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Large Hepatitis Delta Antigen in Packaging and Replication Inhibition: Role of the Carboxyl-Terminal 19 Amino Acids and Amino-Terminal Sequences

CHA-ZE LEE,[1] PEI-JER CHEN,[2,3] AND DING-SHINN CHEN[1,3]*

Department of Internal Medicine,[1] Graduate Institute of Clinical Medicine, College of Medicine,[2] and Hepatitis Research Center,[3] University Hospital, National Taiwan University, Taipei 10016, Taiwan

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Hepatitis delta virus (HDV) encodes two proteins, the small delta antigen (SHDAg) and large delta antigen (LHDAg). The latter is identical to the former except for the presence of additional 19 amino acids at the C terminus. While SHDAg is required for HDV replication, LHDAg inhibits replication and, together with hepatitis B surface antigen (HBsAg), is required for the assembly of HDV. The last 19 C-terminal amino acids of LHDAg are essential for HDV assembly. Most of LHDAg (amino acids 19 to 146 and 163 to 195) had been shown to be dispensable for packaging with HBsAg. To discern whether the last 19 C-terminal amino acids solely constitute the signal for packaging with HBsAg, we constructed two LHDAg deletion mutants and tested their abilities to be packaged with HBsAg in cotransfection experiments. We found that deletion of amino acids 2 to 21 and 142 to 165 did not affect LHDAg packaging. This result suggested that only the last 19 C-terminal amino acids of LHDAg are required for packaging. We further constructed two plasmids which expressed c-H-ras with or without additional 19 C-terminal amino acids identical to those in LHDAg. Only c-H-ras with additional 19 amino acids could be cosecreted with HBsAg in the cotransfection experiment. This result confirmed that the C-terminal 19 amino acids are the packaging signal for HBsAg. We also tested the *trans* activation activity and *trans*-dominant inhibitory activity of the deletion mutants of SHDAg and LHDAg, respectively. In contrast to deletion of amino acids 142 to 165, deletion of amino acids 2 to 21 impaired the *trans*-dominant inhibitory activity of LHDAg. Deletion of amino acids 2 to 21 and 142 to 165 did not affect the *trans* activation activity of SHDAg. This result suggested that a functional domain which is important for the *trans*-dominant inhibitory activity of LHDAg exists in the amino terminus of HDAg.

Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus and can cause fulminant hepatitis and accelerated liver cirrhosis in patients with chronic hepatitis B (36). HDV contains a single-stranded circular RNA genome of 1.7 kb (18, 27, 40), which has extensive intramolecular complementary sequences. Its RNA structure resembles those of viroids, virusoids, and plant satellite virus RNAs. HDV RNA has an autocatalytic cleavage and ligation activity (20, 34, 35, 42–44) and appears to replicate by a rolling-circle mechanism (8, 26). The HDV envelope is provided by hepatitis B virus and contains the large, middle, and small, hepatitis B surface antigens (HBsAg) (1, 11). Although HDV can be assembled with only SHBsAg in the envelope (32, 38), LHBsAg is essential for infectivity (37). HDV RNA encodes two proteins, small hepatitis delta antigen (SHDAg [195 amino acids, 24 kDa]) and large hepatitis delta antigen (LHDAg [214 amino acids, 27 kDa]) (1, 41). These two proteins are identical in sequence, but LHDAg contains 19 additional amino acids at the carboxyl terminus (41). SHDAg is required for HDV RNA replication (19), while LHDAg inhibits RNA replication (5, 13) and is required for HDV assembly (2, 32). The C-terminal 19-amino-acid region of LHDAg contains a prenylation site (12), which is indispensable for HDV packaging (23) and *trans*-dominant inhibitory activity of LHDAg (17). Previously, it was shown that, while C-terminal 19 amino acids (amino acids 196 to 214) of LHDAg are essential for packaging with HBsAg (4, 23), amino acids 19 to 145 and 163 to 195 of LHDAg are dispensable (4, 7, 23, 32). In this study, we show that amino acids 2 to 21 and 143 to 165 of LHDAg are also not required for packaging with HBsAg. Furthermore, addition of C-terminal 19 amino acids of LHDAg to the C terminus of c-H-ras renders this protein able to be cosecreted with HBsAg. Thus, we conclude that C-terminal 19 amino acids of LHDAg are the packaging signal for HBsAg. Besides, we have also found that amino acids 2 to 21 and 143 to 165 are not required for *trans*-activating activity of SHDAg and that amino acids 2 to 21 are important for *trans*-dominant inhibitory activity of LHDAg. This result suggested that a functional domain exists in the N terminus of HDAg.

