Virology 271, 46–57 (2000)
doi:10.1006/viro.2000.0302, available online at http://www.idealibrary.com on IDEAL®

Specific Interaction between the Hepatitis Delta Virus RNA and Glyceraldehyde 3-Phosphate Dehydrogenase: An Enhancement on Ribozyme Catalysis

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Received October 11, 1999; returned to author for revision December 2, 1999; accepted March 8, 2000

Replication of hepatitis delta virus (HDV) RNA occurs in the nuclei of infected cells. The replication is mediated by cellular factors containing an RNA polymerase II-like enzyme activity through a double rolling-circle mechanism and is regulated by delta antigens. In this study, UV cross-linking experiments were carried out to examine interactions between HDV RNA and proteins present in HeLa nuclear extract. Cellular proteins with molecular mass of 23 (p23), 36 (p36), 38 (p38), and 58 (p58) kDa bound to full-length HDV RNA of both genomic and antigenomic strands. Deletion analysis on the antigenomic strand mapped the interacting domain within a 79-nucleotide fragment but not at the ends of the rod-shaped viral RNA structure. The specificity of the RNA-protein interactions was demonstrated by competition experiments and the specific HDV RNA-binding proteins were purified through column chromatography. Electrophoresis mobility shift assay with the purified fractions demonstrated that the interaction between p36 and HDV RNA was relatively stable even in the presence of 0.5 M NaCl. Biochemical analysis including protein microsequencing identified the p36 as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). RNase footprinting indicated that the UC-rich domain between nucleotides 379 and 414 of the HDV antigenomic RNA was involved in the GAPDH binding. Functional studies further demonstrated an enhancing effect of GAPDH on the ribozyme activity of HDV antigenomic RNA. In addition, in the presence of HDV RNA cellular GAPDH relocalized from the cytoplasm to the nucleus where HDV replication occurs. These results suggest that GAPDH is involved in the replication of HDV.

Key Words: hepatitis delta virus; HDV RNA-binding protein; glyceraldehyde 3-phosphate dehydrogenase (GAPDH); GAPDH-binding site; HDV ribozyme; relocalization of GAPDH.

INTRODUCTION

Hepatitis delta virus (HDV) is the causative agent of acute and chronic liver diseases including fulminant hepatitis and accelerated liver cirrhosis (Hoofnagle, 1989). HDV particle contains a single-stranded circular RNA genome of 1.7 kilobases with 60% of G+C content and approximately 70% of intramolecular base pairing that appears as a double-stranded, unbranched, rod-shaped structure under nondenaturing conditions (Kos *et al.,* 1986; Makino *et al.,* 1987a; Wang *et al.,* 1986). HDV is a satellite virus of hepatitis B virus (HBV) as it requires HBV for virion production and transmission (Sureau *et al.,* 1993; Wang *et al.,* 1991). However, replication of HDV RNA genome can occur in the absence of HBV (Kuo *et al.,* 1989). The HDV genomic RNA is associated with delta antigen (HDAg) to form ribonucleoprotein (RNP) complex in virion and transfected cells (Chang *et al.,* 1988, 1995; Lin *et al.,* 1990; Ryu *et al.,* 1993). There are two forms of delta antigen, small (HDAg-S) and large HDAg (HDAg-L). The HDAg-L is derived from the HDAg-S as a result of RNA editing on the antigenomic strand (Casey and Gerin, 1995). A double-stranded RNA-adenosine deaminase, or a closely related host factor, was suggested to be responsible for the editing event (Polson *et al.,* 1996).

HDV RNA replicates through a double rolling-circle mechanism with a host RNA polymerase II-like enzyme activity to generate multimeric viral RNA precursors (Macnaughton *et al.,* 1991; Fu and Taylor, 1993; Macnaughton *et al.,* 1993b). The viral RNA precursors are cleaved into unit-length molecules by the intrinsic ribozyme activity of HDV RNA (Kuo *et al.,* 1988b; Sharmeen *et al.,* 1988; Wu *et al.,* 1989). The ribozyme activity requires a double pseudoknot structure and is important for the viral replication (Ferre-D'Amare *et al.,* 1998; Macnaughton *et al.,* 1993b). Putative RNA promoters involved in HDV replication and transcription have been characterized (Beard *et al.,* 1996; Macnaughton *et al.,* 1993a; Tai *et al.,* 1993; Wang *et al.,* 1997). But how a host DNA-dependent RNA polymerase is capable of directing RNA synthesis with RNA templates remains unclear. Cellular double-stranded RNA-dependent protein kinase (also known as DAI and p68 kinase) was found to be specifically associated with HDV RNA (Circle *et al.,* 1997). Nucleolin and a cellular homolog of HDAg, delta-interacting protein A (DIPA), were demonstrated to interact

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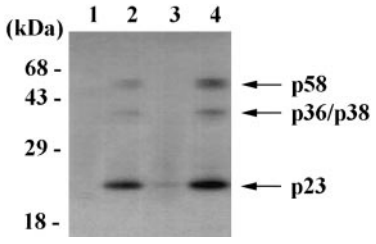

FIG. 1. Detection of cellular proteins binding to HDV RNA by UV cross-linking experiments. Full-length HDV genomic (lanes 1 and 2) and antigenomic (lanes 3 and 4) RNAs were subjected to UV cross-linking in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of HeLa nuclear extract. Arrows indicate proteins that cross-linked to the HDV RNAs. Numbers on the left indicate the sizes (in kilodaltons) of standard protein markers.

with HDAg and regulate HDV replication (Brazas and Ganem, 1996; Lee *et al.,* 1998). In addition, HDAg can function as an RNA chaperone and modulate the ribozyme activity of HDV RNA (Huang and Wu, 1998). Previous studies also proposed the involvement of cellular factors in the regulation of HDV ribozyme (Lazinski and Taylor, 1995; Macnaughton *et al.,* 1993b).

In this study, we demonstrated the specific binding of cellular proteins p23, p36, p38, and p58 to both HDV genomic and antigenomic RNAs. Amino-terminal sequencing identified the p36 as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH specifically bound to the UC-rich domain of a 79-nucleotide (nt) fragment, but not to the ends of the rod-shaped structure, of the viral antigenomic RNA. In addition, cellular GAPDH colocalized with HDV RNA in the nuclei of transfected cells and was capable of enhancing the ribozyme activity of an HDV antigenomic RNA. These results suggested possible roles of GAPDH involved in the multiplication of HDV.

RESULTS

Interactions between cellular proteins and HDV RNAs

For identification of putative cellular proteins that interact with HDV RNA and participate in the viral multiplication, UV cross-linking experiments were carried out with a nuclear extract prepared from HeLa cells. Two bands of approximately 23 and 58 kDa and a doublet of 36 and 38 kDa, designated p23, p58, p36, and p38, respectively, were detected with both full-length HDV genomic and antigenomic RNAs (Fig. 1). The signals were not detected if the UV irradiation step was omitted or if the reaction mixtures were treated with proteinase K prior to gel electrophoresis (data not shown), indicating that the signals were derived from RNA-protein complexes formed via non-covalent linkage.

