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# Production of Hepatitis Delta Virus and Suppression of Helper Hepatitis B Virus in a Human Hepatoma Cell Line

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**The hepatitis delta virus (HDV) is a defective virus with a coat composing of the surface antigen of its helper virus, hepatitis B virus (HBV). Replication of HDV in the absence of HBV has been shown in cell cultures by transient transfection of the HDV plasmid. However, the formation and release of HDV virions have not been observed. In this report, a human hepatoma cell line HuH-7 was transiently cotransfected with HDV and HBV plasmids. The production of monomeric and multimeric antigenomic RNAs of HDV in the transfected cells indicated replication of the HDV genome. The major 3.5- and 2.1-kb RNAs of HBV were also expressed. Virions of both HDV and HBV were released from the cotransfected cells, as shown by the detection of monomeric genomic HDV RNA and partially double-stranded HBV DNA in the culture medium. Thus, this is the first report that describes the assembly and the release of HDV viral particles in an in vitro cell culture. The HDV virions released possessed physicochemical properties identical to those of the HDV virions found in infected human serum. Furthermore, expression of both the 3.5- and 2.1-kb RNAs of HBV was shown to be dramatically decreased by the presence of HDV, indicating suppression of the expression of HBV genes by HDV. The amount of HBV virions released was similarly suppressed by HDV. Cotransfection of HBV with an expression plasmid of the HDV delta antigen remarkably reduced the levels of the 3.5- and 2.1-kb HBV RNAs, indicating that suppression of the expression of HBV RNAs by HDV occurs via the action of the delta antigen. This HBV- and HDV-cotransfected human hepatoma cell line should provide an excellent system for the study of the function of the delta antigen and the interaction between HDV and its helper, HBV.**

The hepatitis delta virus (HDV) is a 36-nm particle with an envelope composed of surface antigen of the hepatitis B virus (HBV). Within the envelope are the HDV-specific delta antigen and a single-stranded RNA genome 1.7 kb in size (20, 23, 28, 33). The 1.7-kb HDV RNA, which is in a closed circular conformation, folds into an unbranched rod structure (20, 33). This genomic 1.7-kb RNA is also present in infected liver cells along with the antigenomic RNA (8). The replication of the HDV RNA genome is considered to proceed via an RNA-directed RNA synthesis mechanism, probably via a rolling-circle model (5). At least five potential open reading frames are present in the genomic and antigenomic RNA strands. However, delta antigen is the only known HDV protein and is encoded by the largest open reading frame present in the antigenomic strand (23, 33). Multiple forms of delta antigen ranging from 20 to 30 kDa have been reported (3, 4, 34). Delta antigen has recently been shown to be phosphorylated at serine residues and to have RNA-binding activity (7). Furthermore, delta antigen is essential for the replication of the HDV genome (19).

HDV is a defective virus, the coat of which is provided by HBV (4, 26). Therefore, HBV seems to be functionally important for both HDV entry and release from host cells. Kuo et al. (19) have recently demonstrated the replication of the HDV genome in the absence of HBV in the human hepatoma HuH-7 and the monkey kidney COS7 cell lines on transfection with a plasmid containing a trimeric HDV genome. Although these results appear to indicate that replication of HDV genome does not require HBV, the influence of HBV on the HDV replication is not clear. Furthermore, the assembly and the release of HDV virions did not occur in that system, which is consistent with the proposed role of HBV.

Clinically, coinfection of healthy persons by HDV and HBV or superinfection of chronic HBV carriers by HDV results in fulminant hepatitis, severe chronic hepatitis, and liver cirrhosis (9, 12, 15, 29). Interestingly, suppression of HBV replication is frequently observed in HBV carriers superinfected by HDV (2, 16, 27, 35, 36). However, these observations do not necessarily indicate the direct interactions between HDV and HBV due to the possible involvement of many host confounding factors. Therefore, we are interested in establishing an in vitro cell culture system in which both HBV and HDV are able to replicate to study the interaction between these two viruses.

By transient transfection of the human hepatoma cell line HuH-7 with a plasmid, pA3HBV3.8, containing the 3.8-kb HBV DNA sequence, which is able to produce the pregenomic HBV RNA, we have previously demonstrated the replication of HBV genome and the release of HBV virions. In this study, plasmid pSVL-D3, containing the trimeric HDV cDNA, was cotransfected with plasmid pA3HBV3.8 into HuH-7 cells. We demonstrated that both HDV and HBV would complete their replication cycle and that viral particles of both viruses were released into the medium. We further demonstrated that HDV suppressed the expression of HBV RNAs through the action of its delta antigen.

