

## Oligomerization of Hepatitis Delta Antigen Is Required for both the *trans*-Activating and *trans*-Dominant Inhibitory Activities of the Delta Antigen

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**Two forms of hepatitis delta antigen (HDAg) have different roles in the replication cycle of hepatitis delta virus (HDV); the small form *trans* activates HDV RNA replication, whereas the large form suppresses it but is needed for virion assembly. To understand the mechanism of these regulatory activities, we studied the possible HDAg oligomerization and its role in HDV replication. In this report, we provide direct biochemical evidence for the *in vitro* and *in vivo* formation of homodimers and heterodimers between these two HDAg species. By deletion mutagenesis, we showed that this protein interaction is mediated by the leucine zipper-like sequence residing in the N-terminal one-third of HDAg. Furthermore, site-specific mutants with various substitutions on two of the leucine residues in this stretch of sequence had reduced or no ability to form HDAg dimers. Correspondingly, the small HDAg with mutations in the leucine zipper-like sequence had reduced abilities to *trans* activate HDV RNA replication. Similar mutations on the leucine zipper-like sequence of the large HDAg also resulted in loss of the ability of large HDAg to inhibit HDV RNA replication. The *in vivo* biological activities of both forms of HDAg (*trans* activation and *trans*-dominant inhibition of HDV RNA replication, respectively) correlated with the extent of HDAg oligomerization *in vitro*. Thus, we conclude that the small HDAg participates in HDV RNA replication as an oligomer form and that the large HDAg inhibits HDV RNA replication as a result of its complex formation with small HDAg. A "black sheep" model for the mechanism of *trans*-dominant inhibition by the large HDAg is presented.**

Human hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus (HBV) and requires HBV for transmission (27). HDV is a 36-nm particle consisting of an envelope with HBV surface antigen, an internal HDV-specific protein, i.e., hepatitis delta antigen (HDAg), and a single-stranded, circular RNA genome of about 1.7 kb (16, 18, 24, 30). HDAg consists of two protein species of 27 and 24 kDa (1, 2, 26, 33), termed large and small HDAg, respectively. These proteins are identical in sequence, except that the large HDAg contains an additional 19 amino acids at its C terminus. These two HDAGs are encoded by two different HDV RNA species (31). Both HDAGs are phosphoproteins, with an RNA-binding activity specific for HDV RNA (4, 6, 15, 22). However, they have significantly different roles in the HDV life cycle. The small HDAg promotes HDV RNA replication (17), whereas the large HDAg inhibits HDV RNA replication (5) and is required for HDV assembly (3, 28). The mechanisms for these contrasting activities of the large and small HDAg are not clear. Both proteins also serve as internal structural components of the HDV virion (1-3, 27).

Several functional domains on the HDAg have been identified. The N-terminal one-third contains a nuclear localization signal, which is located between amino acids 69 and 88 (32). This region contains two stretches of basic amino-acid-rich sequence and is responsible for the nuclear targeting of HDAg (32) and probably HDV RNA as well (13). The middle one-third of HDAg contains two arginine-rich motifs (amino acids 97 to 107 and 136 to 146), which constitute the RNA-binding domain (21). In addition, in the N-terminal one-third upstream of the nuclear targeting signal

is a stretch of leucine zipper-like sequence (amino acids 30 to 51) (Fig. 1). This structural motif has a high alpha-helical content, with four leucine residues located at every seventh position in a common plane, allowing possible interdigitation of the zipper-like regions between different molecules (20). From the study of the mechanism of nuclear transport of HDAg, we have previously suggested that HDAg could form aggregates through this leucine zipper-like sequence and be transported as a protein complex into the nuclei (32). The possible complex formation among HDAg molecules has also been suggested from the functional studies of HDAg (5). However, the significance of this leucine zipper-like sequence in HDV replication cycle was not clear. In this report, we have provided direct biochemical evidence that HDAg forms homodimers and heterodimers both *in vitro* and *in vivo*. Furthermore, by site-specific mutagenesis of the leucine zipper-like sequence, we have shown that the leucine zipper-mediated interaction between HDAGs is important for the *trans*-activating activity of the small HDAg as well as for the *trans*-dominant inhibitory function of the large HDAg. Thus, we conclude that HDAg participates as an oligomer form in HDV RNA replication and that the inhibitory activity of the large HDAg is due to its role as a "black sheep" in this HDAg complex.

### MATERIALS AND METHODS

**Cell line and DNA transfection.** The monkey kidney cell line COS 7 (12) was used as the recipient for DNA transfection studies. The cell line was maintained in Dulbecco's modified minimum essential medium containing 10% fetal calf serum. DNA plasmids were transfected into COS 7 cells by using lipofectin, according to the manufacturer's direc-

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Wild type L E R D L R K L K K K I K K L E E D N P W L  
 pBLGGL . . . . . G . . . . . G . . . . .  
 pBLGVL . . . . . G . . . . . V . . . . .

