JOURNAL OF VIROLOGY, July 1996, p. 4205–4209 Vol. 70, No. 7
0022-538X/96/$04.00+0

Hepatitis Delta Antigens Enhance the Ribozyme Activities of Hepatitis Delta Virus RNA In Vivo

KING-SONG JENG, PEI-YUN SU, AND MICHAEL M. C. LAI*

Howard Hughes Medical Institute and Department of Molecular Microbiology and Immunology, University of Southern California School of Medicine, Los Angeles, California 90033-1054

Received 22 January 1996/Accepted 28 March 1996

The mechanism of regulation for the ribozyme activity of hepatitis delta virus (HDV) RNA in infected cells is unknown. Previously, we developed a direct assay capable of detecting the ribozyme activity of HDV dimer or trimer RNAs in vivo (K.-S. Jeng, A. Daniel, and M. M. C. Lai, J. Virol. 70:2403–2410, 1996). In this study, we used this method to examine the effects of hepatitis delta antigen (HDAg) on the ribozyme activities of HDV RNA in vivo. The HDV multimer cDNAs were cotransfected with plasmids encoding either HDV small delta antigen (SHDAg) or large delta antigen (LHDAg), and the self-cleavage of the primary transcripts from the HDV cDNA was analyzed at day 2 posttransfection. The results were as follows. (i) Both HDAgs, particularly LHDAg, enhanced the self-cleavage activity of HDV RNA; however, HDAgs are not required for HDV RNA cleavage. (ii) HDAg could not restore the ribozyme activity of mutant HDV RNAs which have lost the ribozyme function. (iii) The enhancement of ribozyme activity by HDAg does not require HDV RNA replication. (iv) RNA-binding activity of HDAg is required for the enhancement of RNA cleavage. (v) The self-ligation activities of HDV ribozyme also were enhanced by HDAg. These results suggest that HDAg can regulate the cleavage and ligation of HDV RNA during the HDV life cycle.

Hepatitis delta virus (HDV) is a satellite virus that propagates only in the presence of hepatitis B virus (34) and is the causative agent of acute and chronic liver diseases, often leading to fulminant hepatitis (14). HDV contains a single-stranded circular RNA genome of 1.7 kb, which is predicted to fold into an unbranched, rod-like structure because of the high degree of intramolecular base pairing (17, 30, 40). The HDV genome is complexed with the only HDV-encoded protein, hepatitis delta antigen (HDAg), forming a ribonucleoprotein core (6, 25, 35). The ribonucleoprotein core is surrounded by an outer envelope of hepatitis B surface antigen, which is provided by its helper, hepatitis B virus (3, 34). HDAg is encoded by the antigenomic-sense HDV RNA and usually exists as two related protein species, a 27-kDa large form (LHDAg) and a 24-kDa small form (SHDAg) (1, 3, 41). The two forms are identical in sequence (195 amino acids), but LHDAg contains 19 additional amino acids at the carboxyl terminus; this difference arises from an RNA editing event during HDV replication, in which an amber termination codon is replaced, thereby extending the length of the HDAg-coding open reading frame (26). The two proteins have many similar biochemical characteristics and yet have very different biological roles in the viral life cycle (21): SHDAg is required for HDV RNA replication in a *trans*-acting manner (19), while LHDAg inhibits HDV RNA replication (7) but is required for virus assembly (5).

HDV RNA replication is thought to be carried out by cellular DNA-dependent RNA polymerase II (12, 28) through a double-rolling-circle mechanism (4). It involves the autocatalytic self-cleavage and self-ligation activities, which are parts of the intrinsic properties of the HDV genomic- and antigenomic-sense RNAs (20, 33, 36, 37, 43). Presumably, these ribozyme activities are involved in the cleavage of the multiple-unit-length RNA replication intermediates into monomeric RNA and subsequent ligation of this monomeric RNA into circular RNA. Indeed, mutations at the autocatalytic cleavage sites of HDV RNA affected ribozyme activity and inhibited HDV RNA replication correspondingly (29). More recently, we have further generated ribozyme structural mutants and demonstrated that all of the mutants that lost ribozyme activity in vivo also lost replication ability, indicating that ribozyme activity is required for HDV RNA replication (16).