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## MATERIALS AND METHODS

**Plasmids.** Plasmid pSVL-D3 contains a trimer of the head-to-tail HDV genome driven by the late promoter of simian virus 40 (SV40) to direct the synthesis of the genomic HDV RNA (19, 30). The plasmid pSVL-Ag is an SV40 late promoter-based plasmid that contains the 1.1-kb *Xba*I (nucleotide [nt] 781)-*Bgl*II (nt 224) fragment of HDV sequence spanning the open reading frame of the delta antigen (nt 1598 to 954). The SV40 late promoter transcribes a 1.8-kb RNA containing the 1.1-kb HDV RNA and 0.7-kb SV40 sequences for the synthesis of delta antigen (19). Plasmid pA3HBV3.8 contains an HBV DNA sequence larger than the unit length of the HBV genome spanning from the *Bam*HI site (nt 1403, subtype *adw*, *Eco*RI site at nt 1) to the *Bgl*II site (nt 1990). A trimer of polyadenylated sequence (designated A3) of SV40 was inserted upstream from the HBV sequence to prevent readthrough transcription in pA3HBV3.8. Plasmid pG4B-D3 (19) contains a trimer of the head-to-tail HDV genome driven by the SP6 or the T7 phage promoter to produce strand-specific probes by in vitro transcription. Plasmid pSV2CAT (6) was used as an internal control for transfection efficiency.

**Transfections.** A human hepatoma HuH-7 (24) cell line was used for DNA transfection. HuH-7 cells ($5 \times 10^6$) were seeded in each 15-cm petri dish and cultured in the Dulbecco modified Eagle medium (Flow Laboratories, Australia) supplemented with 10% fetal calf serum (Boehringer Biochemical, Mannheim, Germany) for 24 h and then transfected with a mixture of plasmids as follows: 20 μg of pA3HBV3.8, 20 μg of pUC19, and 10 μg of pSV2CAT; 20 μg of pA3HBV3.8, 20 μg of pSVL-D3, and 10 μg of pSV2CAT; 20 μg of pA3HBV3.8, 20 μg of pSVL-Ag, and 10 μg of pSV2CAT; or 20 μg of pSVL-D3, 20 μg of pUC19, and 10 μg of pSV2CAT. The transfection was performed by the calcium phosphate-DNA coprecipitation method (6, 14). The medium was changed every 3 days. On days 3, 6, and 9 after transfection, the medium was collected and cellular RNA was extracted.

**Northern blot hybridization of cellular HBV and HDV RNAs.** Cellular RNA was extracted by guanidinium isothiocyanate-cesium chloride centrifugation as previously described (11). Fifteen micrograms of total RNA for each sample was used in Northern blot hybridization as previously described (25, 35). The strand-specific HDV RNA probes for the detection of genomic and antigenomic HDV RNAs were prepared by in vitro transcription of pG4B-D3 (19) with a SP6/T7 transcription kit (Boehringer). The HBV DNA probe was prepared from pTWL1 (21) by using a Multiprime DNA labeling system (Amersham International Plc., United Kingdom). The hybridization signals of HDV RNAs, HBV RNAs, and HBV DNA were quantitated by scanning densitometry (Zeineh Soft Laser Scanning Densitometer, model SL-2D UV; Biomedical Instruments Inc., Fullerton, Calif.).

**Northern and Southern blot hybridizations of HDV RNA and HBV DNA in virus particles isolated from the culture medium.** A sample (1 ml) of culture medium collected from transfected cells was ultracentrifuged over a 20% sucrose cushion (20% sucrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 0.1% bovine serum albumin) at 40,000 rpm at 4°C for 5 h in a Beckman SW41 rotor. The pellet was then digested overnight with 0.4 ml of lysing solution (0.2 M NaCl, 20 mM sodium EDTA, 2% sodium dodecyl sulfate, 0.5 mg of proteinase K, 50 μg of tRNA, and 50 mM HEPES [pH 7.4]) and then precipitated with ethanol. The pellet was dissolved in deionized water and analyzed by Northern blot hybridization with strand-specific HDV RNA probes and by Southern blot hybridization with the HBV DNA probe.