FIG. 1. The leucine zipper-like sequence in HDAG. The leucine residues at each seventh position of the leucine zipper region are underlined. The mutated amino acids in the mutant HDAGs are shown. The amino acid sequence is that of the Southern California isolate (24).

tions (Bethesda Research Laboratories Life Technologies, Inc.) with slight modifications. Briefly, cells were grown in 60-mm-diameter tissue culture dishes to 85% confluence. For each plate of cells, 15  $\mu$ g of DNA in 50  $\mu$ l of distilled H<sub>2</sub>O was mixed with 50  $\mu$ g of lipofectin and added directly onto the plate. After incubation at 37°C for 12 to 16 h, the lipofectin-containing medium was removed and replaced with Dulbecco's modified minimum essential medium containing 10% fetal calf serum. The cells were incubated for 4 more days before RNA extraction.

**Construction of HDAG-truncated plasmids.** Leucine zipper deletion mutant TDL<sub>1</sub>, which expresses amino acids 67 to 214 of HDAG, was constructed by first synthesizing a DNA fragment by polymerase chain reaction (PCR) amplification of the desired region on the plasmid PECE- $\delta$ -BE (4). The two primers used in PCR were no. 314 (TCGAAGATGACGGGGCACCCCGCGCAAGAAGCTCCGGATGGAC, with the first 12 nucleotides homologous to nucleotides 1607 to 1596 corresponding to the initiation codon of HDAG and the remaining 33 nucleotides homologous to nucleotides 1371 to 1404 of HDV RNA) and no. 86 (GACGCGAGACGCAACCTGTGAGTC, complementary to nucleotides 918 to 942 of HDV RNA). The amplified fragment was cloned into the *Sma*I site of the PECE vector (10). The resulting construct was digested with *Sac*I and *Kpn*I to release a 600-bp fragment, which was then made blunt ended and inserted into the *Kpn*I and *Sac*I sites of the Bluescript vector (Stratagene). Another mutant, TN<sub>2</sub>, which expresses amino acids 1 to 88 of HDAG, was constructed from plasmid PB1-3 (4), which encodes the full-length large HDAG in a PT7 vector. Plasmid PB1-3 was digested with *Stu*I and *Pst*I to release a 3.3-kb fragment, which was gel purified, made blunt ended, and self-ligated.

**Construction of site-specific mutants of the N-terminal leucine zipper sequence.** DNA fragments containing site-specific mutations on the N-terminal leucine zipper sequence were synthesized by PCR with primer 86 and degenerate primer 460 (GACCTCCGGAAGGG[C, A, or T]AAAGAAGAAAATCAAGAAAGGAGAGGAAGAC, complementary to nucleotides 1461 to 1505 of HDV RNA, with the exception of the underlined nucleotides). The PCR fragments were gel purified, phosphorylated, made blunt ended, and inserted into a *Sma*I-linearized PECE vector (10). Mutants were identified by DNA sequencing analysis. The mutants selected were further digested with *Mro*I, and the desired DNA fragments were gel purified and cloned into either *Mro*I-digested PECE- $\delta$ -BE or PB1-3 (4), which has the simian virus 40 early promoter or the T7 promoter, respectively, to replace the corresponding wild-type sequences.

**In vitro transcription and translation.** RNA transcripts were synthesized by using T7 RNA polymerase. Each reaction mixture contained 2  $\mu$ g of linearized plasmid DNA, 2.5 mM nucleoside triphosphates, 1 $\times$  transcription buffer (Promega), 0.1 mg of bovine serum albumin per ml, 10 mM dithiothreitol, 1  $\mu$ l of RNasin (Promega), and 1  $\mu$ l of T7 RNA

polymerase and was incubated at 37°C for 1 h. The DNA template was removed by digesting with RQ1 DNase (Promega) for 30 min, and RNA transcripts were extracted with phenol-chloroform and precipitated with ethanol. An optimal amount of RNA (determined by titration) was used in a translation mixture containing 10  $\mu$ l of rabbit reticulocyte lysate (Promega), 1  $\mu$ l amino acid mixtures (minus methionine), and 2  $\mu$ l (10  $\mu$ Ci/ $\mu$ l) of [<sup>35</sup>S]methionine in a final volume of 25  $\mu$ l. Incubation proceeded at 30°C for 1 h.