Deletion mapping of the protein-binding domain on HDV antigenomic RNA

To localize the interacting domain of HDV RNA with the cellular proteins, RNA transcripts representing various regions of the HDV antigenomic RNA were synthesized *in vitro* in the presence of [α-^{32}P]UTP and used as probes in UV cross-linking experiments. Results indicated that HDV antigenomic RNA spanning nt 966–225 (AG966/225) contains protein binding sites, whereas nt 228–963 (AG228/963) failed to interact with the specific cellular proteins (Fig. 2A, lanes 1 and 2). Further dissection of the binding domain demonstrated that both HDV RNA fragments nt 434–225 (AG434/225) and nt 434–356 (AG434/356) were able to interact with the cellular proteins, but the interaction was not detected with HDV AG966/431 RNA (Fig. 2A, lanes 3–5). Competition analysis was performed using ^{32}P-labeled AG434/225 RNA as a probe. The UV cross-linked cellular proteins p58, p36/p38, and p23 were significantly diminished in the presence of increasing amounts of unlabeled AG434/225 RNA, whereas nonhomologous TGEVE2 RNA competitor has little effect (Fig. 2B). These results indicate that the cellular proteins are capable of interacting specifically with HDV RNA at a location outside the ends of the rod-shaped RNA structure.

Purification of the HDV RNA-binding proteins and characterization of the HDV RNA-p36 complexes

The HDV RNA-binding proteins present in the HeLa nuclear extract were purified through column chromatography and examined for their biochemical characteristics. Following an initial step of DEAE-cellulose column chromatography, RNA-binding activity of the HeLa nuclear extract was recovered from the flow-through fraction as monitored by UV cross-linking experiment with HDV AG434/225 RNA as a probe (Fig. 3B, lane INPUT). The flow-through fraction was further passed onto a heparin-Sepharose column and eluted with a discontinuous KCl gradient. Protein fractions were collected and resolved by electrophoresis (Fig. 3A). UV cross-linking experiments revealed that one of the HDV RNA-binding proteins, p36, was present in the 0.2 M KCl fraction, whereas p58, p38, and p23 were present in the 0.3 M KCl fraction (Fig. 3B). Electrophoresis mobility shift assay (EMSA) was further performed with the p36-containing fraction using ^{32}P-labeled HDV AG434/356 RNA as a probe. As shown in Fig. 4A, two complexes were formed (lane C). The complexes were diminished with increasing molar excesses of unlabeled AG434/356 RNA, whereas a nonhomologous TGEVE2 RNA competitor had no effect even at a 1000-fold molar excess (Fig. 4A). By performing binding reaction at various concentrations of NaCl, we found that the interaction between HDV RNA and p36 was relatively stable. A significant amount of the

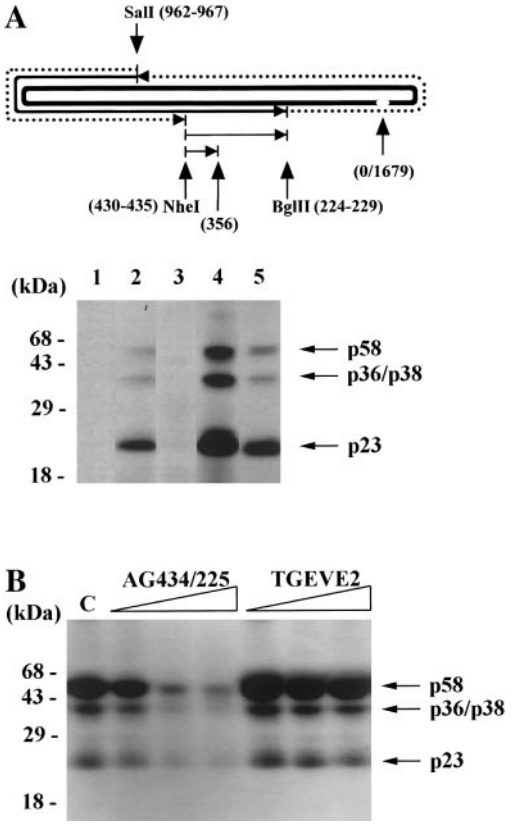

FIG. 2. Deletion mapping of the HDV antigenomic RNA for the binding of the specific cellular proteins. (A) Top: Schematic representation of the RNA fragments used in studying the interactions of HDV antigenomic RNA with specific cellular proteins present in the HeLa nuclear extract. The rod-shaped structure represented by a closed heavy line indicates the antigenomic RNA of HDV. Open oval with nucleotide numbers 0/1679 indicates the artificially designated end of the viral genomic RNA. Nucleotides are numbered according to the genomic orientation. Solid and dotted lines with arrowheads to indicate the directions represent the viral antigenomic RNA fragments that were, according to the present study, capable or unable, respectively, to cross-link the specific HDV RNA-binding proteins. Restriction endonucleases used to generate linear viral cDNA templates for the synthesis of RNA transcripts are indicated along with the positions of their recognition sequences. AG228/963 RNA on the right side of the rod-shaped structure was derived from the *Bgl*II-*Sal*I DNA fragment containing nt 0/1679. AG966/225 RNA was derived from the *Sal*I-*Bgl*II DNA fragment containing nt 356. AG966/431 RNA was derived from the *Sal*I-*Nhe*I DNA fragment. Both AG966/225 and AG966/431 RNAs are located on the left side of the rod-shaped structure. AG434/225 RNA was derived from the *Nhe*I-*Bgl*II DNA fragment, and AG434/356 RNA was derived from the HDV cDNA fragment from *Nhe*I site to nt 356 as indicated. Bottom: UV cross-linking experiments. UV cross-linking experiments were performed with ^{32}P-labeled HDV antigenomic RNA AG228/963 (lane 1), AG966/225 (lane 2), AG966/431 (lane 3), AG434/225 (lane 4), and AG434/356 (lane 5) in the presence of HeLa nuclear extract. (B) Competitive UV cross-linking experiments. Competition analysis was performed with HeLa nuclear extract and ^{32}P-labeled AG434/225 RNA in the presence of various amounts of unlabeled homologous (AG434/225) or nonhomologous (TGEVE2) competitor RNA as indicated. The amounts of competitors used were 50-, 200-, and 400-fold molar excess over the labeled AG434/225 RNA. Lane C represents a control experiment in which no competitor RNA was added. Standard protein markers and proteins that cross-linked to the viral RNAs are indicated.