**Endogenous DNA polymerase assay of HBV particles in culture medium.** The HBV particles in the culture medium were detected by an endogenous DNA polymerase assay that was modified from that reported previously (17). In brief, 10 ml of the culture medium was treated with 1% Nonidet P-40 and then ultracentrifuged. The pellet was suspended and immunoprecipitated with an antibody to the HBV core antigen, and then assayed for HBV repair (17).

**Characterization of HDV RNA-associated particles by CsCl gradient and electron microscopic examination.** A 40-ml sample of pooled day 6 and day 9 media of HuH-7 cells cotransfected with pA3HBV3.8 and pSVL-D3 was ultracentrifuged at 40,000 rpm for 5 h at 4°C. The pellet was suspended in 0.5 ml of 0.85% NaCl–0.01 M phosphate buffer (pH 7.4) and centrifuged on a discontinuous CsCl gradient from 1.14 to 1.40 g/cm$^3$ at 35,000 rpm and 4°C in a Beckman SW41 rotor. Gradient fractions (0.5 ml each) were collected by tapping from the top of the solution. CsCl density was determined with a refractometry radioimmunoassay kit (clinical assay; Connaught Laboratories, Ontario, Canada). The remaining solution of each fraction was diluted and centrifuged again. The pellet was suspended and analyzed for HDV RNA by hybridization. HDV RNA-positive fractions were then examined by electron microscopy after negative staining with 3% uranyl acetate.

## RESULTS

**Replication of HDV in HuH-7 cells.** The HDV plasmid pSVL-D3, which contains a trimeric head-to-tail HDV genome driven by the late promoter of SV40, expresses the genomic strand of the HDV RNA (19). In human hepatoma HuH-7 cells transfected with pSVL-D3, the unit length of the 1.7-kb HDV genomic strand was detected at 3, 6, and 9 days after transfection with a riboprobe containing the antigenomic sequence of HDV (lanes 4, 8, and 12 of Fig. 1A). This 1.7-kb RNA was at the same electrophoretic position as the genome of the HDV virions harvested from HDV-infected human serum (positive control of Fig. 1A). In addition, RNA species of 3.4, 5.1, and 6.8 kb, corresponding to multimers of a 1.7-kb unit, were also detected. These multimers have also been reported previously in COS7 and HuH-7 cells during active replication of HDV (8, 31). If the replication of HDV genome did occur, the antigenomic RNA should be present. Indeed, the monomer and multimers of the HDV antigenomic strand were detected with a riboprobe of genomic sequence (lanes 4, 8, and 12 of Fig. 1B). In addition, a 0.8-kb antigenomic RNA was observed, which probably represented the delta antigen-specific transcript previously reported (8). These results clearly demonstrate the replication of HDV genome in the absence of HBV, which appears on day 3 and is gradually increased through days 6 and 9.

**Replication of HBV and release of HBV virions.** The 3.5- and 2.1-kb HBV RNAs were detected in HuH-7 cells transfected with the HBV plasmid pA3HBV3.8 on days 3, 6, and 9 after transfection (lanes 1, 5, and 9 of Fig. 2A). The levels of both 3.5- and 2.1-kb RNAs on day 9 were slightly lower than those on day 6. The replication intermediates were also detected (data not shown). To determine whether HBV virions were assembled and released from transfected cells, viral particles were pelleted from the culture medium, followed by the endogenous DNA polymerase repair assay. The presence of linear and relaxed circular forms of HBV DNA indicated the release of HBV virions from the trans-