**Glutaraldehyde cross-linking and analysis of translation products.** In vitro-synthesized proteins were cross-linked by using chemical cross-linker glutaraldehyde (Sigma) according to the published method (9). Briefly, protein samples were incubated with glutaraldehyde at a final concentration of 0.005% in reaction buffer containing 75 mM sodium phosphate (pH 7.0), 10 mM EDTA, 20  $\mu$ M leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 20  $\mu$ g of aprotinin per ml at 20°C for 2 h. After incubation, samples were mixed with an equal volume of 2 $\times$  sample buffer (0.075 M Tris-HCl [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, and 3% sodium dodecyl sulfate [SDS]) and separated by electrophoresis on a 5 to 12.5% gradient polyacrylamide gel containing 0.1% SDS (19).

**Preparation of cell lysate and immunoprecipitation.** Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), resuspended in 150  $\mu$ l of lysis buffer (50 mM Tris HCl [pH 8.0], 140 mM NaCl, 0.5% Nonidet P-40, 10 mM EDTA, 20  $\mu$ M leupeptin, 7  $\mu$ g of pepstatin per ml, 2 mM phenylmethylsulfonyl fluoride, and 20  $\mu$ g of aprotinin per ml), and incubated on ice for 30 min. Cell lysates were then passed through a 25-gauge syringe needle 10 times and clarified of cell debris by centrifugation in a microcentrifuge. For immunoprecipitation, protein samples were incubated with a 1:50 dilution of an HDAG-specific antibody in TNEN buffer (20 mM Tris HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) on ice for 2 h. Afterwards, one-tenth volume of Pansorbin (Calbiochem), freshly washed with NDET buffer (1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris HCl [pH 7.4]) containing 0.3% SDS, was added to the sample. Incubation was continued for an additional hour on ice. The reaction mixture was layered onto 900  $\mu$ l of 30% sucrose cushion made in 0.5 $\times$  TNEN buffer and microcentrifuged for 5 min at room temperature. The immune precipitates were washed three times with the same buffer and analyzed by electrophoresis on a 12.5% polyacrylamide gel.

**RNA extraction and Northern (RNA) blot analysis.** Total cellular RNA was extracted from the transfected cells with 4 M guanidinium isothiocyanate and then with phenol-chloroform, according to the published procedure (23). The RNA was precipitated twice with isopropanol in guanidinium isothiocyanate buffer. RNA samples were then diluted threefold in a buffer containing 320 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 8% formaldehyde, and 65.7% formamide and boiled for 10 min before electrophoresis on a 1.2% formaldehyde gel (23). Northern blot hybridization of RNA samples was performed with <sup>32</sup>P-labeled riboprobes made by transcription from a cDNA construct containing monomeric HDV sequence.

**Immunoblotting of HDAG.** Cells were washed twice in PBS and lysed in Laemmli's buffer (19) by boiling for 10 min. Cell debris was removed by microcentrifugation for 10 min at room temperature. Aliquots of supernatant were electrophoresed on 12.5% polyacrylamide gels, and proteins were transferred to Immobilon-NC transfer membranes (Millipore) according to the previously described procedure (29).

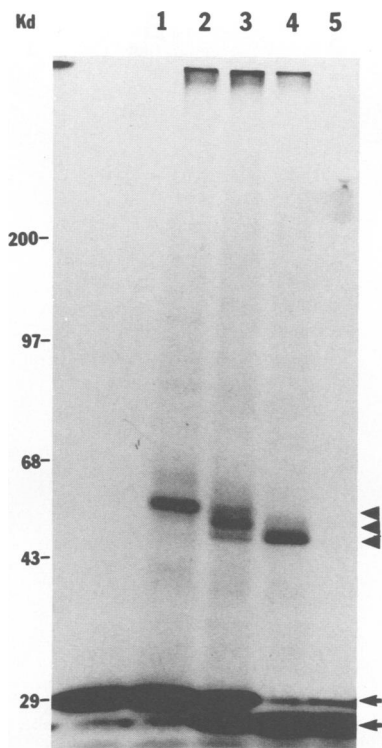


FIG. 2. Glutaraldehyde cross-linking of in vitro-translated HDAGs. Equal amounts of the in vitro-transcribed RNAs encoding the large or small HDAG were translated either individually or together in rabbit reticulocyte lysate containing [ $^{35}$ S]methionine. The translation products were incubated with glutaraldehyde and then separated by electrophoresis on a 12.5% polyacrylamide gel. Lanes: 1, large HDAG; 2, large HDAG + glutaraldehyde; 3, large and small HDAG + glutaraldehyde; 4, small HDAG + glutaraldehyde; 5, small HDAG. Monomer HDAGs are indicated by arrows. Cross-linked HDAG species are indicated by arrowheads.

Briefly, proteins were electrotransblotted onto the membrane in transfer buffer (3 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol) with an electroblotter (Hoefer) for 1 h at 100 V. After transfer, the membrane was incubated in PBS containing 5% nonfat dried milk (Carnation), 0.01% antifoam A, and 0.02% sodium azide for 1 h at room temperature and then incubated in a 1:1,000 dilution of a rabbit antibody specific for HDAG and subsequently detected by  $^{125}$ I-protein A.