HDV ribozyme activity requires a secondary structure with several stem-loops, including a pseudoknot structure (33). This structural requirement has been demonstrated in vitro and in vivo (16, 33, 43). However, this structure is not expected to exist in the native structure of the full-length HDV RNA, which has been demonstrated to form an unbranched, rod-like structure (17). Thus, the ribozyme-active structure probably exists only transiently and may alternate between different RNA conformations. Indeed, the cleavage activity of HDV ribozymes can be enhanced in a stepwise manner by repeated denaturation and renaturation, suggesting that the RNA exists in alternative RNA conformations (42). How the different RNA conformations are regulated during HDV replication is not clear. Conceivably, the formation of the ribozyme-active conformation of the full-length or multiple-length HDV RNA in the cells may be facilitated and regulated by cellular or viral factors. Circumstantial evidence has suggested that cellular factors are involved in the regulation of HDV RNA ribozyme activity (23, 29). However, the identities of these factors have not been determined. In contrast, the viral protein, HDAg, appears not to be required for ribozyme activity in vitro (20, 36, 43) or in vivo (22, 23). However, all of the HDV RNAs used in the previous ribozyme studies were truncated RNA fragments, which may have simpler structures than the full-length HDV RNA and therefore do not require HDAg for their ribozyme activities. The potential effects of HDAg on the ribozyme activity of the full-length or multiple-length HDV RNA have not been studied. Since several other types of ribozymes have been

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, University of Southern California School of Medicine, 2011 Zonal Ave., HMR-401, Los Angeles, CA 90033-1054. Phone: (213) 342-1748. Fax: (213) 342-9555. Electronic mail address: michlai@hsc.usc.edu.

shown to be stimulated by nonspecific RNA-binding proteins (2, 10, 13, 31, 39) and HDAg is an RNA-binding protein (6), it is conceivable that HDAg may modulate HDV ribozyme activity even if it is not required for HDV RNA cleavage. So far, this issue has not been studied in vitro or in vivo.

Recently, we have developed a direct assay that is capable of detecting the ribozyme activity of HDV dimer or trimer RNA in vivo (16). This method enabled us to examine the possible effects of HDAg on the ribozyme activity of HDV RNA. In this report, we have demonstrated that HDAg is not required for HDV RNA cleavage but can enhance ribozyme activity (including both the cleavage and ligation activities) in vivo through its RNA-binding activity. LHDAg is more efficient than SHDAg in enhancing HDV ribozymes. We also found that the enhancement of cleavage by HDAg does not depend on HDV RNA replication. This study thus suggests an additional functional role for HDAg in HDV RNA replication.

MATERIALS AND METHODS

Cell culture and transfection. HuH-7 cells (32) were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 mg of streptomycin per ml, 2 mM L-glutamate, and 1% nonessential amino acids. All transfections were performed by the calcium phosphate coprecipitation method as described previously (16). The transfected cells were harvested, and total RNAs were prepared at day 2 posttransfection.

Plasmids. Most of the plasmids used in this study have been described previously (16, 24). All other constructs are described below. Plasmids pKS/smd2 (sense orientation) and pKS/smd2-ag (antisense orientation) were constructed by digesting plasmid smd2 (24) with *Xba*I and *Bgl*II to isolate the HDV dimer that contains a frameshift mutation in the HDAg-coding region (24); the blunt-ended fragment was then ligated in both orientations into *Sal*I-*Xho*I-digested and blunt-ended pKS/CMV vector (16).

Northern (RNA) blot analysis. Total RNAs were isolated from transfected cells at day 2 posttransfection, using the guanidinium thiocyanate method (9). The RNAs were digested with RQ1 DNase (Promega) to remove the contaminating plasmid DNA, then treated with formaldehyde, and electrophoresed through a formaldehyde-containing 1.2 or 1.5% agarose gel in 3-[*N*-morpholino]-propanesulfonic acid (MOPS) buffer (16). The RNAs were then blotted onto a nitrocellulose membrane (Hybond C Extra; Amersham) and probed with a ^{32}P-labeled antisense HDV RNA or antisense *neo* gene riboprobe or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene DNA probe as described previously (16). The riboprobe for detecting genomic HDV RNA was transcribed with T7 RNA polymerase (Promega) from pBS/HDV1.7(T3G) (16) following linearization with *Eco*RV. The riboprobe for detecting *neo* transcripts was transcribed with T7 RNA polymerase from *Eco*RI-linearized pSP6neo (16). The GAPDH DNA probe was prepared from a *Pst*I-*Xba*I fragment of pHcGAP (38) by using a Random Primed DNA Labeling kit (Boehringer Mannheim). RNA extracted from H1δ9 cells, which expresses and replicates HDV RNA from an integrated cDNA dimer (27), was used as a positive control in all Northern blot analyses.