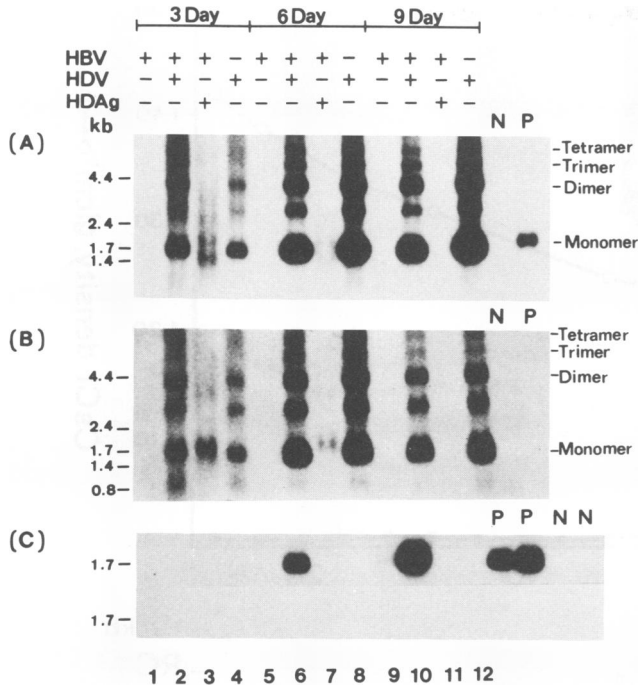


FIG. 1. Northern blot hybridization to detect the genomic and antigenomic HDV RNA. Human hepatoma HuH-7 cells ($5 \times 10^6$) were transfected with the HBV plasmid pA3HBV3.8 (lanes 1, 5, and 9) or the HDV plasmid pSVL-D3 (lanes 4, 8, and 12) alone or cotransfected with pA3HBV3.8 and pSVL-D3 (lanes 2, 6, and 10) or pA3HBV3.8 and the expression plasmid of the delta antigen pSVL-Ag (lanes 3, 7, and 11) as described in Materials and Methods. Plasmid pSV2CAT was cotransfected as a control. On days 3, 6, and 9 after transfection, the media were collected and the cellular RNA was extracted. (A and B) Detection of the intracellular HDV genomic and antigenomic RNA. Total RNA (15 μg) from each sample was loaded in 1% agarose gel for electrophoresis and Northern blot hybridization with strand-specific RNA probes, which were prepared by in vitro transcription from pG4B-D3. (C) Detection of the HDV genomic (upper row) and antigenomic RNAs (lower row) in virions released into the medium. One milliliter of culture medium was centrifuged to pellet viral particles. RNA was extracted, and then Northern blot hybridization was performed with strand-specific RNA probes. A RNA ladder (0.3 to 9.5 kb) was run in parallel to indicate the size of RNA samples. Positive and negative controls were included in A, B, and C. The positive control (P) was a 1-ml serum sample of HBV carrier with HDV superinfection; the negative control (N) was a 1-ml serum sample of HBV carrier without HDV superinfection. Monomer, dimer, trimer, and tetramer correspond to sizes of 1.7, 3.4, 5.1, and 6.8 kb.

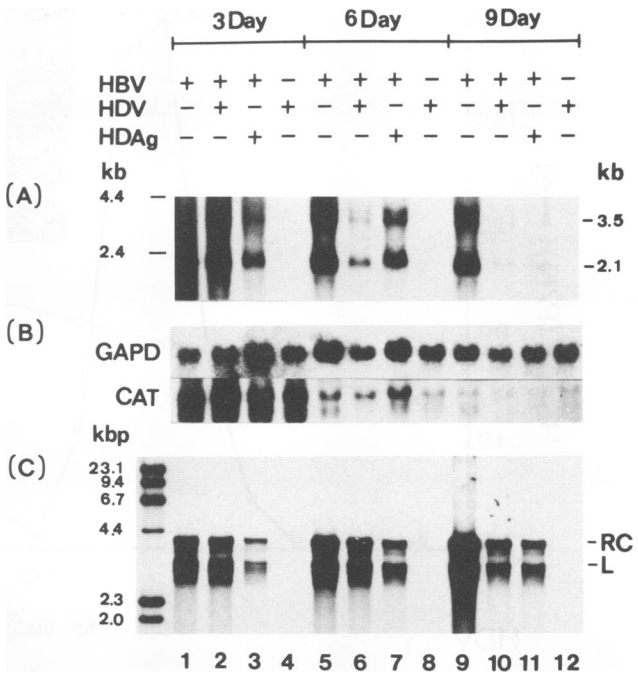


FIG. 2. Northern blot hybridization to detect intracellular HBV RNAs and endogenous DNA polymerase repair assay to detect the HBV DNA in the released viral cores. HuH-7 cells were transfected as described in the legend to Fig. 1. (A) Detection of HBV RNAs. Total RNA (15 μg) was analyzed for the HBV RNAs by Northern blot hybridization with $^{32}$P-labeled HBV DNA as a probe. (B) Detection of glyceraldehyde-3-phosphate dehydrogenase (GAPD) and CAT RNAs. The same blot was rehybridized with the $^{32}$P-labeled glyceraldehyde-3-phosphate dehydrogenase gene and CAT gene as the probes. (C) HBV particles in 10 ml of the culture medium were used for the endogenous DNA polymerase repair assay as described in Materials and Methods. RC and L, Relaxed circle and linear forms of HBV DNA, respectively; HDAg, hepatitis delta antigen.

fected cells (lanes 1, 5, and 9 of Fig. 2C). The release of HBV virions was first observed on day 3, and the level of released virions was elevated on days 6 and 9.