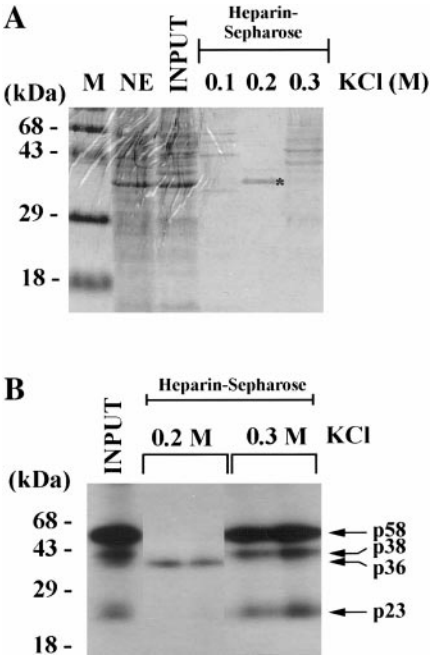

FIG. 3. Purification of cellular proteins that specifically bind to HDV RNA. (A) Coomassie brilliant blue staining. Total HeLa nuclear extract (NE) and protein fractions loaded onto (INPUT) and eluted from heparin-Sepharose column as indicated were resolved by electrophoresis on an SDS-15% polyacrylamide gel followed by Coomassie brilliant blue staining. Standard protein markers (M) are shown. (B) UV cross-linking experiments. UV cross-linking experiments were performed with AG434/225 RNA and protein fractions as indicated. Cross-linked cellular proteins and standard protein markers are indicated. The asterisk in (A) indicates the 36-kDa protein used to carry out microsequence analysis.

complexes was formed even at a salt concentration of 0.5 M (Fig. 4B).

Identification of the HDV RNA-binding protein p36 as GAPDH

As demonstrated in Fig. 3A, the p36 HDV RNA-binding protein appeared to be the major protein present in the 0.2 M KCl fraction following heparin-Sepharose column chromatography. To learn the biochemical characteristics of the p36, the 0.2 M KCl fraction was resolved by electrophoresis on an SDS-15% polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane from which the p36 was excised for microsequence analysis. The amino acid sequence of p36 was compared with known protein sequences in database. As shown in Fig. 5, the NH_2-terminal 21 amino acid residues of the p36 were identical to the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Because no mixed protein sequences were detected, it is likely that the band of p36 represents GAPDH and no other proteins were comigrated with it on the polyacrylamide gel.

To confirm the identity of the p36 as human GAPDH,

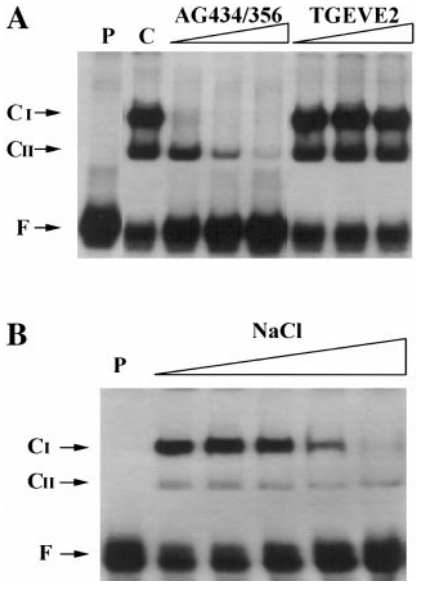

FIG. 4. Specificity and stability of the complexes formed between the p36-containing fraction and HDV AG434/356 RNA. (A) Specificity of the interactions. Competitive EMSA was performed with 0.2 μg of the p36-containing fraction, ^{32}P-labeled HDV AG434/356 RNA, and 20 μg of tRNA in the presence of increasing amount (40-, 200-, and 1000-fold molar excesses) of unlabeled AG434/356 and TGEVE2 competitor RNAs as indicated. Lane C represents a control experiment in which no competitor RNA was added; P, probe only. RNA-protein complexes (CI and CII) and free probe (F) are indicated. (B) Effect of ionic strength on the complexes' formation. Binding reactions were carried out with 0.2 μg of the p36-containing fraction, ^{32}P-labeled HDV AG434/356 RNA, and 3 μg of tRNA at increasing concentrations of NaCl (0.05, 0.1, 0.2, 0.5, and 1 M). The concentration of NaCl in standard binding reaction was 50 mM. RNA-protein complexes (CI and CII) and free probe (F) are indicated. P, probe only.

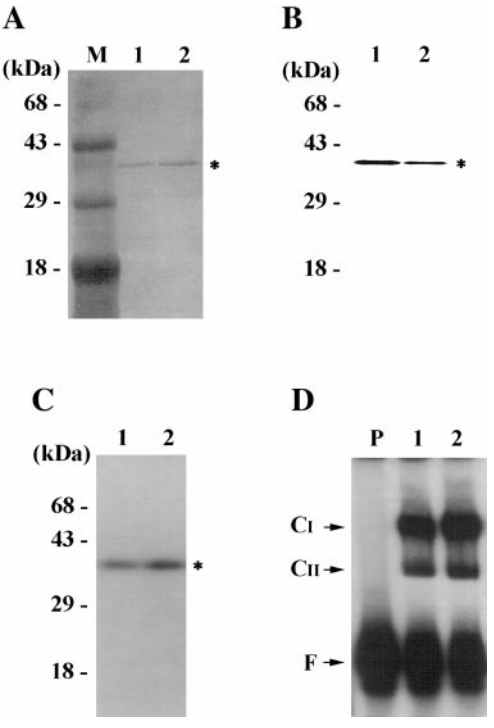

FIG. 6. Similarity of the biochemical and immunogenic characteristics of the HDV RNA-binding protein p36 and human GAPDH. The partially purified p36 (lanes 1) and human erythrocyte GAPDH (lanes 2) were characterized in parallel for their electrophoretic migration pattern on an SDS-15% polyacrylamide gel as detected by silver staining (A), the immunoreactive pattern as detected by monoclonal antibody to rabbit muscle GAPDH (B), and the binding activity to HDV AG434/356 RNA as detected by UV cross-linking (C) and EMSA (D). The amounts of the partially purified p36/human erythrocyte GAPDH used in these studies were 0.1/0.2, 0.5/0.2, 0.2/0.5, and 0.2/0.2 (μg/μg) in (A), (B), (C), and (D), respectively. Six and 3 μg of yeast tRNA were used in the binding reaction of UV cross-linking and EMSA, respectively. The p36/GAPDH (*), standard protein markers (M), RNA-protein complexes (CI and CII), and free probe (F) are indicated. P, probe only.

further analyses were conducted in parallel with the partially purified p36 and a commercial preparation of GAPDH isolated from human erythrocytes. Results demonstrated that the two proteins had similar electrophoretic migration patterns on SDS–polyacrylamide gel (Fig. 6A) and were both recognized by a monoclonal antibody specific for the rabbit muscle GAPDH in immunoblot analysis (Fig. 6B). In addition, the HDV RNA-binding activity of p36 was also detected for the human erythrocyte GAPDH by UV cross-linking analysis (Fig. 6C) and EMSA (Fig. 6D). These data strongly support that the HDV RNA-binding protein p36 is GAPDH.

p36	^{1}GKVKVGVNGFGRIGRVLTRAA^{21}
GAPDH	^{1}GKVKVGVNGFGRIGRVLTRAA^{21}

FIG. 5. Comparison of the NH_2-terminal sequence of the p36 to human GAPDH. The 36-kDa protein used to perform microsequence analysis was purified from the 0.2 M KCl fraction as shown in Fig. 3A. The NH_2-terminal sequences of the p36 and human GAPDH are shown. The amino acid identity implies the p36 as human GAPDH.