RESULTS

HDAgs enhance HDV RNA cleavage in vivo. To test whether HDAg can modulate the ribozyme activity of HDV RNA in vivo, we used a recently established experimental protocol which enabled the detection of the self-cleavage of HDV RNA in vivo (16). In this method, a wild-type HDV trimer cDNA under a cytomegalovirus immediate-early promoter that transcribed a trimer HDV RNA, plus some vector sequence, was cotransfected with an SHDAg- or LHDAg-expressing plasmid, and the RNA was examined at 2 days posttransfection. As shown previously, most of the HDV RNA detected at this time point represents the primary transcript and its cleaved product from the transfected cDNA, and very little RNA was replicated (16). As shown in Fig. 1, the primary transcript from this plasmid was processed into trimer, dimer, and monomer RNAs, the majority of the products being either the primary transcript itself or trimers (lane 1). When the HDV cDNA was cotransfected with an SHDAg-expressing (lane 2) or LHDAg-expressing (lane 3) plasmid, the amounts of the monomer and dimer

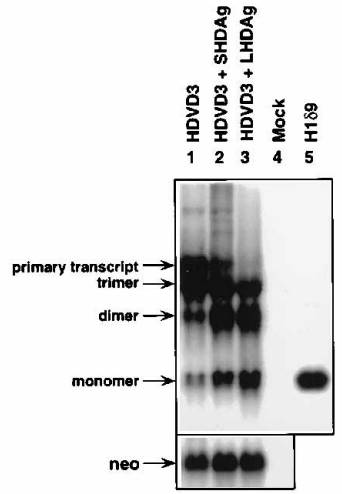

FIG. 1. Effects of HDAg on HDV RNA cleavage in vivo. RNAs were extracted from cells cotransfected with wild-type HDV trimer cDNA and an SHDAg- or LHDAg-expressing plasmid at day 2 posttransfection and separated by electrophoresis on a 1% agarose gel. The RNA blot was probed with a ^{32}P-labeled HDV (top panel) or *neo* (bottom panel) antigenomic-strand riboprobe to detect HDV genomic-strand RNA and *neo* transcripts. The positions of various RNA species are indicated at the left. Lane 4 contained RNA from mock-transfected HuH-7 cells, and lane 5 contained RNA from a stably transformed cell line (H1δ9) producing HDV RNA (27).

RNAs significantly increased. Correspondingly, the amounts of the primary transcript and the trimer HDV RNA, particularly in the presence of a cotransfecting LHDAg, decreased. These results suggest that HDAg enhanced the self-cleavage of HDV RNA in the cells and that LHDAg was more efficient than SHDAg in enhancing HDV RNA cleavage. Since HDV RNA cannot replicate in the presence of LHDAg (7), this result further indicates that the monomer RNAs seen in this experiment represent the cleavage products rather than the replication products of HDV RNA. A control study showed that the expression levels of the *neo* gene were similar in all of the transfected cells, indicating that the transfection efficiencies were similar. We conclude that HDAgs, particularly LHDAg, can enhance the cleavage activity of HDV RNA in vivo.

Effects of HDAg on the cleavage of ribozyme mutants. Previously, we constructed a series of ribozyme structural mutants that have lost the ribozyme activity and some other mutants that have restored the ribozyme activity through compensatory mutations (16). We used these mutants to explore two questions. (i) Can HDAg restore the cleavage activity of the defective ribozyme mutants? (ii) Does the enhancement of HDV RNA cleavage by HDAg depend on HDV RNA replication? Several ribozyme mutants were used. H1(D3), H2(D2), H3-2(D3), and H4(D2) had a mutation in one of the four helices (I, II, III, and IV, respectively) (33) of the HDV ribozyme and were defective in ribozyme activity (D2 and D3 signify a dimer or trimer cDNA construct, and they gave identical results) (16). H1′(D3) and H2′-1(D2) have compensatory mutations (as indicated by the pseudoknot model) in helices I and II, respectively, and have restored ribozyme activity (16). H2′-2(D2) and H2′-3(D2) have compensatory mutations in helix II (as judged from alternative ribozyme structures) but are still defective in ribozyme activity (16). These constructs were cotransfected with either vector (Fig. 2A) or an SHDAg-expressing plasmid (Fig. 2B). As shown in Fig. 2, among all of the ribozyme mutants, only H1′(D3) and H2′-1(D2) can cleave the primary transcripts into monomer HDV RNA (lanes 8 and 9),