* Corresponding author. Phone: 886-2-397-0800, ext. 7120. Fax: 886-2-331-7624.

MATERIALS AND METHODS

Construction of plasmids. All the HDV constructs were made from the cDNA clones of the Southern California isolate of HDV RNA (28). All the mutations were made by the recombinant PCR method described by Higuchi et al. (14). All the mutated cDNA fragments were inserted into the *Bgl*II and *Eco*RI sites of the PECE vector (10).

The deletion mutants of LHDAg and SHDAg were derived from PECE-δ-BE (3) and PECE-Sm (24), which contain the *Bam*HI-*Eco*RI fragment of HDV cDNA and express the wild-type LHDAg and SHDAg, respectively, when transfected into the Huh7 cell line.

p-c-H-ras was made by inserting the c-H-ras-coding region, amplified from a human liver cDNA library, into the PECE vector. p-c-H-ras19 was made by inserting the coding region of c-H-ras and C-terminal 19 amino acids of LHDAg, which were joined by recombinant PCR, into the PECE vector. The resulting mutations are shown in Fig. 1.

The sequences of the resulting plasmids were confirmed by dideoxyribonucleotide chain termination sequencing (33).

DNA transfections and sample harvest. The human hepatoma cell line Huh7 (29) was used as the recipient cells for DNA transfection. The cell line was maintained in Dulbecco's modified Eagle essential medium containing 10% fetal calf serum. For packaging assay, Huh7 cells at 80% confluency in a 10-cm petri dish were transfected with 10 μg of plasmid pS1X (2), an HBsAg expression plasmid, and 10 μg of a plasmid encoding the wild-type LHDAg, mutant LHD-

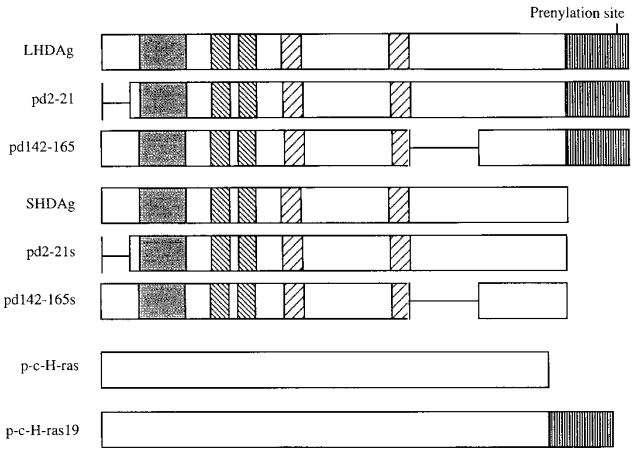

FIG. 1. Mutants and functional domains of HDAg. ■, coil-coil sequence (amino acids 29 to 51); ▧, nuclear localization signal (amino acids 69 to 75 and 85 to 88); ▨, ARM (amino acids 97 to 107 and 136 to 146); ■, packaging-associated sequence (amino acids 196 to 214).

Ags, p-c-H-ras, or p-c-H-ras19 by the calcium phosphate-DNA precipitation method (45). On day 4 after transfection, the cells were scraped off the dish and resuspended in the Laemmli sample buffer (21). The medium was clarified by low-speed centrifugation (10,000 rpm for 30 min in a JA17 rotor; Beckman), and the HBsAg particles were pelleted by high-speed centrifugation (35,000 rpm for 16 h at 4°C in an SW41Ti rotor; Beckman) through a 20% sucrose cushion. The pellet was resuspended in the same sample buffer. For p-c-H-ras and p-c-H-ras19, the pellet was resuspended in 1 ml of RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 0.1 mM phenylmethylsulfonyl fluoride and then incubated overnight with 5 μl of polyclonal goat anti-HBsAg antibody (Dako Japan Co., Kyoto, Japan) and 50 μl of protein G agarose (Boehringer Mannheim) at 4°C. The sample was spinned down, washed three times with RIPA buffer, and resuspended in the Laemmli sample buffer.