## RESULTS

**Dimerization of the large and small HDAGs.** To determine whether HDAG could form oligomers, we first attempted to cross-link HDAG made by in vitro translation. The large and small HDAGs were synthesized separately in a rabbit reticulocyte lysate system with in vitro-transcribed RNA containing an open reading frame for either the large or small HDAG (31). The in vitro-translated products were cross-linked by glutaraldehyde and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 2, in vitro translation yielded primary translation products of 27 and 24 kDa and, in addition, proteins of approximately 54 and 48 kDa, which correspond to dimers of the large and the small HDAGs, respectively (lanes 2 and 4). This result suggests that both the large and the small HDAGs can form

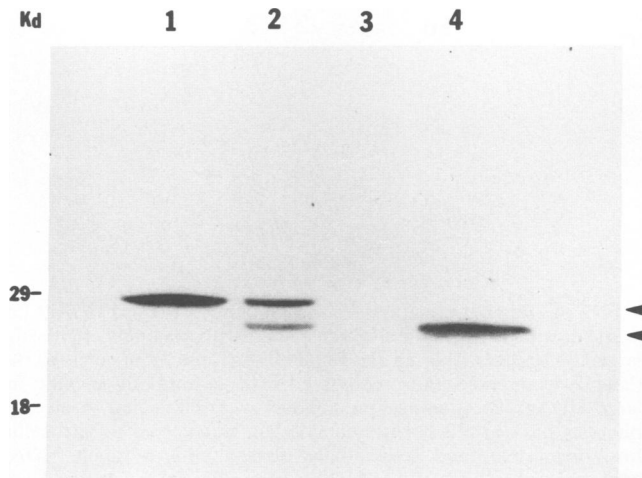


FIG. 3. Immunoprecipitation of in vitro-translated HDAGs. [ $^{35}$ S]methionine-labeled large (lane 1) and small (lane 3) HDAGs or a mixture of the two (lane 2) translated in rabbit reticulocyte lysate were immunoprecipitated with an antibody specific for the large HDAG and electrophoresed on 12.5% polyacrylamide gel. As a control, the in vitro-translated small HDAG without immunoprecipitation was included (lane 4). HDAG species are indicated by arrowheads.

homodimers in vitro. The fractions of the HDAG cross-linked by glutaraldehyde were approximately 20 to 30% of the total proteins, and there were no noticeable differences between the large and small HDAGs in the ability to form homodimers. Occasionally, larger protein complexes corresponding to tetramer or even larger complexes could also be detected (data not shown and see below).

To examine the possible formation of heterodimers between the large and the small HDAGs, an in vitro translation reaction with a mixture of equal amounts of the two RNA species was performed and the translation products were cross-linked by glutaraldehyde. Three cross-linked bands were detected which have sizes corresponding to those predicted for the large HDAG homodimer, large and small HDAG heterodimer, and small HDAG homodimer, respectively (Fig. 2, lane 3). The relative ratio of the three cross-linked protein species was 1/2/1 (large homodimer-heterodimer-small homodimer), suggesting that the binding affinity between the large and small HDAGs was similar to the homologous interaction of the respective proteins. The heterodimer formation was not observed when the large and small HDAGs were mixed after separate in vitro translation experiments (data not shown). We further examined the specificities of the cross-linked protein species. First, we performed in vitro translation of the large and small HDAGs in the absence of radiolabel, and then we detected cross-linked products by immunoblot analysis with an HDAG-specific antibody. All three dimerized proteins were detected, indicating that they were authentic HDAG dimers (data not shown). Second, we precipitated the in vitro-translated protein products with an antibody generated against the synthetic peptide of the C-terminal 19 amino acids of the large HDAG (14a). Figure 3 shows that only the large HDAG, but not the small HDAG, was precipitated (lanes 1 and 3, respectively). In contrast, when the in vitro translation products of the RNA mixture were tested, both the large HDAG and the small HDAG were precipitated even in the absence of prior glutaraldehyde treatment, suggesting

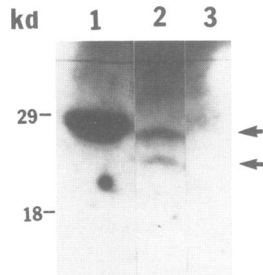


FIG. 4. Immunoprecipitation and immunoblotting of HDAGs expressed in cells. COS 7 cells transfected with plasmids expressing large HDAG (lane 1), large and small HDAG (lane 2), or small HDAG (lane 3) were lysed and precipitated with an antibody specific for large HDAG. The immune precipitates were subjected to electrophoresis on a 12.5% polyacrylamide gel, transferred to nitrocellulose membrane, and sequentially blotted with a rabbit HDAG antibody and  $^{125}\text{I}$ -protein A. HDAGs are indicated by arrows.

that the small HDAG was complexed with the large HDAG and that this interaction was not an artifact of glutaraldehyde cross-linking.