**Release of HDV virions in the presence of HBV.** When HBV plasmid pA3HBV3.8 and HDV plasmid pSVL-D3 were cotransfected into HuH-7 cells, the levels of both genomic and antigenomic HDV RNA were not significantly altered when compared with levels after pSVL-D3 single transfection (lanes 2, 6, and 10 of Fig. 1A and B). These results indicate that HBV does not modulate the replication of the HDV genome.

To investigate whether the HDV virions were assembled and released in this system, the culture medium of cotransfected HuH-7 cells was subjected to ultracentrifugation, followed by RNA extraction. A single 1.7-kb genomic strand of HDV RNA, but not the antigenomic strand, was detected in viral particles released from cells that were transfected with both pSVL-D3 and pA3HBV3.8 on days 6 and 9 (lanes 6 and 10 of Fig. 1C). Because the HDV virions contain only the genomic RNA, these results clearly show the assembly and release of HDV virions into the culture medium. The 1.7-kb genomic RNA was not detected in the culture medium in the absence of HBV plasmid (lanes 8 and 12 of Fig. 1C), demonstrating that HBV is required for the assembly and release of HDV.

**Characterization of released HDV virions.** To further characterize the released HDV virions, the pelleted viral particles from the culture medium of HuH-7 cells cotransfected with HDV and HBV were subjected to isopycnic ultracentrifugation in a discontinuous CsCl density gradient. Fractions were collected and analyzed for HBV surface antigen, HDV RNA, and HBV DNA. HDV RNA was detected in fractions of densities between 1.21 to 1.24 g/cm$^3$, with a peak in fractions at a density of 1.23 g/cm$^3$. HBV DNA was detected in fractions at densities between 1.21 to 1.28 g/cm$^3$, with a peak fraction at a density around 1.23 g/cm$^3$ (Fig. 3). These are consistent with previous reports (4, 30). Samples from the HDV RNA-positive fractions were examined under an electron microscope. As shown in the inset of Fig. 3, the 36-nm HDV particles were the predominant particles in