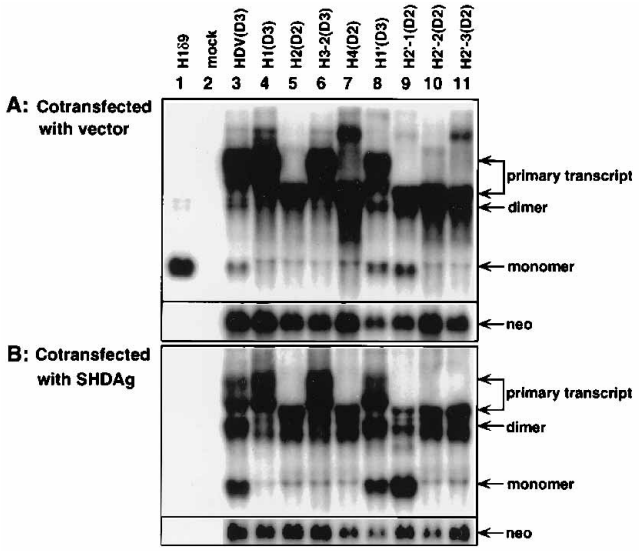

FIG. 2. Effects of SHDAg on the cleavage of ribozyme mutants. HDV dimer and trimer cDNAs carrying various ribozyme mutations (16) (see text) were cotransfected with vector (A) or an SHDAg-expressing plasmid (B). RNA analysis was performed as for Fig. 1. The arrows indicate the positions of various RNA species. HDV(D3) is a wild-type trimer HDV cDNA.

consistent with previous findings (16). A faint RNA band slightly larger than the monomer RNA probably represents the rRNA. In the presence of a cotransfecting HDAg, the amounts of monomer RNA of H1′(D3) and H2′-1(D2) increased, and their primary transcripts correspondingly decreased, indicating that HDAg enhanced the cleavage activity. None of the other ribozyme mutants yielded any monomer RNA in the presence of HDAg, indicating that HDAg could not restore the cleavage activity of the defective ribozymes. Interestingly, the previous study had shown that neither H1′(D3) nor H2′-1(D2) can replicate RNA despite their restored ribozyme activities (16), and yet the current finding shows that their ribozyme activities were enhanced in the presence of HDAg, suggesting that the enhancement of RNA cleavage by HDAg does not depend on HDV RNA replication. Taken together, these data clearly show that the enhancement of RNA cleavage by HDAg does not depend on RNA replication and that HDAg cannot restore the cleavage activity of the defective ribozymes.

HDAgs are not required for the cleavage of the full-length HDV RNA in vivo. Previous studies have suggested that HDAg is not required for the ribozyme activity of the small fragments of HDV RNA in vitro (20, 36, 43) or in vivo (22, 23). However, whether the in vivo cleavage activity of the full-length HDV RNA depends on HDAg is unknown. This is a significant issue since the conformation of the full-length HDV RNA is significantly different from that of the RNA containing only the minimum HDV ribozyme domain, and since all of the HDV RNA mutants used in previous in vivo ribozyme studies (16, 29) have a functional HDAg. Therefore, whether the in vivo cleavage activity of HDV RNA detected in the assays described above depended on the presence of HDAg is not certain. To address this question, a replication-defective HDV dimer cDNA, smd2, which has a frameshift mutation in the HDAg-coding region (24), was cotransfected with and without HDAg. As shown in Fig. 3, in the absence of HDAg, smd2 yielded, in addition to the primary transcript, a small amount of the monomer or dimer HDV RNA at 26 h posttransfection (lane 2), which slightly increased at 50 h posttransfection (lane 7), indicating that HDAg is not required for the cleavage of HDV RNA in vivo. The amounts of the cleaved products (monomer and dimer) noticeably increased when smd2 was cotransfected with HDAg (lanes 3, 4, 8, and 9), particularly at 50 h posttransfection. It is particularly significant that in the presence of the LHDAg, the amounts of the dimer and monomer RNAs were even larger than those in the presence of SHDAg. LHDAg is known to inhibit RNA replication (7); thus, the increase of the monomer and dimer HDV RNAs could have resulted only from the increase of cleavage efficiency. This result also confirmed the previous finding (Fig. 1) that LHDAg is more efficient than SHDAg in enhancing HDV RNA cleavage. We conclude that HDV RNA cleavage occurs at low efficiency in the absence of HDAgs and that HDAgs, particularly LHDAg, indeed enhance the cleavage of HDV RNA in vivo.