RNase footprinting analysis of the GAPDH binding sites on HDV antigenomic RNA

To map the binding sites of GAPDH on HDV antigenomic RNA, RNase footprinting analysis was conducted with ribonucleoprotein complexes assembled from GAPDH and d3 RNA that contains HDV antigenomic RNA nt 434–356 flanking by extra sequences derived from the cloning vector. The 79-nt RNA domain has been shown to be responsible for the interaction between HDV antigenomic RNA and the p36 (Figs. 2 and 4). The reactions were performed with RNase T1 followed by primer extension. Strand scission caused by RNase T1 treatment resulted in an arrest of the primer-extended synthesis at the nucleotide on the 3′-side of the cleaved phosphodiester bond. As shown in Fig. 7, in the presence of 10^{-1} U/μl of RNase T1, cleavage occurred preferentially at G379, G396, G397, G402, and G414 in the absence of GAPDH. GAPDH reduced significantly the RNase T1 reactivity at all these positions, indicating an involvement

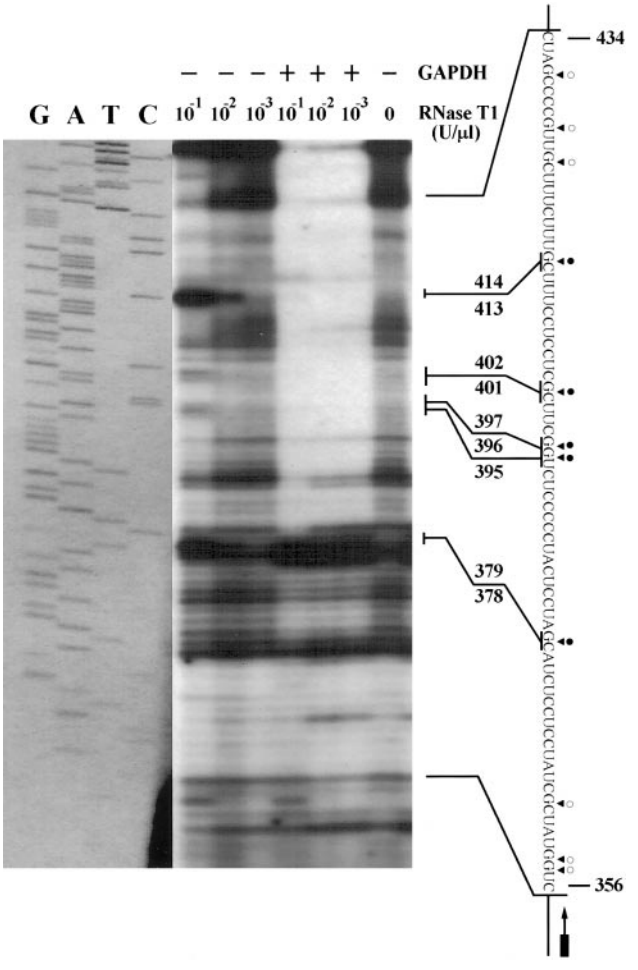

FIG. 7. Footprinting analysis of the HDV RNA bound by GAPDH. The GAPDH binding sites on the HDV antigenomic RNA were determined by RNase T1-primer extension analysis. The binding reaction was performed with d3 RNA encompassing HDV antigenomic RNA nt 434–356 in the absence (−) or presence (+) of GAPDH. RNase T1 digestion was carried out at various concentrations as indicated. The primer-extended products were analyzed on a 6% polyacrylamide gel containing 8 M urea. Sequencing ladders derived from cDNA of the d3 RNA were run in parallel to serve as size markers. Autoradiography is shown. Positions of the 3′-terminal residue of the primer-extended products and their neighboring guanosine residues protected by GAPDH are indicated along with the sequence of the d3 RNA. Filled bar (■) represents the primer and arrow points out the direction of primer extension. Arrowheads (◂) indicate potential RNase T1 cleavage sites, but experimental results demonstrated that only some were completely digested (●). Open circles (○) indicate those less sensitive or insensitive to RNase T1. A doublet at G402 may be explained by the stuttering effect of reverse transcriptase.

of nt 379–414 of the HDV antigenomic RNA in the binding of GAPDH. In addition, this defined GAPDH-binding domain is highly UC-rich. Many primer extension pause sites detected in the absence of GAPDH disappeared at a higher concentration of RNase T1 (10^{-1} U/μl) and in the presence of GAPDH, suggesting that the HDV RNA may form a highly ordered structure that was modified upon GAPDH binding and RNase T1 cleavage.

GAPDH relocalized from cytoplasm to nucleus and colocalized with HDV RNA in Huh 7 cells

GAPDH is a primarily cytoplasmic phosphoprotein with the ability to shuttle between the cytoplasm and nucleus (Sirover, 1999). To examine possible functions of the interaction between GAPDH and HDV RNA, Huh 7 cells were transfected with plasmid pSVD2 that represents the dimeric HDV cDNA (Chang *et al.,* 1994), and proceeded to fluorescence *in situ* hybridization/immunofluorescence staining. Interestingly, we found that GAPDH relocalized from the cytoplasm to the nucleus where the HDV RNA replicates (Fig. 8). These results indicate an association of GAPDH and viral RNA *in vivo* and strongly suggest that GAPDH plays an important role in the replication of HDV by interacting with the viral RNA.

GAPDH enhanced the ribozyme activity of HDV RNA

GAPDH is a multifunctional protein (Sirover, 1997). It was previously demonstrated to bind to ribozyme directed against mRNA of tumor necrosis factor alpha (TNFα) and increase the *in vitro* cleavage rate of the hammerhead-type ribozyme of TNFα mRNA (Sioud and Jespersen, 1996). HDV ribozyme possesses a double pseudoknot structure different from hammerhead (Ferre-D'Amare *et al.,* 1998). Although HDAg was recently demonstrated to be capable of modulating the *cis*-cleavage activity of HDV RNA (Huang and Wu, 1998), cellular factors that regulate HDV ribozyme activity have not been clearly demonstrated. In this study, we made an attempt to elucidate the biological significance of the interaction between GAPDH and HDV RNA by examining the possible role of GAPDH on the catalysis of HDV ribozyme. As shown in Fig. 9, the *cis*-cleavage activity of the HDV antigenomic RNA AG966/225 increased from 34 to 62% following a 40-min incubation with 50 ng/μl of GAPDH. The enhancing effect of GAPDH on the catalysis of HDV ribozyme was reproducible and was not observed with bovine serum albumin (BSA). These results indicate the specificity of the enhancing effect of GAPDH and a role of GAPDH involved in HDV multiplication.

DISCUSSION

In this study, we have identified the HDV RNA-binding protein p36 as GAPDH. GAPDH/p36 specifically interacted with HDV RNA *in vitro* and relocalized from the cytoplasm to the nucleus during HDV replication. In addition, GAPDH enhanced the ribozyme activity of the viral antigenomic RNA. These results suggest a possible role of GAPDH involved in HDV multiplication.