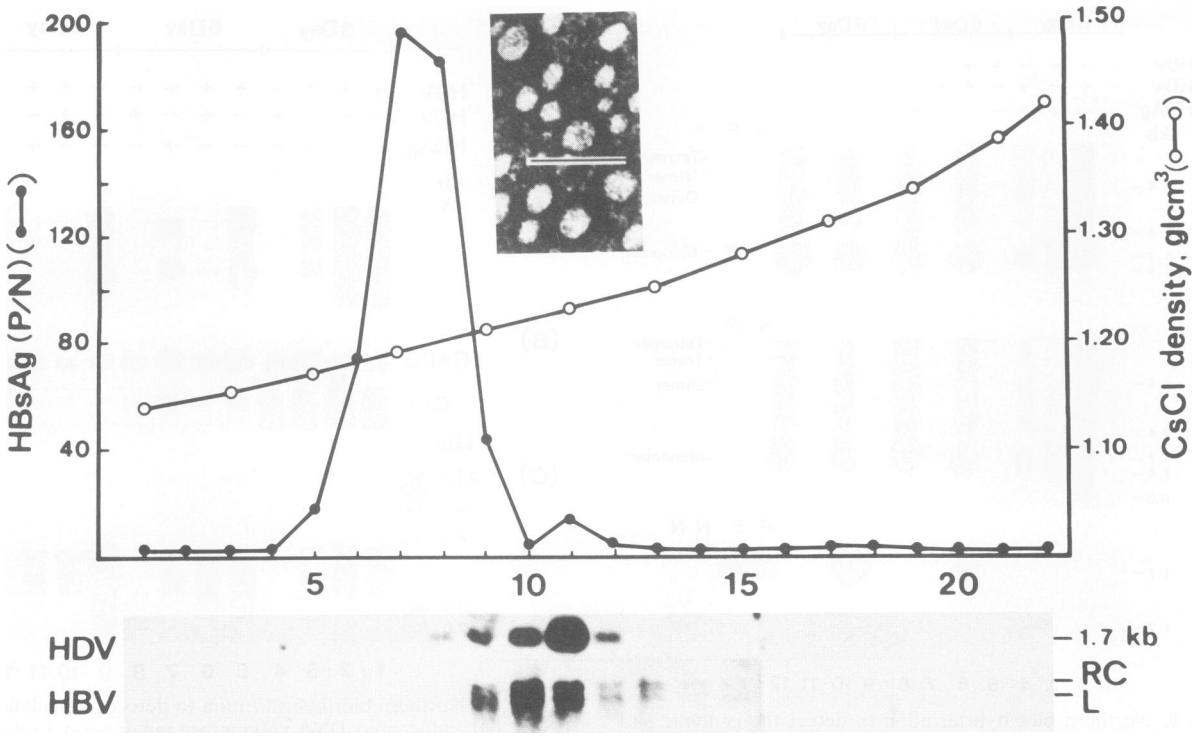


FIG. 3. Characterization of HDV particles in the culture medium by isopycnic banding and electron microscopic examination. Pooled medium (40 ml) was collected from HuH-7 cells cotransfected with pA3HBV3.8 and pSVL-D3 as described in Materials and Methods. The medium was centrifuged, suspended, and then centrifuged on a discontinuous CsCl gradient from 1.14 to 1.40 g/cm$^3$. Fractions were collected by tapping from the top of the solution. CsCl density (○) was determined by refractometry. The HBV surface antigen (HBsAg) (●) in each fraction was assayed by radioimmunoassay and represented as a P/N ratio. The remaining solution in each fraction was analyzed for HDV RNA by Northern blot hybridization and HBV DNA by Southern blot hybridization. The autoradiograms were exposed for 1 and 3 days for HDV RNA and HBV DNA, respectively. (Inset) HDV RNA positive fractions ranged from 1.21- to 1.24-g/cm$^3$ density gradients were examined by electron microscopy after negative staining with 3% uranyl acetate (bar, 100 nm). The larger particles are the 36-nm HDV virus particles, whereas the smaller particles are the 22-nm HBV surface antigen particles.

these samples, but the 22-nm HBV surface antigen particles were also observed. Although the 42-nm HBV particles with the characteristic double-shelled structure are not shown in the inset of Fig. 3, they were also detected in a much lower amount. Therefore, the physicochemical properties of HDV particles released into medium are similar to those reported previously in infected chimpanzees and woodchucks (26, 27).

**Suppression of the expression of HBV RNAs by HDV.** The HDV plasmid pSVL-D3 was cotransfected with the HBV plasmid pA3HBV3.8 into HuH-7 cells. On days 3, 6, and 9 after transfection, the levels of both the HBV 3.5- and 2.1-kb RNAs were significantly decreased in comparison with the transfection with the HBV plasmid alone (lanes 2, 6, and 10 of Fig. 2A). The reduction was most marked on day 9, with a 12- and 7-fold decrease in the levels of 3.5- and 2.1-kb RNAs, respectively. The striking differences in the levels of the 3.5- and 2.1-kb RNAs were not due to the variations in the quantity of RNA loaded or transfection efficiency, as shown by rehybridization of the same nitrocellulose filter with a probe specific for the endogenous housekeeping glyceraldehyde dehydrogenase gene (32) (upper row of Fig. 2B) and a probe specific for the cotransfected chloramphenicol acetyltransferase (CAT) gene (lower row of Fig. 2B). The amount of CAT transcript decreased on day 6 and further decreased on day 9 due to gradual degradation of the transfected CAT DNA. Thus, our results showed that the expression of HBV RNAs was suppressed by the expression of the HDV genes. Furthermore, the level of released HBV virions reflected by the endogenous DNA polymerase repair activity (lanes 2, 6, and 10 of Fig. 2C) and the hybridization signals of Southern blots (data not shown) were suppressed 6-fold by HDV, probably as a result of the decreased amount of 3.5-kb RNA.