RNA-binding activity of HDAg is required for the enhancement of cleavage. We next examined the possible mechanism by which HDAgs enhance the cleavage of HDV RNA. Since several nonspecific RNA-binding proteins have been shown to enhance the activity of various ribozymes (2, 10, 13, 31, 39) and HDAg has an RNA-binding activity (6, 8, 24), we examined whether the RNA-binding activity of HDAg is involved in enhancing the cleavage. The replication-defective HDV dimer cDNA, smd2, was cotransfected with two RNA-binding mutants, A1 and A2, of HDAg (24). The A2 mutant retains partial RNA-binding and *trans*-activating activities, while the A1 mutant has lost both (24). As shown in Fig. 3, the A2 mutant enhanced the cleavage of HDV RNA (lanes 5 and 10), although the extent of enhancement was not as high as that of the wild-type SHDAg or LHDAg (lanes 3, 4, 8, and 9). In contrast, the A1 mutant did not enhance the cleavage of HDV RNA (lanes 6 and 11). These results suggest that the enhancement of HDV RNA cleavage by HDAg requires the RNA-binding activity of HDAg.

HDAgs also enhance the circularization of HDV RNA. The ribozymes of HDV include both self-cleavage and ligation activities. Presumably, the ligation activity is involved in the ligation of the cleaved monomer HDV RNA into a circular RNA. To explore whether HDAg can also modulate the ligation activity of HDV ribozyme, the HDV RNAs from the

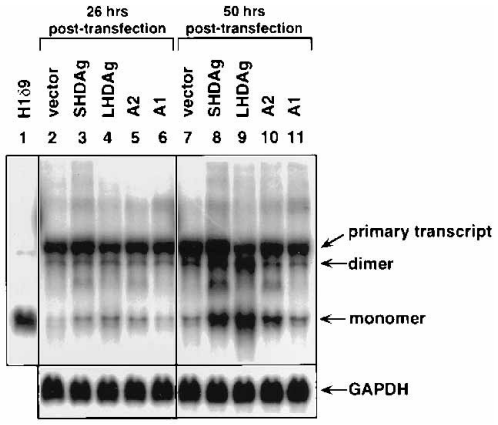

FIG. 3. Effects of HDAg and its RNA-binding mutants on the cleavage of the replication-defective HDV RNA. HuH-7 cells were cotransfected with mutant HDV dimer cDNA smd2, which is defective in RNA replication (24), and various HDAg-expressing plasmids. RNA was extracted at either 26 or 50 h posttransfection and analyzed as for Fig. 1. The control blot (bottom) was probed with a ^{32}P-labeled GAPDH gene. A2 and A1 are SHDAg mutants that have partial or no RNA-binding activities, respectively (24).

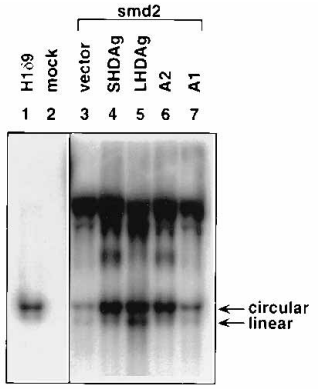

FIG. 4. Effects of HDAg on the ligation activity of HDV RNA ribozyme. The RNA samples were from the experiment shown in Fig. 3. RNAs were treated with formaldehyde and separated by electrophoresis in a 1.5% agarose gel containing 2 M formaldehyde in MOPS buffer. The method of detection was the same as for Fig. 1, using ^{32}P-labeled antigenomic sense HDV RNA as a probe. The circular and linear RNAs are indicated.

transfected cells were separated by electrophoresis in a high-percentage agarose gel under denaturing conditions to separate the circular RNA from linear RNA (16). As shown in Fig. 4, the amounts of the circular monomer RNA in the presence of HDAg were significantly higher than in its absence (lanes 3 to 5). Also, the ratio of circular HDV RNA to linear HDV RNA in the presence of HDAg appears to be slightly higher than in the absence of HDAg. Similarly, the A2 mutant also enhanced the amounts of the circular HDV RNA (lane 6), whereas the A1 mutant failed to enhance the formation of either circular or linear monomer RNA (lane 7). These results indicate that HDAg also enhanced the ligation activity of HDV ribozyme.