For testing *trans* activation activity of mutant SHDAgs, 5 μg of pCDm2G (39), a plasmid containing a head-to-tail dimer of HDV DNA with a frameshift in the HDAg-coding region, was cotransfected with 5 μg of a plasmid encoding one of the various SHDAg mutants into Huh7 cells. For testing *trans*-dominant inhibitory activity of mutant LHDAgs, 5 μg of pSVL(D3) (19), a plasmid containing a head-to-tail trimer of HDV DNA, was cotransfected with 5 μg of a plasmid encoding one of the various LHDAg mutants into Huh7 cells. The total cellular RNA and protein were extracted by the modified acid guanidinium isothiocyanate method (9) using the TRIzol reagent (BRL/Life Technology, Gaithersburg, Md.) 4 days posttransfection.

Immunoblot analysis. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and electrotransferred to nitrocellulose membranes. The membranes were first incubated with 5% nonfat milk and then with human anti-HDAg antibody or monoclonal mouse anti-c-H-ras antibody (Dako Japan Co.). An alkaline phosphatase-conjugated rabbit anti-human (Promega, Madison, Wis.) or goat anti-mouse (Promega) immunoglobulin antibody was used as the second antibody. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for color development.

Northern (RNA) blot analysis. RNA samples (20 μg) were electrophoresed in a formaldehyde gel and transferred to a nitrocellulose membrane. The membrane was baked at 80°C for 2 h, prehybridized for 6 h, and hybridized at 65°C overnight with digoxigenin-labeled antigenomic HDV RNA. The membrane was washed twice at room temperature and twice at 68°C and then detected with alkaline phosphatase-conjugated goat anti-digoxigenin antibody (Boehringer Mannheim). Lumi-phos (Boehringer Mannheim) was used as a substrate for chemiluminescence development according to the manufacturer's instructions.

RESULTS

Only C-terminal 19 amino acids are essential for LHDAg to be packaged by HBsAg. It has previously been shown that, while C-terminal 19 amino acids (196 to 214) of LHDAg are essential for HBsAg packaging (4, 23), amino acids 19 to 146 and 163 to 195 are dispensable (4, 7, 23, 32). To determine whether the remaining regions of amino acids 1 to 18 or 147 to 162 are required for packaging, we constructed two LHDAg mutant plasmids, pd2-21 and pd142-165, which expressed mutant LHDAgs with a specific deletion of amino acids 2 to 21 or 142 to 165, respectively. These mutants were cotransfected with pS1X into Huh7 cells. The medium was then tested for the presence of mutant LHDAgs. As expected, the wild-type LHDAg, but not SHDAg, was found in the medium (Fig. 2, lanes L and S). Both of the deletion mutant proteins were also secreted into medium as efficiently as the wide-type LHDAg (Fig. 2, lanes 2-21 and 142). Therefore, we conclude that amino acids 2 to 195 of LHDAg, in contrast to C-terminal 19 amino acids (positions 196 to 214), are not required for LHDAg to be packaged by HBsAg.

C-terminal 19 amino acids of LHDAg direct packaging of a foreign protein by HBsAg. The result obtained in the previous experiment indicated that the C-terminal 19-amino-acid region of LHDAg contains the essential signal required for LHDAg to be packaged by HBsAg. To test whether this signal sequence was also sufficient to allow packaging of foreign protein sequences by HBsAg, the nucleotide sequence of the C-terminal 19 amino acids of LHDAg was inserted into a plasmid, p-c-H-ras (Fig. 1), which expresses c-H-ras in animal cells. c-H-ras was chosen as the testing protein, because its molecular mass (21 kDa) is about the same as that of HDAg and it, like LHDAg, is an isoprenylated protein. This construct, p-c-H-ras19, or parental plasmid p-c-H-ras, was cotransfected with pS1X (2), an HBsAg-expressing plasmid, into Huh7 cells. As demonstrated by the result of a Western blot (immunoblot) with an anti-c-H-ras antibody, c-H-ras and c-H-ras19 were present in the cell (Fig. 3). In the medium, however, only the chimeric c-H-ras containing the C-terminal 19 amino acids of LHDAg was found to be cosecreted with HBsAg whereas c-H-ras without these 19 amino acids was not (Fig. 3). This result confirmed the previous report that isoprenylation is not sufficient for packaging with HBsAg (23) and demonstrated that the C-terminal 19 amino acids of LHDAg are indeed sufficient to direct packaging of c-H-ras by HBsAg.