GAPDH was previously demonstrated to interact with AU-rich mRNAs through the dinucleotide-binding Rossmann fold (Nagy and Rigby, 1995). In agreement with the observation, we found that the formation of HDV RNA-

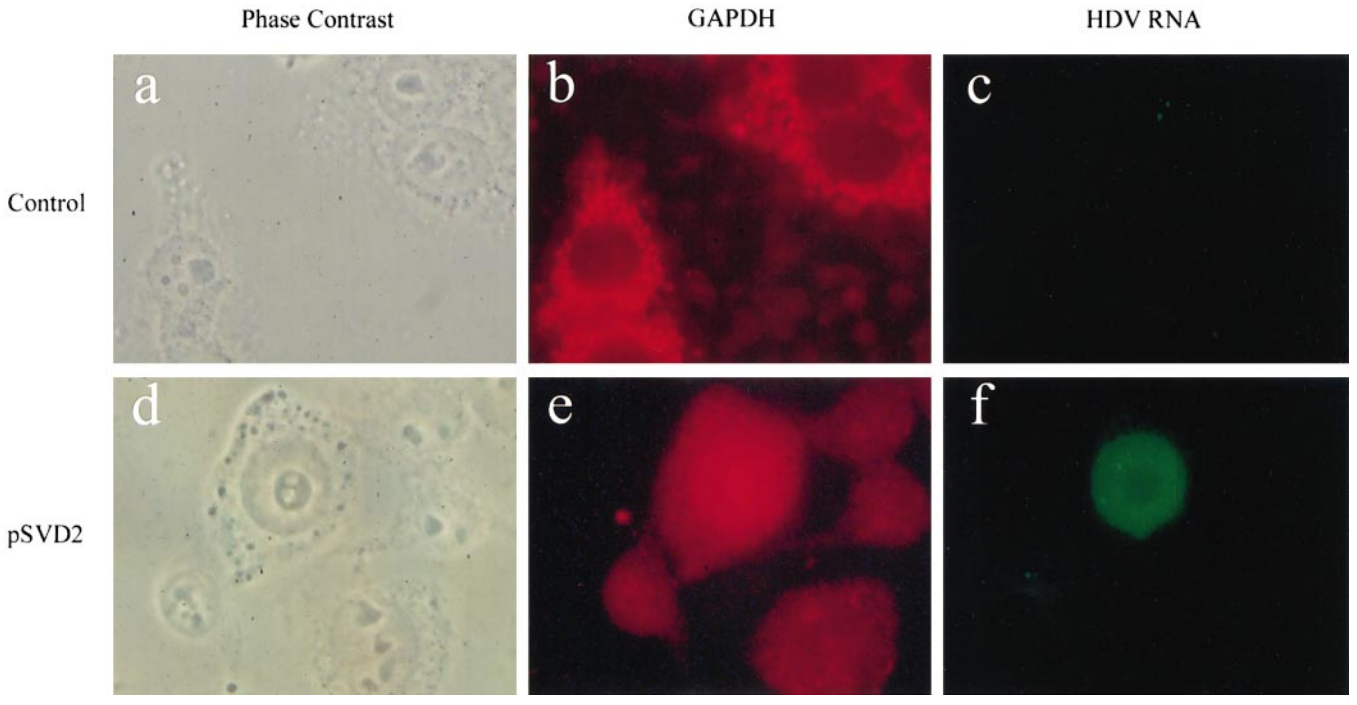

FIG. 8. Fluorescence *in situ* hybridization/immunofluorescence staining detected nuclear colocalization of GAPDH and HDV RNA in transfected Huh 7 cells. Huh 7 cells were seeded onto coverslips and allowed to adhere overnight before transfection of plasmid pSVD2 that represents the dimeric HDV cDNA. Untreated Huh 7 cells (a, b, and c) and the pSVD2-transfected cells (d, e, and f) were fixed 2 days posttransfection and proceeded to fluorescence *in situ* hybridization/immunofluorescence staining using mouse mAb against GAPDH (b and e) and goat antiserum against biotin (c and f) as the primary antibodies. The cellular GAPDH and the HDV RNA that hybridized to the biotin-labeled probe were then visualized by using Texas Red-conjugated horse anti-mouse antibodies and FITC-conjugated rabbit anti-goat antibodies, respectively, as the secondary antibodies. Phase-contrast micrographs (a) and (d) represent cells of the same fields as (b) and (c), and (e) and (f), respectively.

p36/GAPDH complexes was diminished in the presence of poly(U) and poly(A), with the former demonstrating a stronger effect (data not shown); however, comparison among GAPDH-associated RNA fragments (De *et al.,* 1996; Nagy and Rigby, 1995; Singh and Green, 1993; Sioud and Jespersen, 1996) did not reveal any apparent sequence similarity. Similar to the GAPDH-interacting RNAs, sequence diversity was also observed for RNAs that interact with polypyrimidine tract-binding protein (PTB) (Garcia-Blanco *et al.,* 1989; Hellen *et al.,* 1994; Jang and Wimmer, 1990; Mulligan *et al.,* 1992). Higher-ordered structures with U-rich motifs in the RNAs may be important for the initial recognition of the RNA-binding proteins. GAPDH was demonstrated to form complexes with RNAs of hepatitis A virus (HAV) and human parainfluenza virus type 3 (HPIV3) (De *et al.,* 1996; Schultz *et al.,* 1996). However, HDV RNA has a unique characteristic to form two complexes with GAPDH rather than one (Fig. 6). Since GAPDH can exist as tetramer, monomer, and possibly dimer in an equilibrium (Constantinides and Deal, 1969), it is likely that HDV RNA interacted with GAPDH of different forms.

Previous studies have identified a number of cellular proteins that associated with both HDAg and HDV RNA and participated in the viral replication (Brazas and Ganem, 1996; Lee *et al.,* 1998; Polson *et al.,* 1996). In this study, p23, p36/GAPDH, p38, and p58, present in the HeLa nuclear extract were identified to interact with both HDV genomic and antigenomic RNAs (Fig. 1). These proteins are also present in the nuclear extracts of Huh 7 and HepG2 cells (data not shown) and are likely to be common cellular factors. Although functional roles of p36/GAPDH in the nuclei are not fully understood, at least four glycolytic enzymes, including GAPDH, were detected in cell nuclei and found to interact with DNA *in vitro* (Ronai, 1993). GAPDH was also demonstrated to interact with tRNA (Singh and Green, 1993), mRNA (Nagy and Rigby, 1995), microtubules (Kumagi and Sakai, 1983), red cell membrane (Kant and Steck, 1973), and several proteins involved in neurodegenerative disorders (Burke *et al.,* 1996; Koshy *et al.,* 1996; Schulze *et al.,* 1993). Multiple functions have been attributed to GAPDH in addition to glycolysis. These include DNA replication (Baxi *et al.,* 1995), DNA repair (Meyer-Siegler *et al.,* 1991), protein phosphorylation (Kawamoto and Caswell, 1986), and neuronal apoptosis (Ishitani *et al.,* 1996; Sunaga *et al.,* 1995). GAPDH from rabbit muscle was demonstrated to greatly stimulate RNA polymerase II transcription when injected into *Xenopus laevis* oocytes (Morgenegg *et al.,* 1986). GAPDH was also demonstrated to destabi-

