translation of HDAg from a cloned HDV genome and its expression in eucaryotic cells. The expression of the HDAg in transfected cells allowed us to study the translation, processing, and localization of this protein in nonhepatic cells and in the absence of HBV coinfection. These studies were not previously possible with the HDV infection of humans, chimpanzees, or woodchucks. This report showed that HDAg is a nuclear phosphoprotein with possible RNA-binding activity. This finding supports the potential role of HDAg in HDV RNA replication, which appears to take place in the nucleus (12, 36).

Both in vitro translation in rabbit reticulocyte lysates and expression in COS 7 cells of our cDNA clones which contain the ORF encoding the HDAg yielded a single protein of 26 kDa. This finding contrasts with the previous reports that the HDAg in the serum or liver of infected humans or chimpanzees consisted of two protein species (2, 3, 25, 40). The sizes of these two protein species differed from 24 to 30 kDa, depending on different reports (2, 3, 25, 40). Thus, we could not determine whether the HDAg detected in vitro and in vivo in our experiments corresponds to the larger or smaller protein of the previously identified HDAg species. It is not clear how the two HDAg proteins found in infected liver were derived. Our study in this report suggested that the two HDAg proteins were not derived from the differential uses of alternative AUGs in the same or different reading frames, since the monomeric antigenomic HDV RNAs did not synthesize any additional protein. Also, there are no other optimal AUGs around the first AUG of the ORF-2, as predicted from the sequence data of the human- and chimpanzee-adapted HDV RNAs (23, 38). It is possible that one of these two proteins is synthesized from a spliced mRNA, or, alternatively, from a heterogeneous virus population present in HDV stocks. Another possibility which could not be ruled out is that the second protein is a liver-specific translation product. These possibilities are currently under investigation.

The finding that monomeric antigenomic RNA was less efficient in translating the HDAg than ORF-2 alone suggests that the upstream region may interfere with translation. Thus, in the HDV-infected hepatocytes, there may be a processed mRNA corresponding to this ORF. In fact, a 0.8-kb poly(A)-containing, anti-genomic RNA has been detected in HDV-infected chimpanzee liver (6), and a poly(A) addition signal is present at the 3′ end of ORF-2 (23, 38). Whether this subgenomic RNA is the mRNA for the HDAg in hepatocytes is not yet clear. Another relevant issue is that the sequence context at the AUG site for the ORF-2 is good but not optimum, based on the Kozak consensus sequences (17, 18). Other AUG sites in the antigenomic sense have even better sequence context for translation but were not utilized for translation. Thus, other factors may exist for the regulation of translation of the HDAg.

It should also be noted that the HDV *Pst*I-to-*Pst*I monomer used in this study may not reflect the "true" 5′ end of endogenous HDV antigenomic RNA. HDV RNA is circular (6), and, although monomer-length antigenomic RNA has been detected in HDV-infected liver (6), the exact 5′ end of this RNA has not yet been determined. Thus, HDV antigenomic RNAs initiating from other regions may be differentially processed or translated. This possibility is currently under investigation.

The phosphorylation of pp26 is the only posttranslational modification of the HDAg observed so far. It is unlikely that it is glycosylated, since no conserved N-glycosylation sites are found in our cloned HDV cDNAs (23). In vitro translation studies performed in the presence of microsomal membrane fractions also ruled out the possibility that HDAg is an integral membrane protein (data not shown). Thus, phosphorylation appears to account for most of the differences between the predicted and apparent molecular sizes (24 versus 26 kDa) of the HDAg. Phosphorylation of HDAg, coupled with its nuclear localization and RNA-binding activity, suggests that it may play an important role in the replication and pathogenesis of the HDV. Phosphorylation has been suggested to exert a regulatory role in the selection of the negative-stranded RNA genome in mature Sendai virions (20) and in the regulation of overall RNA synthesis or the switch from transcription to replication in vesicular stomatitis virus-infected cells (7). Phosphorylation also affects the specific binding of the viral phosphoprotein to the RNA genome in RNA tumor viruses (31).

It is likely that the HDAg is a nucleocapsid protein which interacts directly with the viral RNA. This possibility is consistent with the RNA-binding activity of HDAg demonstrated in this study (Fig. 7). Alternatively, the HDAg may interact with HBsAg, which, in turn, may regulate the maturation of HDV virus particles. Furthermore, the localized HDAg accumulation within some nuclei of the transfected COS 7 cells (Fig. 6b) suggests that HDAg may interact with certain organelles, such as nucleoli, of the nucleus. It should be noted that the study of RNA-binding by HDAg performed in this report demonstrated only the strong affinity of HDAg for RNAs in general. We have not examined whether this binding is specific for HDV RNA or possibly

some cellular RNAs. Further investigations of the structure and functions of HDAg in the presence and absence of helper virus HBV in hepatocytes will shed further light on the mechanism of HDV RNA replication.

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