Deletion of amino acids 2 to 21 impairs the *trans*-dominant inhibitory function of LHDAg. Four functional domains of LHDAg have been defined. The coil-coil sequence mediates the oligomerization of HDAg. The nuclear localization signal targets HDAg to the nucleus (47). The middle-one-third domain contains two arginine-rich motifs (ARMs) and is required for binding with HDV RNA (24, 25). The C-terminal

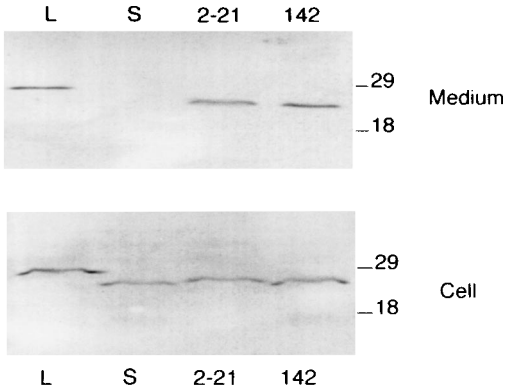

FIG. 2. Packaging assay of the deletion mutants of LHDAg. Huh7 cells were cotransfected with various mutant LHDAg-expressing plasmids and pS1X, an HBsAg-expressing plasmid. The cells and media were harvested 4 days posttransfection. Western blot analyses of LHDAg mutants expressed in the cells (A) and secreted to the media (B) were performed with a human anti-HDAg antibody. The positions of molecular mass markers (in kilodaltons) are indicated. Lanes: L, LHDAg; S, SHDAg; 2-21, pd2-21; 142, pd142-165.

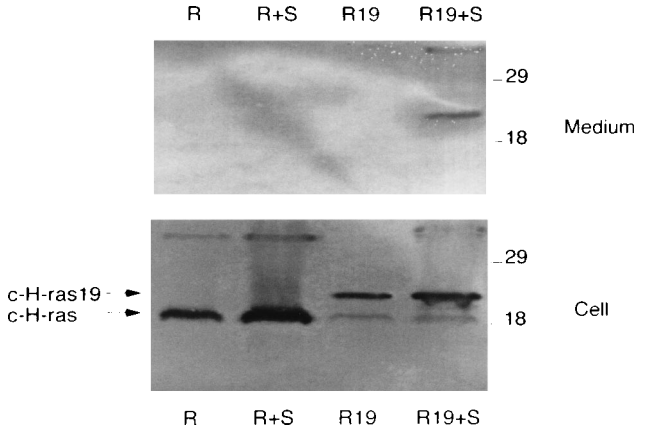

FIG. 3. Packaging assay of c-H-ras protein and a mutant c-H-ras protein with additional C-terminal 19 amino acids identical to those in LHDAg. Huh7 cells were cotransfected with a c-H-ras-expressing plasmid or a mutant c-H-ras-expressing plasmid and pS1X, an HBsAg-expressing plasmid. The cells and media were harvested 4 days posttransfection. Western blot analysis of c-H-ras or mutant c-H-ras expressed in the cells (A) and secreted to the media (B) were performed with a monoclonal mouse anti-c-H-ras antibody. The positions of molecular mass markers (in kilodaltons) are indicated. Lanes: R, p-c-H-ras; R+S, p-c-H-ras plus pS1X; R19, p-c-H-ras19; R19+S, p-c-H-ras19 plus pS1X. The small bands in lanes R19 and R19+S represent the ras proteins produced by Huh7 cells.