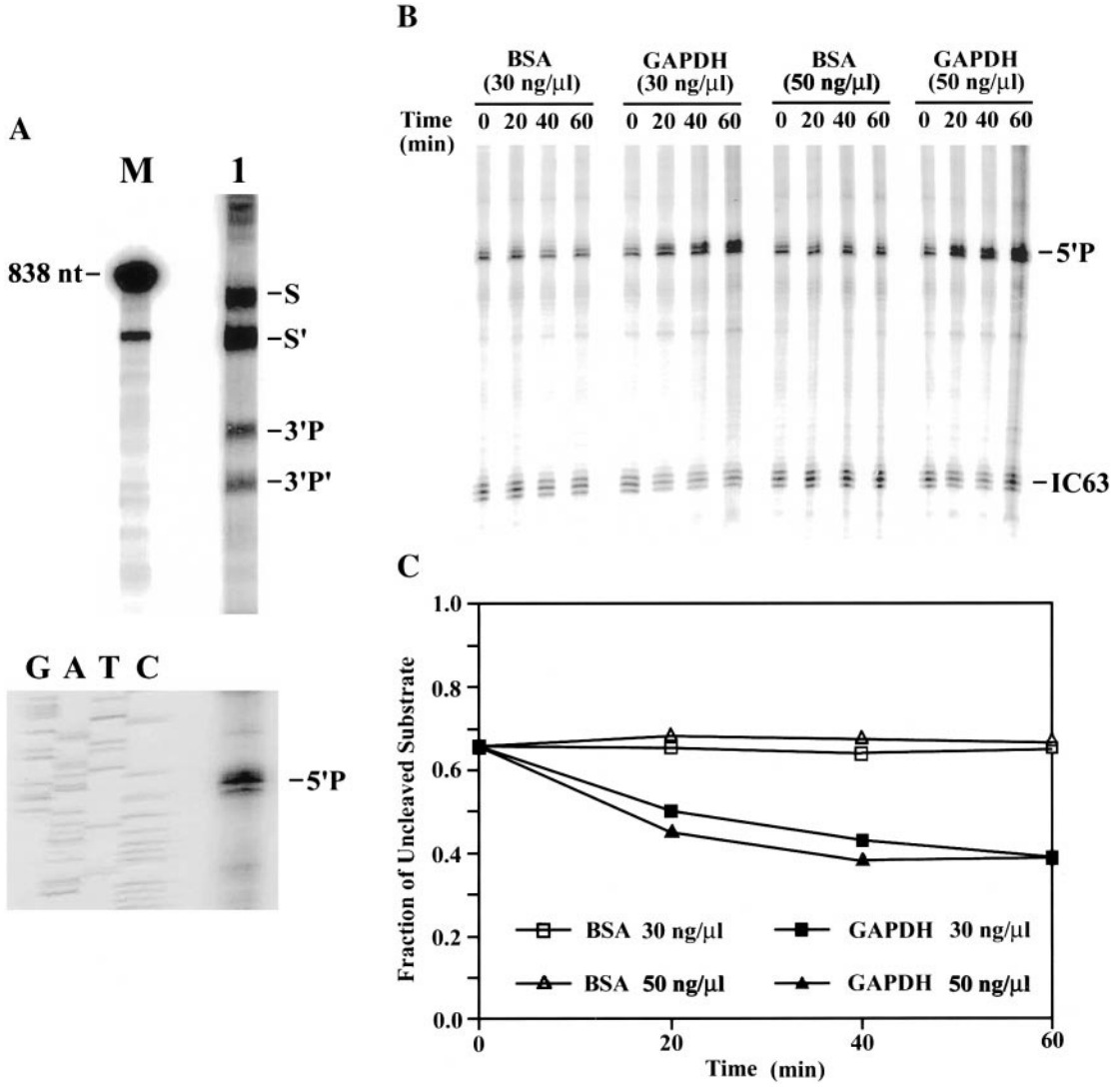

FIG. 9. Enhancing effect of GAPDH on the *cis*-cleavage activity of HDV antigenomic RNA. (A) *Cis*-cleavage activity of the HDV antigenomic RNA AG966/225. HDV antigenomic RNA AG966/225 was *in vitro* transcribed in the presence of [α-^{32}P]UTP and its *cis*-cleavage activity was examined by resolving the transcription products on a 4% polyacrylamide gel containing 7 M urea. Autoradiography is shown. S, 3′P, and 5′P represent the AG966/225 RNA substrate, the 3′ and 5′ cleavage products, respectively. S′ and 3′P′ are possibly the conformers of S and 3′P, respectively. The 838-nt RNA marker (lane M) was synthesized with T7 RNA polymerase in the presence of [α-^{32}P]UTP from plasmid pGEM4Z-C191 (Yen *et al.,* 1995) linearized with *Eco*RI restriction endonuclease. Sequencing ladders derived from M13mp18 DNA were run in parallel to serve as size markers. (B) The 5′ *cis*-cleavage products of the HDV AG966/225 RNA in the presence of GAPDH. HDV antigenomic RNA AG966/225 and the internal control RNA (IC63) were cosynthesized *in vitro* and incubated with various amounts of GAPDH and BSA for various time periods as indicated. The *cis*-cleavage products were analyzed on a 6% polyacrylamide gel containing 7 M urea. Autoradiography is shown. The multiple bands of cleavage products and IC63 resulted from the multiple sites of initiation and runoff of T7 RNA polymerase (Milligan *et al.,* 1987). (C) The relative ribozyme activity of the HDV AG966/225 RNA in the presence of GAPDH. Radioactivities of the cleavage products and uncleaved substrates were quantitated by PhosphorImager (Molecular Dynamics) and normalized against the uncleavable internal control IC63 RNA. The fraction of uncleaved substrate was calculated by dividing the molar ratio of the sum of the cleavage products and uncleaved substrates into the uncleaved substrates and was plotted against the incubation time. The fraction of uncleaved substrate at 0 min of incubation was 66%.

lize helix and folded structures (Karpel and Burchard, 1981; Schultz *et al.,* 1996) and to enhance the ribozyme activity of hammerhead RNA (Sioud and Jespersen, 1996).

Given that HDV RNA appeared as a double-stranded, rod-shaped structure under nondenaturing conditions, it is reasonable to expect that during HDV multiplication, cellular helicases are involved in unwinding the double-stranded RNA template and facilitating the release of progeny RNA from template following replication. Previous studies also suggested that cellular factors are involved in regulating the ribozyme activity of the HDV genomic strand, possibly by preventing its association with the ribozyme attenuator (Lazinski and Taylor, 1995). Although HDV ribozyme is different from hammerhead or hairpin ribozyme, it is worth noting that GAPDH in-

creased the *in vitro* cleavage rate of the hammerhead ribozyme of TNFα mRNA, presumably via the RNA unfolding activity of GAPDH (Sioud and Jespersen, 1996).