**Suppression of the expression of HBV RNAs by delta antigen.** Since the delta antigen is the only known protein of HDV, its role on the suppression of the expression of HBV RNAs by HDV was investigated by cotransfection of the HBV plasmid pA3HBV3.8 and the HDV delta antigen plasmid pSVL-Ag. Plasmid pSVL-Ag has been shown to transcribe a 1.8-kb RNA from the SV40 late promoter and to synthesize the delta antigen in HuH-7 cells (19). In HuH-7 cells cotransfected with pA3HBV3.8 and pSVL-Ag, the 1.8-kb RNA for the synthesis of delta antigen was detected (lanes 3 and 7 of Fig. 1B). A much lower level of this 1.8-kb RNA was detected on day 6 than on day 3, most probably due to gradual degradation of the transfected pSVL-Ag DNA residing within the cells. Compared with the transfection with the HBV plasmid alone, the levels of the 3.5- and 2.1-kb HBV RNAs were suppressed 17- and 9-fold, respectively, by the presence of the delta antigen expression plasmid (lanes 3, 7, and 11 of Fig. 2A). Furthermore, the level of HBV virions released into the culture medium was also suppressed ninefold (lanes 3, 7, and 11 of Fig. 2C). These results clearly demonstrate that the HDV delta antigen alone can suppress the expression of HBV RNAs.

## DISCUSSION

Transfection of human hepatoma HuH-7 cells and monkey kidney COS7 cells with a trimeric HDV genome could lead to the replication of HDV RNA genome (19), indicating that HDV RNA replication is not liver specific. It also indicates that the HBV proteins or HBV nucleotide sequences are not essential for the RNA-directed HDV genome replication (19). However, the assembly and release of HDV virions were not observed, probably due to the lack of the HBV surface proteins. In the present study, cells of the human hepatoma HuH-7 cell line were cotransfected with HDV cDNA and HBV DNA. Not only was the HDV genome able to replicate, but also HDV virions were released. The released HDV virions have physicochemical characteristics similar to those reported previously for HDV virions (26, 27). The concentration of the released HDV virions in the medium was comparable to those of HDV-infected patients who still have active replication of HDV (35). Therefore, we have successfully established a cell culture system in which HDV replication proceeds to completion.

In cells cotransfected with HBV DNA and HDV cDNA, the intracellular levels of the genomic and antigenomic strands of HDV RNA were low on day 3 and were gradually increased on days 6 and 9. Surprisingly, despite the apparent replication of the HDV genome, there was no release of HDV virions on day 3. The levels of the genomic and antigenomic strands of the HDV RNA were similar on days 6 and 9, but the level of released HDV virions on day 9 was higher than that on day 6. Therefore, the assembly and release of HDV virions appear to be a very slow process. Since the levels of genomic and antigenomic HDV RNAs were not altered by the cotransfection of HBV DNA, the replication of the HDV genome was apparently not modulated by the replication of the HBV or HBV proteins. This supports the hypothesis that the role of HBV in the HDV replication cycle is probably limited to the entry or release of the HDV virus particles.

In HBV carriers superinfected with HDV, the HBV DNA is usually not detected in the sera during the acute stage (15, 27, 35). Although the suppression of the HBV replication has been suggested, there is no direct supporting evidence (35). Since we have successfully established a cell culture system in which the replication cycles of both HDV and HBV proceed to completion, the interaction between HBV and HDV without the confounding host factors can now be studied. We found that expression of the HBV 3.5- and 2.1-kb RNAs was dramatically decreased by cotransfection with the HDV cDNA. Moreover, the level of HBV virions released was also significantly reduced, probably as a result of the reduced level of 3.5-kb RNA. The suppression was most remarkable on day 9 posttransfection. At present, we do not know why this suppression occurs so late, despite of the much earlier replication of the HDV genome. Although expression of the HBV RNAs is suppressed, whether the suppression occurs at the level of transcription, posttranscription, or intracellular amplification via the formation of covalently closed circles (1) remains to be elucidated.

HDV replication appears to occur in the nucleus of the host cell (13, 31). The delta antigen has been shown to be essential for the replication of HDV genome (19), which is consistent with its nuclear localization and RNA binding activity (6). We now further show the suppression of HBV RNA expression by delta antigen. The mechanism of this suppressive effect is a very interesting question. One possibility is that delta antigen may bind to the HBV RNAs to affect their stabilities, which in turn reduces the steady-state level of HBV RNAs. The second possibility is that a direct or indirect binding of the delta antigen to the regulatory sequences of HBV DNA leads to a reduced transcription. However, binding of the delta antigen to a DNA sequence has not been shown. Alternatively, the delta antigen may compete with the HBV 5′-terminal binding protein in binding to the HBV RNA or DNA, consequently reducing the HBV replication activities, since binding of HBV 5′-terminal binding protein to the minus strand of HBV DNA is required for the replication of HBV DNA (10). Although there is no direct evidence to indicate that the same HBV sequence is bound by the HDV delta antigen and the HBV 5′-terminal binding protein, the possibility of a competition between these two proteins has been raised by a recent report by Khudyakov and Markhov (18). They proposed a functional similarity between these two proteins based on the striking structural homology between the 5′-terminal binding protein and predicted delta antigen with a partially frame shifted sequence.