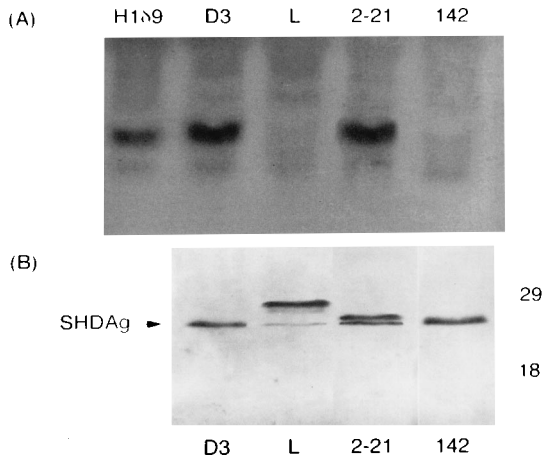

FIG. 4. *trans*-dominant inhibitory activity of mutant LHDAgs with a replication-competent HDV trimer and mutant LHDAg-expressing plasmids. (A) Huh7 cells were cotransfected with HDV trimer DNA [pSVL(D3)] and various mutant LHDAg DNAs as described in Results. Cellular RNA was extracted 4 days posttransfection, separated in a formaldehyde gel, transferred to a nylon membrane, and incubated with digoxigenin-labeled antigenomic HDV RNA. (B) Western blot analysis of LHDAg mutants expressed in the cells were performed with a human anti-HDAg antibody. In lane 142, SHDAg and mutant protein of pd142-165 migrated very close to each other and appeared as one band. The positions of molecular mass markers (in kilodaltons) are indicated. H1δ9 is a COS7 cell line stably transformed with HDV which serves as a size marker for monomer HDV RNA. Lanes: D3, pSVL(D3); L, LHDAg plus pSVL(D3); 2-21, pd2-21 plus pSVL(D3); 142, pd142-165 plus pSVL(D3).

19-amino-acid region contains an isoprenylation site and is essential for HDV assembly (12, 23). The LHDAg mutants described above had deletions outside these functional domains. To test whether these deletion mutants affected the *trans*-dominant inhibitory function of LHDAg, we cotransfected wild-type LHDAg, pd2-21, or pd142-165 with pSVL (D3) (19), a replication-competent trimer of unit-length HDV, into Huh7 cells. Figure 4A shows that, while LHDAg and pd142-165 completely inhibited the replication of HDV RNA, pd2-21 still allowed a significant level of HDV RNA replication. The efficiencies of transfection were checked by detecting the expressed proteins with a human anti-HDAg antibody (Fig. 4B) and were shown to be similar. This result suggested that N-terminal sequence contains a functional domain which is important for the *trans*-dominant inhibitory function of LHDAg.

Amino acids 2 to 21 and 142 to 165 of SHDAg are not required for *trans* activation of HDV replication. On the basis of the above experiment we expected to see a similar effect in *trans* activation activity of SHDAg. So, we constructed corresponding deletion mutants of SHDAg, pd2-21s and pd142-165s, and tested their *trans* activation activities by cotransfecting them with pCDm2G (39), a replication-defective HDV dimer, into Huh7 cells. To our surprise, pd2-21s could rescue HDV RNA replication as effectively as wild-type SHDAg and pd142-165s (Fig. 5A, lane S, 2-21s, and 142s). In contrast, an ARM mutant, PECE-A1 (24), was unable to *trans* activate HDV RNA replication (Fig. 5A, lane A1). The transfection efficiencies were similar, as shown in Fig. 5B. We further tested whether pd2-21 can inhibit the *trans* activation activities of pd2-21s by cotransfecting pd2-21 with pCDm2G and pd2-21s into Huh7 cells. Again, no inhibition of HDV RNA replication could be seen when the ratio of pd2-21 to pd2-21s was either 5 to 5 μg (Fig. 6A, lane 2-21a) or 10 to 5 μg (Fig. 6A, lane 2-21b). The transfection efficiencies were similar, as shown in Fig. 6B. Therefore, we proposed that a functional domain resides between amino acids 2 and 21 and that this domain is related to the *trans*-dominant inhibitory function of LHDAg but is dispensable for *trans* activation activity of SHDAg.