We next examined the possible complex formation between the large and small HDAGs in the cells.  $N_{63}$  cells, a COS 7 cell line stably expressing HDV RNA and synthesizing a small HDAG (30a), were transfected with an expression plasmid, PECE- $\delta$ -BE, which expresses the large HDAG (4). Cell lysates were made at 48 h posttransfection, and proteins were immunoprecipitated with the antibody specific for the C-terminal 19 amino acids of the large HDAG (14a). The immunoprecipitated proteins were detected by immunoblotting with an antibody recognizing both forms of HDAG. Figure 4 shows that in the cells expressing both forms of HDAG, the small HDAG was coprecipitated with the large HDAG (lane 2). In contrast, in the cells expressing either HDAG alone, only the large HDAG was precipitated (lanes 1 and 3). Thus, we concluded that the large and small HDAGs were complexed with each other in the cells.

**Leucine zipper motif is responsible for HDAG dimerization.** To determine the sequence of HDAG responsible for the dimerization of HDAG, we first constructed the HDAG-truncated plasmids  $TN_2$ , which encodes the N-terminal one-third of HDAG, and  $TDL_1$ , which encodes the middle and C-terminal one-third of HDAG. The truncated HDAGs were translated in vitro and subjected to glutaraldehyde cross-linking treatment. Figure 5A shows that the primary translation product of  $TN_2$ , which was expected to synthesize a truncated HDAG of 9 kDa, migrated as a protein of 14 kDa. The reason for this abnormal electrophoretic behavior is not clear. One possible explanation is that the N-terminal domain of HDAG is highly charged. When the translation products were treated with glutaraldehyde (lane 2), protein bands of 21 kDa, 33 kDa, and higher, probably corresponding to dimer, trimer, and larger complexes of HDAG, were also detected. Again, the migration rates of these proteins were slightly slower than the predicted rates. Nevertheless, these protein bands could be precipitated by an HDAG-specific antibody (data not shown). In contrast,  $TDL_1$ , which does not have the N-terminal leucine zipper sequence, formed only a trace amount of HDAG dimer (Fig. 5B). Therefore, the N-terminal one-third, most likely the leucine zipper-like sequence, was responsible for HDAG oligomerization.

To substantiate the role of the leucine zipper-like se-

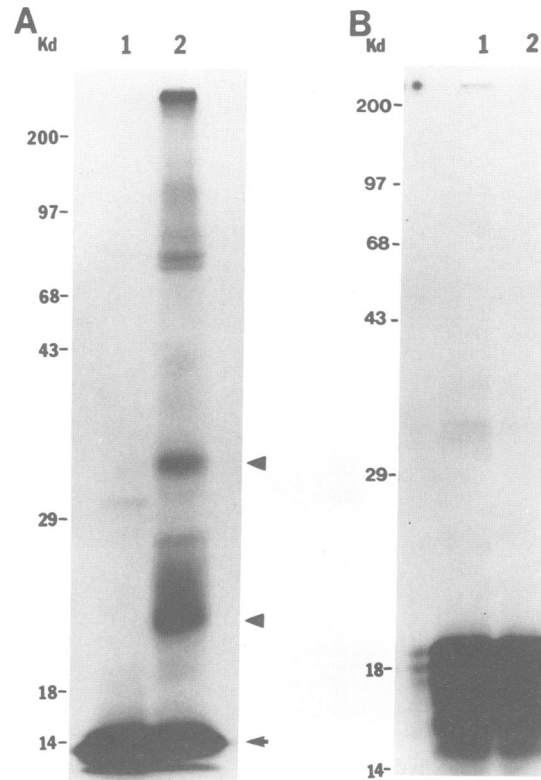


FIG. 5. Dimerization of the truncated HDAG proteins. (A)  $TN_2$ , the  $^{35}\text{S}$ -labeled in vitro-translated N-terminal portion of HDAG, was treated (lane 2) or untreated (lane 1) with glutaraldehyde. (B)  $TDL_1$ , the middle plus the carboxyl portion of HDAG, was treated (lane 1) or untreated (lane 2) with glutaraldehyde. Cross-linking was followed by electrophoresis on SDS-polyacrylamide gels. HDAG monomers are indicated by arrows, and possible oligomers are indicated by arrowheads.