DISCUSSION

The results presented in this report first showed that HDV RNA ribozyme activity was stimulated by the presence of HDAgs, particularly LHDAg. This study was made possible by the development of an in vivo ribozyme assay system (16) which allows the direct detection, without the requirement of RNA replication, of the primary transcript from a multimer HDV cDNA construct and its cleavage product. The results demonstrated that in the presence of HDAg, the amounts of monomer HDV RNA increased significantly, while the amounts of the primary transcript decreased correspondingly. Since this result was seen in the presence of the LHDAg, which should inhibit HDV RNA replication (7), and also with mutant HDV RNAs that cannot replicate, the increase of the monomer HDV RNA most likely was the result of the enhanced RNA cleavage. This result also is in agreement with a previous study in which the presence of HDAg was shown to increase the amount of the HDV RNA when a relatively large, but not the small, HDV subgenomic cDNA fragment was used for transfection (22). However, that finding was interpreted to indicate that HDAg can stabilize HDV RNA (22). Since in that study the primary transcript was not observed, it was not possible to distinguish between the possibilities that HDAg enhanced RNA cleavage or stabilized RNA. The finding presented here, that the accumulation of the monomer HDV RNA was accompanied by the decrease of the primary transcript, would be most consistent with the enhancement of RNA cleavage, rather than the stabilization of RNA, by HDAg. Although we cannot completely rule out the possibility that HDAg also selectively stabilizes some HDV RNA species, the most logical interpretation of the data presented here and in previous studies is that HDAg enhances the HDV ribozyme activity. This newly identified functional role of HDAg adds to the growing list of demonstrated functions for HDAg (21).

The mechanism of the ribozyme enhancement by HDAg is not known. The data presented here showed that this activity correlated with the RNA-binding activity of HDAg. HDAg has been shown to bind to several regions of HDV RNA, probably recognizing a secondary structure unique to HDV RNA rather than specific HDV nucleotide sequences (8, 18, 25). Conceivably, the binding of HDAg would alter the conformation of HDV RNA, facilitating the formation of the ribozyme-active structure. The protein binding to HDV RNA may be crucial for an active HDV ribozyme in vivo, since the full-length HDV RNA tends to form a rod-like structure (17), which will prevent the formation of the ribozyme-active structure. The requirement of the RNA-binding activity of HDAg for the ribozyme enhancement activity is reminiscent of the previous demonstration that several catalytic RNAs can be modulated by nonspecific cellular or viral RNA-binding proteins. For examples, the group I intron ribozyme can be enhanced by the *Neurospora* CTY-18 protein (31), and the human immunodeficiency virus p7 nucleocapsid protein can increase the efficiency of cleavage by a hammerhead ribozyme (39). Other proteins, such as heterogeneous nuclear ribonucleoprotein A1 and *Escherichia coli* ribosomal protein S12, also have been shown to enhance the efficiency of cleavage by a hammerhead ribozyme (2, 10, 13). However, these modulating factors are heterologous to the assay systems and thus not relevant to the in vivo regulation of these ribozymes. Our finding here represents one of the first biologically relevant proteins shown to regulate a ribozyme in vivo.

Our finding also shows that HDAg is not an absolute requirement for HDV RNA cleavage in vivo. Even in the absence of HDAg, a low level of HDV RNA cleavage could be detected (Fig. 3). It is conceivable that certain cellular RNA-binding proteins may specifically or nonspecifically bind to HDV RNA and induce a basal level of HDV ribozyme activity in the absence of HDAg. Recently, we have indeed detected a cellular protein capable of binding to the sequence around HDV ribozyme in vitro (11). Nevertheless, in the absence of HDAg, the extent of HDV RNA cleavage in vivo was very low (Fig. 3). Therefore, HDAg likely plays a significant role in facilitating HDV RNA cleavage during HDV replication.

It is not clear why LHDAg is much more efficient than SHDAg in this ribozyme enhancement function. LHDAg and SHDAg have very similar RNA-binding activities (15). It is possible that LHDAg recruits certain cellular factors, such as helicase-like proteins to unwind the rod-like structure, to facilitate the formation of the ribozyme-active secondary structure. Preliminary data showed that this ribozyme enhancement activity of HDAg was present in several different cell lines studied; however, there was some difference in the efficiency of this activity among the different cell types, suggesting the possible contributions of cellular factors to this activity (data not shown). This conclusion is also consistent with the previous finding from the cleavage-site mutants of HDV RNA that the ribozyme activity may vary with different host cells (29). Since LHDAg is more abundant later in the viral life cycle (26) and promotes viral assembly (5), the ribozyme-enhancing activity of LHDAg may serve to produce monomer HDV RNA in preparation for viral assembly. This interpretation is in agreement with our finding here that LHDAg promotes the formation of circular RNA as well. Thus, HDAg participates in an ever-enlarging list of activities in the HDV replication cycle.