DISCUSSION

LHDAg has two biological activities that differ from those of SHDAg. First, it is involved in HDV virion assembly (2, 32). The C-terminal 19 amino acids together with prenylated residues are essential for HDV packaging and protein-protein interaction between LHDAg and HBsAg (16, 23). Two possible mechanisms can explain this LHDAg-HBsAg interaction: the protein-protein interaction is mediated directly through prenylated C-terminal 19 amino acids, or prenylation alters HDAg conformation to allow another part of LHDAg to interact with HBsAg. This report clearly suggests that, except for C-terminal 19 amino acids, no other part of LHDAg is required for interaction with HBsAg and that this stretch of amino acids can solely mediate interaction with HBsAg.

Second, while SHDAg *trans* activates HDV replication, LHDAg inhibits HDV replication (5). Two functional domains have been shown to be important for this inhibitory activity (4, 7, 17, 22, 46). The coil-coil sequence (amino acids 29 to 47) mediates the oligomerization of HDAg (4, 7, 17, 22, 46), and deletion of this sequence makes LHDAg unable to inhibit HDV replication (22, 46). It has also been shown that amino acids 1 to 88 of HDAg alone can inhibit HDV replication (22). The C-terminal 19-amino-acid region of LHDAg contains a CXXX motif and is prenylated (12). This prenylated C-terminal 19-amino-acid region masks a specific conformation in SHDAg (15), which is required for *trans* activation activity of HDAg (17). This conformation has been mapped by an SHDAg-specific monoclonal antibody to the C-terminal 32 amino acids (164 to 195) of SHDAg (15). In this report we have shown that the deletion mutants of SHDAg, pd2-21S and pd142-165S, still retain the *trans* activation activity. Together with the previously identified functional domains in SHDAg, the C-terminal 30 amino acids of SHDAg very likely represent a necessary, and possibly the *trans*-activation, domain of SHDAg.

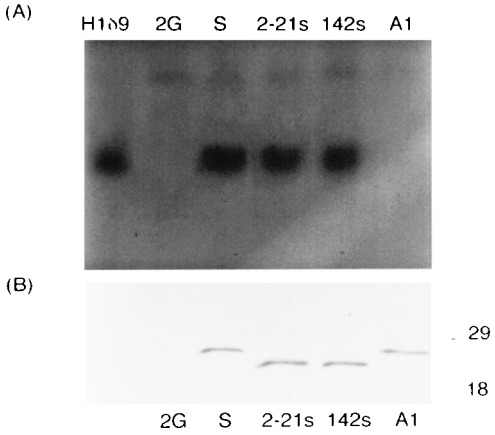

FIG. 5. *trans* activation activity of mutant SHDAgs with a replication-defective HDV dimer and mutant SHDAg-expressing plasmids. (A) Huh7 cells were cotransfected with HDV dimer DNA (pCDm2G) and various mutant SHDAg DNAs as described in Results. Cellular RNA was extracted 4 days posttransfection, separated in a formaldehyde gel, transferred to a nylon membrane, and incubated with digoxigenin-labeled antigenomic HDV RNA. (B) Western blot analyses of SHDAg mutants expressed in the cells were performed with a human anti-HDAg antibody. The positions of molecular mass markers (in kilodaltons) are indicated. H1δ9 is a COS7 cell line stably transformed with HDV which serves as a size marker for monomer HDV RNA. Lanes: 2G, pCDm2G; S, SHDAg plus pCDm2G; 2-21s, pd2-21s plus pCDm2G; 142s, pd142-165s plus pCDm2G; A1, PECE-A1 plus pCDm2G. PECE-A1 is an ARM mutant of SHDAg.