quence in the oligomerization of HDAG, site-specific mutations were performed on the leucine residues of this region (Fig. 1). One of the mutants, pBLGGL, had the middle two leucine residues replaced with glycines, and another mutant, pBLGVVL, had these two leucines replaced with glycine and valine, respectively. The mutant proteins were translated in vitro, cross-linked with glutaraldehyde, separated by SDS-PAGE, and detected by immunoblotting with a rabbit antibody specific for HDAG. The results showed that these two mutant proteins had greatly reduced abilities to form dimers; the dimer of pBLGVVL was approximately 50%, and that of pBLGGL was less than 5% that of wild-type HDAG (Fig. 6). In this gel, protein bands corresponding to trimers and tetramers were also observed; their amounts were also reduced with mutant HDAGs. We concluded that the leucine zipper-like sequence in the N-terminal one-third of HDAG was responsible for HDAG dimerization.

**A functional leucine zipper sequence on the small HDAG is required for the *trans* activation of HDV replication.** It has previously been shown that the small HDAG *trans* activates the replication of HDV RNA (17). To determine whether the leucine zipper-like sequence is required for this *trans*-activating activity, two mutants were constructed, pSGGL and pSGVL, which have glycine/glycine and glycine/valine, respectively, replacing the middle two leucine residues. The two mutants were assayed for their abilities to complement

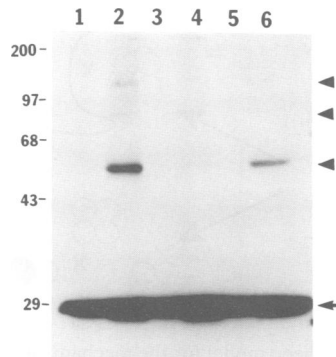


FIG. 6. Oligomerization in vitro of wild-type and site-specific mutant HDAGs. In vitro-translated wild-type HDAG (lanes 1 and 2) and mutants pBLGGL (lanes 3 and 4) and pBLGVL (lanes 5 and 6) were untreated (lanes 1, 3, and 5) or treated with glutaraldehyde (lanes 2, 4, and 6) and analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and incubated sequentially with rabbit HDAG antibody and  $^{125}\text{I}$ -protein A. HDAG monomers are indicated by the arrow, and cross-linked species are indicated by arrowheads.

the defect of a replication-defective HDV dimer, Smd2 (21), which has a frame-shift mutation in the HDAG coding region. As seen in Fig. 7, HDV dimer mutant Smd2 could not replicate by itself (lane 1), but it was able to replicate when cotransfected with the wild-type small HDAG (lane 2). In contrast, pSGGL failed to promote RNA replication of Smd2 (lane 3). HDAG mutant pSGVL, which retained approximately half of the dimerization activity, partially *trans* activated Smd2 RNA replication to an extent corresponding to its relative degree of dimerization activity (Fig. 7, lane 4). Thus, we conclude that oligomerization of the small HDAG is

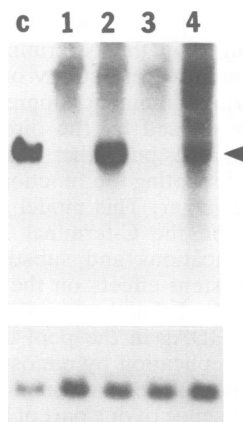


FIG. 7. Northern blot analysis of the *trans*-activation activities of the small HDAG leucine zipper mutants. COS 7 cells were cotransfected with equal amounts of the HDV replication-defective dimer Smd2 and plasmids expressing the wild-type small HDAG (lane 2) or the small HDAG leucine zipper mutants pSGGL (lane 3) or pSGVL (lane 4). As a control, Smd2 was cotransfected with salmon sperm DNA (lane 1). RNAs were extracted 4 days posttransfection, electrophoresed on 1.2% formaldehyde gels, transferred to Hybond-C membranes (Amersham), and incubated with  $^{32}\text{P}$ -labeled HDV antigenomic-sense RNA. The HDV monomer RNA is indicated by an arrowhead. The lower panel is an internal control for RNA amounts, using a  $^{32}\text{P}$ -labeled rRNA probe. Lane C, monomer HDV RNA from a stable HDV transformant cell line.