ACKNOWLEDGMENTS

We thank Daphne Shimoda for editorial assistance.

K.-S.J. and P.-Y.S. are research associates and M.M.C.L. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Bergmann, K. F., and J. L. Gerin.** 1986. Antigens of hepatitis delta virus in the liver and serum of humans and animals. J. Infect. Dis. **154:**702–706.
2. **Bertrand, E. L., and J. J. Rossi.** 1994. Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonuclearprotein A1. EMBO J. **13:**2904–2912.
3. **Bonino, F., K. H. Heermann, M. Rizzetto, and W. H. Gerlich.** 1986. Hepatitis delta virus: protein composition of delta antigen and its hepatitis B virus-derived envelope. J. Virol. **58:**945–950.
4. **Branch, A. D., and H. D. Robertson.** 1984. A replication cycle for viroids and other small infectious RNA's. Science **223:**450–455.
5. **Chang, F.-L., P.-J. Chen, S.-J. Tu, C.-J. Wang, and D.-S. Chen.** 1991. The large form of hepatitis delta antigen is crucial for the assembly of hepatitis delta virus. Proc. Natl. Acad. Sci. USA **88:**8490–8494.
6. **Chang, M.-F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. C. Lai.** 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. J. Virol. **62:**2403–2410.
7. **Chao, M., S.-Y. Hsieh, and J. Taylor.** 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. J. Virol. **64:**5066–5069.
8. **Chao, M., S.-Y. Hsieh, and J. Taylor.** 1991. The antigen of hepatitis delta virus: examination of in vitro RNA-binding specificity. J. Virol. **65:**4057–4062.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Coetzee, T., D. Herschlag, and M. Belfort.** 1994. Escherichia coli proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones. Genes Dev. **8:**1575–1588.
11. **Daniel, A., and M. M. C. Lai.** Unpublished data.
12. **Fu, T.-B., and J. Taylor.** 1993. The RNAs of hepatitis delta virus are copied by RNA polymerase II in nuclear homogenates. J. Virol. **67:**6965–6972.
13. **Herschlag, D., M. Khosla, Z. Tsuchihashi, and R. L. Karpel.** 1994. An RNA chaperone activity of non-specific RNA binding proteins in hammerhead ribozyme catalysis. EMBO J. **13:**2913–2924.
14. **Hoofnagle, J. H.** 1989. Type D (delta) hepatitis. JAMA **261:**1321–1325.
15. **Hwang, S. B., C.-Z. Lee, and M. M. C. Lai.** 1992. Hepatitis delta antigen expressed by recombinant baculoviruses: comparison of biochemical properties and post-translational modifications between the large and small forms. Virology **190:**413–422.
16. **Jeng, K.-S., A. Daniel, and M. M. C. Lai.** 1996. A pseudoknot structure is active in vivo and required for hepatitis delta virus RNA replication. J. Virol. **70:**2403–2410.
17. **Kos, A., R. Dijkema, A. C. Arnberg, P. H. van der Merde, and H. Schellekens.** 1986. The HDV possesses a circular RNA. Nature (London) **323:**558–560.
18. **Kumar, P. K. R., S. B. Hwang, M. M. C. Lai, and A. D. Ellington.** Unpublished data.
19. **Kuo, M. Y.-P., M. Chao, and J. Taylor.** 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. J. Virol. **63:**1945–1950.
20. **Kuo, M. Y.-P., L. Sharmeen, G. Dinter-Gottieb, and J. Taylor.** 1988. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. J. Virol. **62:**4439–4444.
21. **Lai, M. M. C.** 1995. The molecular biology of hepatitis delta virus. Annu. Rev. Biochem. **64:**259–286.
22. **Lazinski, D. W., and J. M. Taylor.** 1994. Expression of hepatitis delta virus RNA deletions: *cis* and *trans* requirements for self-cleavage, ligation, and RNA packaging. J. Virol. **68:**2879–2888.
23. **Lazinski, D. W., and J. M. Taylor.** 1995. Intracellular cleavage and ligation of hepatitis delta virus genomic RNA: regulation of ribozyme activity by *cis*-acting sequences and host factors. J. Virol. **69:**1190–1200.