In this study, we consistently detected a twofold enhancing effect of GAPDH on the *cis*-cleavage activity of the HDV antigenomic RNA. This result is significant as in the *in vitro* transcription system, we consistently observed a basal *cis*-cleavage activity at 30–35% (Fig. 9). In addition, a complete cleavage is unlikely to happen, as uncleaved products are always found in the replication cycle of HDV RNA (Makino *et al.,* 1987b). Furthermore, the effect of GAPDH on the catalysis of HDV ribozyme was not observed with BSA (Fig. 9), indicating that the enhancing effect of GAPDH is specific. Athough the underlying mechanism of the enhancing effect remains to be elucidated, it is likely that GAPDH acts as a molecular chaperone to unwind the double-stranded rod-shaped RNA structure and render the nascent viral RNA in a conformation with double pseudoknot structure suitable for the cleavage. In agreement with the role of the glycolytic enzyme GAPDH in regulating HDV ribozyme activity important for the viral multiplication, an earlier study with transgenic mice system has demonstrated that the replication of HDV RNA occurred in most tissues but exhibited at a higher level in the skeletal muscle (Polo *et al.,* 1995), a tissue that undergoes extensive glycolytic process. Further studies are required to elucidate possible roles of the other three HDV RNA-binding proteins identified in this study on the multiplication of HDV.

MATERIALS AND METHODS

Cell line and culture condition

HeLa cells (a human cervical carcinoma cell line) adapted to suspension culture were grown at 37°C in RPMI medium supplemented with 5% fetal calf serum plus 100 units of penicillin and 100 μg of streptomycin/ml.

Preparation of HeLa nuclear extract

Nuclear extract was prepared from HeLa cells grown to mid-log phase, followed the procedures as described previously (Dignam *et al.,* 1983). In brief, cells were harvested by centrifugation for 10 min at 1000 *g* and washed twice with cold phosphate-buffered saline (PBS). The cell pellet was resuspended in five packed cell volumes of buffer A containing 10 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT), and allowed to stand for 10 min at 4°C. The cells were then collected and disrupted using a Dounce homogenizer in two packed cell volumes of the buffer A. Nuclei were pelleted from the homogenate by centrifugation at 1000 *g* for 10 min then 25,000 *g* for 20 min. The nuclei were resuspended in buffer C containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM DTT, stirred for 30 min and subjected to centrifugation for 30 min at 25,000 *g*. The supernatant was collected and dialyzed against buffer D containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT supplemented with 0.1 M KCl for 5 h. The dialysate was further clarified by centrifugation for 20 min at 25,000 *g* and the resulting nuclear extract was frozen as aliquots to store at −80°C.

Plasmid construction

Plasmid p4B. Plasmid p4B contains a single copy of the full-length HDV cDNA and was used to generate HDV AG228/963 RNA transcript. For construction of plasmid p4B, a 1679-bp *Bgl*II DNA fragment was isolated from pD2 (Chang *et al.,* 1993), treated with the Klenow fragment of DNA polymerase I, and subcloned into the unique *Xba*I site, which had been blunted with the Klenow fragment of DNA polymerase I, of pGEM4Z vector (Promega).

Plasmids pBN and pBNd3. Plasmid pBN contains cDNA representing HDV RNA sequence nt 225–434 (nucleotides are numbered as described previously [Kuo *et al.,* 1988a]) and was used to generate HDV AG434/225 RNA. For construction of plasmid pBN, a 210-bp *Bgl*II-*Nhe*I DNA fragment was isolated from pD1 (Chang *et al.,* 1993), treated with the Klenow fragment of DNA polymerase I, and subcloned into modified pGEM4Z from which the polylinker sequence from *Eco*RI to *Xba*I site had been deleted and the remaining DNA blunted with the Klenow fragment of DNA polymerase I. Plasmid pBNd3 contains cDNA representing HDV RNA nt 356–434 and was used to generate HDV AG434/356 and d3 RNA. For construction of plasmid pBNd3, plasmid pBN was digested with *Sal*I plus *Pst*I, treated with exonuclease III and mung bean exonuclease, and self-ligated after a treatment of T4 DNA polymerase.

Preparation of RNA transcripts

RNA transcripts were synthesized *in vitro* from linearized DNA templates containing the promoter sequences for SP6 or T7 RNA polymerase. *In vitro* transcription was performed as described previously (Chang *et al.,* 1993). To generate ^{32}P-labeled RNA probes, *in vitro* transcription was performed in the presence of [α-^{32}P]UTP (3000 Ci/mmol).

HDV G-RNA and AG-RNA. HDV G-RNA and AG-RNA represent the full-length HDV genomic and antigenomic RNA, respectively. The G-RNA was synthesized with SP6 RNA polymerase from plasmid pD1 linearized with *Bam*HI, whereas the AG-RNA was synthesized with T7 RNA polymerase from plasmid pD1 linearized with *Hin*dIII.

AG966/431 and AG966/225. AG966/431 and AG966/225, representing HDV antigenomic RNA nt 966–431 and 966–225 (nucleotides are numbered according to the genomic orientation), were synthesized with T7 RNA polymerase from plasmid pD1 linearized with *Nhe*I and *Bgl*II, respectively.

AG228/963, AG434/225, and AG434/356. AG228/963 represents HDV antigenomic RNA nt 228–1 plus nt 0/1679–963, and AG434/225 represents HDV antigenomic RNA nt 434–225. These two RNA transcripts were synthesized with SP6 RNA polymerase from *Sal*I-linearized plasmid p4B and pBN, respectively. AG434/356 representing HDV antigenomic RNA nt 434–356 was synthesized with SP6 RNA polymerase from plasmid pBNd3 linearized with *Hin*dIII.

d3 RNA. For synthesis of d3 RNA transcript, a 144-bp DNA fragment was first generated by polymerase chain reaction using pBNd3 as the template and SP6 and T7 promoter sequences as the primers. *In vitro* transcription was performed with SP6 RNA polymerase. The d3 RNA encompasses HDV antigenomic RNA nt 434–356 flanking by an extra 11 nt at the 5′-end and 37 nt at the 3′-end that were derived from the vector sequence of plasmid pBNd3.

TGEVE2 RNA. The 159-nt TGEVE2 RNA was prepared as previously described (Yen *et al.,* 1995) and was used as a nonhomologous competitor to study the specific interactions between HDV RNA and cellular proteins.

IC63 RNA. The 63-nt IC63 RNA was synthesized with T7 RNA polymerase from *Eco*RI-linearized pGEM4Z and was used as an internal control in the *cis*-cleavage reaction of HDV ribozyme.

UV cross-linking experiment

UV cross-linking experiments were performed with [α-^{32}P]UTP-labeled RNA (1×10^6 cpm) and 20 μg of HeLa nuclear extract, or indicated amounts of human erythrocyte GAPDH (Sigma) and protein fractions of HeLa nuclear extract purified through column chromatography. The binding reactions were carried out at 30°C for 30 min in a buffer containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.02% FicoII, 0.02% polyvinylpyrrolidone, 0.02% BSA, and 20 μg or otherwise indicated amounts of yeast tRNA. The reaction mixtures were transferred into wells of a microtiter plate seated on ice, UV-irradiated (254 nm, Model UVGL-58, Ultra-Violet Products, Inc.) for 30 min at a distance of 4 cm, followed by a digestion at 37°C for 30 min with 30 μg RNase A (Sigma) and 30 units RNase T1 (Roche Molecular Biochemicals). The UV cross-linked products were separated on an SDS-15% polyacrylamide gel. The gel was fixed, dried, and exposed to X-ray film. In competition experiments, various amounts of unlabeled competitor RNA were added into the reaction mixtures 10 min prior to the addition of ^{32}P-labeled RNA probe.