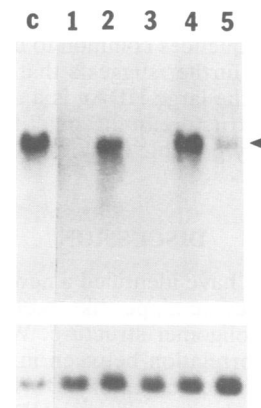


FIG. 8. Northern blot analysis of the *trans*-dominant suppression activities of the large HDAG leucine zipper mutants. COS 7 cells were cotransfected with equal amounts of the replication-competent HDV dimer PYJ-1 and plasmids expressing wild-type large HDAG (lane 1), with large HDAG leucine zipper mutants pLGGL (lane 2) or pLGVL (lane 3), or with plasmids encoding only the N-terminal one-third of HDAG (lane 5). As a control, PYJ-1 was cotransfected with salmon sperm DNA (lane 4). RNAs were extracted 4 days posttransfection, electrophoresed on 1.2% formaldehyde gels, transferred to Hybond-C membranes (Amersham), and incubated with  $^{32}\text{P}$ -labeled HDV antigenomic-sense RNA. HDV monomer RNA is indicated by an arrowhead. The lower panel is an internal control of rRNA probed with a  $^{32}\text{P}$ -labeled rRNA probe. Lane C, monomer HDV RNA from a stable HDV transformant cell line.

a prerequisite for its activity in up-regulating HDV RNA replication.

**Leucine zipper mutations impair the *trans*-dominant suppressor function of large HDAG.** To test the possibility that the leucine zipper-like sequence is also required for the suppression of HDV replication by large HDAG, we introduced similar site-specific mutations to the leucine zipper region of large HDAG. Two mutants, pLGGL and pLGVL, with reduced dimerization activities were constructed and assayed for their *trans*-dominant suppression function on HDV RNA replication. Figure 8 shows that when a replication-competent HDV cDNA dimer construct, PYJ-1, was cotransfected with wild-type large HDAG, the replication of HDV RNA was completely inhibited (Fig. 8, lane 1). In contrast, when the pLGGL mutant, which could not dimerize, was cotransfected with PYJ-1, the level of HDV RNA replication was significantly restored (lane 2), almost to the same level as in the absence of large HDAG (lane 4). The other leucine zipper mutant pLGVL, which retained partial dimerization activity, strongly inhibited HDV RNA replication (lane 3). Analysis of the RNA expression levels of the transfected plasmids in the cells showed that they had similar transfection efficiencies (data not shown). Thus, the suppressor activities of the various large HDAG constructs paralleled their dimerization capacities in vitro. We conclude that dimerization of HDAG is crucial for the *trans*-dominant suppressor activity of large HDAG. These results suggested that the large HDAG may interact with an oligomer structure of the small HDAG and thus alter its conformation, resulting in loss of the function of small HDAG. To corroborate this interpretation, we cotransfected the replication-competent HDV dimer and a plasmid expressing only the N-terminal one-third of HDAG. Northern blot analysis showed that this portion of HDAG was sufficient to suppress HDV RNA

replication (Fig. 8, lane 5). Since this truncated protein represented only sequences common to both small and large HDAGs, this result further suggests that inhibition of HDV RNA synthesis by the large HDAG is a result of the disruption of the regular structure of the small HDAG oligomer but is not caused by any possible inhibitory sequence unique to large HDAG.

### DISCUSSION

In this report, we have identified a new functional domain of HDAG, i.e., a leucine zipper-like sequence which allows HDAG to form an oligomer structure. We have also established a positive correlation between *in vitro* HDAG oligomerization and *trans* activation of HDV RNA replication by the small HDAG and between *in vitro* HDAG oligomerization and inhibition of HDV RNA replication by the large HDAG. Thus, HDAG appears to function as an oligomer form. Although the leucine zipper-like sequence in HDAG is relatively short, our data suggested strongly that this domain is responsible for the oligomer formation of HDAG. Mutations of at least two leucine residues in this stretch of sequence are required to inhibit completely HDAG oligomerization. Furthermore, the large and small HDAGs have the same binding affinities, and the heterodimers and homodimers of these proteins are formed at equal efficiencies. However, heterodimer formation *in vitro* required cotranslation of the large and small HDAG mRNAs prior to glutaraldehyde cross-linking; mixing of the HDAGs translated separately did not lead to heterodimer formation. The most likely explanation is that the HDAG complexes have a high affinity and once formed will not readily exchange with other proteins. This is consistent with our previous finding that HDAG complexes can be formed in the cytoplasm (32), suggesting that oligomerization occurs rapidly after translation. It should be noted that the leucine zipper-like sequence in the N-terminal one-third is not tightly conserved in different HDV isolates (7, 18, 30). Some leucine residues in the key leucine zipper positions are diverged in some isolates. However, most of these mutations are conservative, resulting in substitutions with amino acids of similar properties. Furthermore, as evidenced from the site-specific mutants reported here, a mutation on a single leucine residue is not sufficient to eliminate the dimerization activity of HDAG. Thus, all of the HDAGs in different HDV isolates are expected to retain the dimerization activity.