It is surprising that pd2-21, which contains two domains mentioned above, is unable to suppress HDV replication. There are two possible explanations: the deletion of amino acids 2 to 21 may alter the conformation of LHDAg, rendering it unable to form an oligomer with SHDAg, or this region may contain a functional domain that is required for the inhibitory activity. Using synthetic peptides, Rozzelle et al. demonstrated that all of amino acids 12 to 60 of HDAg are required for multimer formation (31). Lack of amino acids 12 to 21 would then render pd2-21 unable to form a multimer. The intact *trans* activation activity of pd2-21s argues against this possibility. Poisson et al. showed that amino acids 2 to 27 bind specifically to HDV RNA and that amino acids 2 to 10, of which five are basic, share sequence similarity with amino acids 97 to 107 (30), the first ARM of the RNA-binding domain of HDAg. By using TrpE-HDAg fusing protein, Lin et al. showed that the RNA-binding domain of HDAg lies between amino acids 79 and 146 (25). However, they did not assess the role of the N-terminal 10 amino acids, because their fusion proteins did not contain these amino acid sequences (25). Chao et al. demonstrated that HDAg binds to the rod structure of HDV RNA (6). However, HDAg also can bind to a small RNA fragment with a stem-and-loop structure containing the ribozyme domain of HDV genomic RNA (24). These findings suggest that HDAg might recognize a double-stranded RNA structure unique to HDV RNA. SHDAg has to bind to HDV RNA through two ARMs in the middle-one-third domain to exert its *trans* activation activity. Our report suggested that LHDAg may also have to bind to a specific region of HDV RNA through the putative N-terminal RNA-binding domain to exert its inhibitory activity. The currently accepted mechanism of inhibitory activity of LHDAg is disruption of the conformation of the HDAg complex. It should be noted that all the supporting experiments for this model used LHDAg mutants with at least 18 preserved N-terminal amino acids. Whether there exists a functional domain in N-terminal sequences of HDAg will require further study.

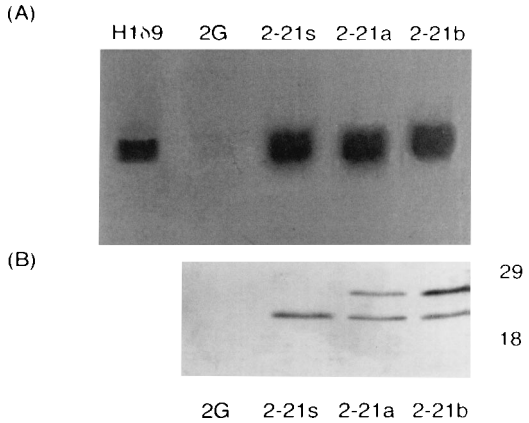

FIG. 6. Effect of pd2-21 on the *trans* activation activity of pd2-21S. (A) Huh7 cells were cotransfected with 5 μg of HDV dimer DNA (pCDm2G), 5 μg of pd2-21S, and either 5 μg or 10 μg of pd2-21. Cellular RNA was extracted 4 days posttransfection, separated in a formaldehyde gel, transferred to a nylon membrane, and incubated with digoxigenin-labeled antigenomic HDV RNA. (B) Western blot analyses of HDAg mutants expressed in the cells were performed with a human anti-HDAg antibody. The positions of molecular mass markers (in kilodaltons) are indicated. H1δ9 is a COS7 cell line stably transformed with HDV which serves as a size marker for monomer HDV RNA. Lanes: 2G, pCDm2G; 2-21s, pd2-21s plus pCDm2G; 2-21a, pCDm2G plus pd2-21s plus pd2-21 (5 μg); 2-21b, pCDm2G plus pd2-21s plus pd2-21 (10 μg).

Lazinski and Taylor showed that deletion of amino acids 146 to 163 destroyed the *trans* activation activity of SHDAg and rendered the LHDAg unstable (22). In our experiment, however, deletion of amino acids 142 to 165 did not affect the *trans* activation activity of SHDAg or the stability of LHDAg. Their constructs differ in six amino acids from ours; such a difference might have a profound effect on protein conformation, which could explain the discrepancy. The middle one-third of HDAg is an RNA-binding domain and contains two ARMs (24). While site-specific mutations of both upstream (amino acids 139 and 140) and downstream (amino acids 142 and 143) basic amino acids of the second ARM affect the RNA-binding activity of HDAg, only mutation of upstream amino acids impaired the *trans* activation activity of SHDAg (24). The intact *trans* activation activity of pd142-165S is compatible with this finding.

Assembly of infectious HDV required major HBsAg, large HBsAg, and possibly middle HBsAg (37). By adding the packaging signal of LHDAg to the C terminus of a foreign protein, we conferred on this protein the ability to be packaged with HBsAg. If the recombinant protein is a DNA- or RNA-binding protein, it possibly could bring the related nucleic acid sequences with it and be assembled with hepatitis B envelope protein into infectious particles. This provides a basis for developing vectors for transfer of foreign proteins or genes into cells and test animals. In view of the high tissue and species specificities of HDV, this possibility appears very attractive.

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