24. **Lee, C.-Z., J.-H. Lin, M. Chao, K. McKnight, and M. M. C. Lai.** 1993. RNA-binding activity of hepatitis delta antigen involves two arginine-rich motifs and is required for hepatitis delta virus RNA replication. J. Virol. **67:**2221–2227.
25. **Lin, J.-H., M.-F. Chang, S. C. Baker, S. Govindarajan, and M. M. C. Lai.** 1990. Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. J. Virol. **64:**4051–4058.
26. **Luo, G., M. Chao, S.-Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor.** 1993. A specific base transition occurs on replicating hepatitis delta virus RNA. J. Virol. **64:**1021–1027.
27. **Macnaughton, T. B., E. J. Gowans, A. R. Jilbert, and C. J. Burrell.** 1990. Hepatitis delta virus RNA, protein synthesis and associated cytotoxicity in a stably transfected cell line. Virology **177:**692–698.
28. **Macnaughton, T. B., E. J. Gowans, S. P. McNamara, and C. J. Burrell.** 1991. Hepatitis delta antigen is necessary for access of hepatitis delta virus RNA to the cell transcriptional machinery but is not part of the transcriptional complex. Virology **184:**387–390.
29. **Macnaughton, T. B., Y.-J. Wang, and M. M. C. Lai.** 1993. Replication of hepatitis delta virus RNA: effect of mutations of the autocatalytic cleavage sites. J. Virol. **67:**2228–2234.
30. **Makino, S., M.-F. Chang, C.-K. Shieh, T. Kamahora, D. M. Vannier, S. Govindarajan, and M. M. C. Lai.** 1987. Molecular cloning and sequencing of a human hepatitis delta virus RNA. Nature (London) **329:**343–346.
31. **Mohr, G., A. Zhang, J. A. Gianelos, M. Belfort, and A. M. Lambowitz.** 1992. The neurospora CYT-18 protein suppresses defects in the phage T4 td intron stabilizing the catalytically active structure of the intron core. Cell **69:**483–494.
32. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated function in chemically defined medium. Cancer Res. **42:**3858–3863.
33. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) **350:**434–436.
34. **Rizzetto, M., B. Hoyer, M. G. Canese, J. W.-K. Shih, R. H. Purcell, and J. L. Gerin.** 1980. δ agent: association of δ antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees. Proc. Natl. Acad. Sci. USA **77:**6124–6128.
35. **Ryu, W.-S., H. J. Netter, M. Bayer, and J. Taylor.** 1993. Ribonucleoprotein complexes of hepatitis delta virus. J. Virol. **67:**3281–3287.
36. **Sharmeen, L., M. Y.-P. Kuo, G. Dinter-Gottieb, and J. Taylor.** 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. J. Virol. **62:**2674–2679.
37. **Sharmeen, L., M. Y.-P. Kuo, and J. Taylor.** 1989. Self-ligating RNA sequences on the antigenome of human hepatitis delta virus. J. Virol. **63:**1428–1430.
38. **Tso, J. Y., X.-H. Sun, T.-H. Kao, K. S. Reece, and R. Wu.** 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and evolution of the gene. Nucleic Acids Res. **13:**2485–2502.
39. **Tsuchihashi, Z., M. Khosla, and D. Herschlag.** 1993. Protein enhancement of hammerhead ribozyme catalysis. Science **262:**99–102.
40. **Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton.** 1986. Structure, sequence and expression of the hepatitis delta viral genome. Nature (London) **323:**508–514.
41. **Weiner, A. J., Q.-L. Choo, K.-S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton.** 1988. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides $p24^{\delta}$ and $p27^{\delta}$. J. Virol. **62:**594–599.
42. **Wu, H. N., and M. M. C. Lai.** 1990. RNA conformational requirements of self-cleavage of hepatitis delta virus RNA. Mol. Cell. Biol. **10:**5575–5579.
43. **Wu, H. N., Y.-J. Lin, F.-P. Lin, S. Makino, M.-F. Chang, and M. M. C. Lai.** 1989. Human hepatitis δ virus RNA subfragments contain an autocleavage activity. Proc. Natl. Acad. Sci. USA **86:**1831–1835.
44. **Wu, H. N., Y.-J. Wang, C.-F. Hung, H.-J. Lee, and M. M. C. Lai.** 1992. Sequence and structure of the catalytic RNA of hepatitis delta virus genomic RNA. J. Mol. Biol. **223:**233–245.