Deletions and site-specific mutations of HDAG showed that the N-terminal portion of HDAG has a major role in HDAG oligomerization. However, the middle portion plus the C-terminal portion of HDAG retained some residual oligomerization activity. This result suggests that regions other than the N-terminal leucine zipper sequence may contribute to a small extent to the dimerization of HDAG. Computer analysis showed that the middle portion of HDAG has a helix-loop-helix motif (data not shown), a structural motif that has been shown to mediate dimerization (25). This motif may play a more significant role in HDAG dimerization in some HDV isolates.

Glutaraldehyde cross-linking studies revealed the formation of larger protein complexes, including trimers and tetramers, from both full-length and deletion mutants of HDAG which contain the N-terminal leucine zipper-like sequence. It is possible that even larger protein complexes also exist. The failure to detect these larger HDAG complexes by *in vitro* cross-linking studies could be due to the limitation of glutaraldehyde, which cross-links only residues

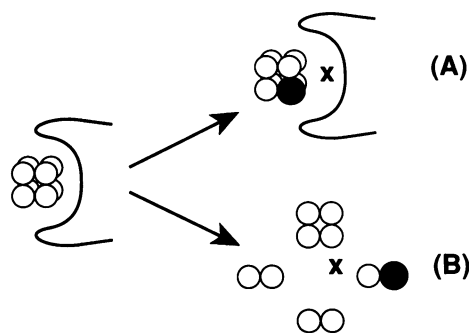


FIG. 9. Black sheep model for the mechanism of *trans*-dominant suppressor activity of large HDAG. Small HDAG (open circles) forms an oligomer which interacts with cellular factors. The presence of a large HDAG (black sheep) (solid circle) in this complex disrupts the correct conformation of this complex and prevents its interaction with the factor (A) or prevents the oligomerization of small HDAG (B). The number of HDAG molecules in each complex is speculative.

within a certain distance; alternatively, the larger protein aggregates may be assembled only in association with HDV RNA.

Our results also demonstrated that the leucine zipper-like sequence on the small HDAG is indispensable for its activity in HDV RNA replication and the corresponding sequence on the large HDAG is required for its *trans*-dominant suppressor activity. These findings strongly suggest that the oligomerization of the small HDAG may be a prerequisite for its function. The small HDAGs may interact with one another, forming a structurally symmetrical complex which acts as a positive regulator. If this structure is disrupted, for example, by the large HDAG entering the complex through the leucine zipper sequence the symmetrical conformation will be altered and, as a result, the complex will become inactive. This interpretation is supported by the finding that a portion of HDAG containing only the N-terminal one-third of the protein also has a suppressor activity on HDV RNA replication. Thus, the *trans*-dominant suppressor activity of the large HDAG is not caused by the direct function of the C-terminal 19 amino acids but rather is due to its interaction with small HDAG, disrupting the functional conformation of the small HDAG oligomer. This model explains the earlier mutagenesis study on the C-terminal 19 amino acids, in which different truncations and substitutions resulted in variable and inconsistent effects on the *trans*-dominant inhibitory activities (11). It has been estimated that the presence of 12% large HDAG in the pool of small HDAG can inhibit HDV RNA replication by almost 90% (5). If all the large HDAGs form complexes with small HDAGs, this result suggests that the presence of one part of large HDAG in eight parts of small HDAG is sufficient to disrupt the function of the small HDAG. Together these results suggest a black sheep model (Fig. 9), in which a bad component of a multi-subunit complex can disrupt the function of the whole complex by either altering the conformation of the functional complex (Fig. 9A) or by preventing the formation of such a functional complex (Fig. 9B). This mechanism of negative regulation of a multi-subunit complex is common among prokaryotic and eukaryotic regulatory proteins (14). An alternative mechanism is that host factors may interact with small HDAG through compatible leucine zipper-like sequences to promote HDV RNA replication. The large HDAG

may compete with cellular factors and therefore block the functional interaction between small HDAg and host factors. Additional studies are needed to rule out this possibility. It has recently been shown that the N-terminal one-third of HDAg is required for packaging of the small HDAg and for the *trans*-dominant inhibitory activity of the large HDAg (8). This result is consistent with the idea presented here that interaction between the two forms of HDAg is needed for their respective activities.

The mechanism by which small HDAg promotes HDV RNA replication remains unclear. There is no evidence that HDAg contains an RNA polymerase activity; thus, HDV RNA replication is most likely carried out by cellular RNA polymerase II, as suggested from the sensitivity of HDV RNA synthesis to  $\alpha$ -amanitin (23a). In the absence of HDAg, there was a basal level of HDV RNA replication, since the transfected genomic-sense HDV cDNA, which does not encode HDAg, could replicate, giving rise to antigenomic HDV RNA (17) and HDV RNA synthesis could proceed in cell extracts without HDAg (23a). However, HDAg greatly increased the efficiency of this process. Therefore, the hypothetical host factors could be RNA polymerase and transcription factors, which may interact with HDAg. Additional studies are required to resolve this issue.

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