Electrophoresis mobility shift assay (EMSA)

To perform electrophoresis mobility shift assay, protein-RNA binding reactions were carried out as described in the UV cross-linking experiments, except that [α-^{32}P]UTP-labeled RNA probe at 2×10^4 cpm with comparable specific activity was used. The reaction mixtures were subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel [acrylamide:bisacrylamide weight ratio of 79:1 in 0.5× TBE (1× TBE: 90 mM Tris–borate, 2 mM EDTA, pH 8.0) and 5% glycerol] and the RNA-protein complexes were visualized by autoradiography. Competition study was performed with unlabeled RNA as described for the UV cross-linking competition experiments.

Purification of HDV RNA-binding proteins

For purification of cellular proteins that interact with HDV RNA, HeLa nuclear extract that was prepared and dialyzed against buffer D supplemented with 0.1 M KCl as described earlier was loaded onto a DEAE-cellulose (Whatmann) column preequilibrated with the same buffer. Because the flow-through fraction was found to contain HDV RNA-binding activity, it was loaded further onto a heparin-Sepharose (Amershan Pharmacia Biotech) column preequilibrated with the 0.1 M KCl-supplemented buffer D. The column matrix was washed with 20 column volumes of the same buffer and cellular proteins were eluted with 4 column volumes of each of buffer D containing 0.2, 0.3, and 0.4 M KCl. Fractions were collected and examined for their binding activities to HDV RNA.

Protein sequence analysis

Cellular proteins partially purified through heparin-Sepharose column were precipitated by trichloroacetic acid, resolved by electrophoresis on an SDS-15% polyacrylamide gel, and electrotransferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was stained with Coomassie brilliant blue, and the major band corresponding to a 36-kDa protein was excised. The protein was sequenced by using a model 477A sequenator (Applied Biosystems) equipped with an online 120A phenylthiohydantoin amino acid derivative analyzer.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Chang *et al.,* 1993). Briefly, proteins were electrophoresed on an SDS-15% polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane. Immunoreactive bands were visualized by successive incubations of the membrane with mouse monoclonal antibody to GAPDH of rabbit muscle (Biodesign International) and horseradish peroxidase-conjugated

goat anti-mouse IgG antibody. Color development was performed using 3,3′-diaminobenzidine tetrahydrochloride as a substrate.

RNase footprinting analysis

RNase footprinting-primer extension analysis was carried out as described previously (Ito and Lai, 1997; Kolupaeva *et al.,* 1996) with modifications. In brief, the binding reactions were performed with HDV d3 RNA and the human erythrocyte GAPDH at 30°C for 30 min as described in the UV cross-linking experiments, except that 3 μg of yeast tRNA was added. A 10-min incubation was followed after an addition of various amounts of RNase T1 (Roche Molecular Biochemicals) and the resulting RNA mixtures served as templates to perform primer extension in analyzing the sizes of the RNA products. Annealing reactions of the primer extension were performed at 85°C for 3 min with a [γ-^{32}P]ATP-labeled primer containing sequences of T7 RNA polymerase promoter, in an annealing buffer containing 40 mM Tris–HCl, pH 7.5, 25 mM $MgCl_2$, and 50 mM NaCl. The annealing reaction mixtures were incubated at 37°C in a period of 20–30 min, then kept on ice for 5 min prior to the extension reaction with avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) at 37°C for 30 min in a buffer containing 50 mM Tris–HCl, pH 7.5, 75 mM KCl, 10 mM DTT, 100 μg of BSA/ml, and 1 mM dNTP. The reaction was terminated by adding a stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. Aliquots of 5 μl were boiled for 3 min, and subjected to electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Fluorescence *in situ* hybridization/immunofluorescence staining

Fixation/permeabilization of cultured cells was performed as previously described (Carmo-Fonseca *et al.,* 1991). Briefly, cells on coverslips were washed twice in PBS, pH 7.4, permeabilized by covering with a CKS buffer (100 mM NaCl, 300 mM sucrose, 10 mM 1,4-piperazinediethanesulfonic acid, pH 6.8, 3 mM $MgCl_2$) containing 0.5% Triton X-100 and 1 mM PMSF for 3 min on ice and fixed in the CKS buffer containing 3.7% paraformaldehyde for 10 min at room temperature. To detect HDV RNA, the fixed cells were washed in PBS and incubated with HDV AG434/225 RNA prelabeled with biotin-14-CTP (Life Technologies, Inc.). The final concentration of the biotinylated RNA probe was 30 nM, and the *in situ* hybridization was carried out in a reaction buffer containing 40% formamide, 4× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1 M potassium phosphate buffer, pH 7.0, 300 μg/ml sonicated salmon sperm DNA, and 300 μg/ml yeast tRNA at 42°C overnight in a humidified chamber. The cells were then rinsed with 2× SSC, followed by extensive washing three times with 50% formamide/2× SSC at 45°C for 3 min each, and once with PN buffer (1 M NaH_2PO_4, 1 M Na_2HPO_4, 1% NP-40, pH 8.0) for 5 min at room temperature prior to double immunofluorescence staining. Hybridization of the biotin-labeled probe to the HDV RNA was detected by goat antiserum against biotin (Vector) and fluorescence isothiocyanate (FITC)-labeled rabbit anti-goat antibodies (Jackson ImmunoResearch Laboratories, Inc.). Cellular GAPDH was detected by mouse mAb to GAPDH (Biodesign) and Texas Red-labeled horse anti-mouse antibodies (Vector). Photographs were taken using a Zeiss Axiophot microscope equipped with epifluorescence.

Cis-cleavage reaction of HDV ribozyme

Cis-cleavage reaction was carried out as previously described (Huang and Wu, 1998) with modifications. Briefly, HDV antigenomic RNA AG966/225 and an internal control RNA IC63 were cosynthesized *in vitro* with T7 RNA polymerase and incubated with various amounts of GAPDH or BSA in a total volume of 20 μl containing 40 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.02 mM EDTA, 2% glycerol, and 12 mM $MgCl_2$. At 20-min intervals, 4-μl aliquots were removed from the reaction mixtures and 5 μl of a stop solution containing 50 mM EDTA, 7 M urea, 0.005% bromphenol blue, and 0.005% xylene cyanol were added. The reaction products were resolved on polyacrylamide gels containing 7 M urea. Radioactivities of the cleavage products and uncleaved substrates were quantitated by PhosphorImager (Molecular Dynamics) and normalized against the uncleavable internal control IC63 RNA.

ACKNOWLEDGMENTS

We are grateful to Hsueh-Liang Lee, Ying-Tai Peng, and Ming-Der Lin for technical assistance and to Shao-Chun Lu, Ta-Hsiu Liao, Chung-Hsun H. Wu, and Huey-Nan Wu for helpful discussion. This work was supported in part by research grants (NSC88-2316-B-002-021 and NSC89-2316-B-002-013) from the National Science Council of the Republic of China.

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