In natural infection, the delta antigen appears as two species ranging from 24 to 30 kDa (3, 4, 34). Recently, Luo et al. (22) demonstrated that the larger delta antigen resulted from a A-to-G substitution at the termination codon induced by a RNA duplex unwindase during the replication of the HDV genome. In our cotransfection experiment, the plasmid expressing the delta antigen encodes the small delta antigen. The large delta antigen should not have been produced, because there was no replication of the HDV genome. Therefore, it appears that the small delta antigen is sufficient for the suppression of the expression of HBV RNAs. The small delta antigen has also been shown to be sufficient for HDV genome replication (19). Moreover, since delta antigen is posttranslationally phosphorylated (6), the role of phosphorylation in the suppressive effect of the delta antigen remains to be understood.

In conclusion, a cell culture system in which both HDV and HBV simultaneously complete their replication cycles has been established. This allows further investigation of the mechanism of the HDV RNA replication in the presence of its helper HBV and of the interaction between HDV and HBV as an example for the understanding of the relationship between a defective virus and its helper virus.

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## REFERENCES

1. **Aldrich, C. E., L. Coates, T. Wu, J. Newbold, B. C. Tennant, J. Summers, C. Seeger, and W. Mason.** 1989. In vitro infection of woodchuck hepatocytes with woodchuck hepatitis virus and ground squirrel hepatitis virus. Virology **172**:247–252.
2. **Arico, S., M. Aragona, M. Rizzetto, F. Caredda, A. Zanetti, G. Marinucci, S. Diana, P. Farci, M. Arnone, N. Caporaso, A. Ascione, P. Dentico, G. Pastore, G. Raimondo, and A. Craxi.** 1985. Clinical significance of antibody to the hepatitis delta virus in symptomless HBsAg carriers. Lancet **ii**:356–358.
3. **Bergmann, K., and J. Gerin.** 1986. Antigens of hepatitis delta

virus in the liver and serum of humans and animals. J. Infect. Dis. **154**:702–706.
4. **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.
5. **Branch, A. D., and H. D. Robertson.** 1984. A replication cycle for viroids and other small infectious RNA's. Science **223**:450–455.
6. **Chang, H. K., C. K. Chou, C. Chang, T. S. Su, C. P. Hu, M. Yoshida, and L. P. Ting.** 1987. The enhancer sequence of human hepatitis B virus can enhance the activity of its surface gene promoter. Nucleic Acids Res. **15**:2261–2268.
7. **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.
8. **Chen, P. J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor.** 1986. The structure and replication of the genome of the hepatitis delta virus. Proc. Natl. Acad. Sci. USA **83**:8774–8778.
9. **Fattovich, G., S. Boscaro, F. Noventa, E. Pornaro, D. Stenico, A. Alverti, A. Ruol, and G. Realdi.** 1987. Influence of hepatitis delta virus infection on progression to cirrhosis in chronic hepatitis type B. J. Infect. Dis. **155**:931–935.
10. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56**:651–693.
11. **Glisin, V., R. Crkvenjako, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry. **13**: 2633–2637.
12. **Govindarajan, S., B. Lim, and R. L. Peters.** 1984. Immunohistochemical localization of the delta antigen associated with hepatitis B virus in liver biopsy sections embedded in Araldite. Histopathology **8**:63–67.
13. **Gowans, E. J., F. Negrs, B. M. Baroudy, B. E. Korba, F. Bonino, A. Ponzetto, R. H. Purcell, and J. L. Gerin.** 1987. Molecular markers of WHV and HDV in single cells, p. 591–600. *In* W. Robinson, K. Koike, and H. Will (ed.), Hepadna viruses. Alan R. Liss, Inc., New York.
14. **Graham, F., and A. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–457.
15. **Hadler, S. C., M. D. Monzon, A. Ponzetto, E. Anzola, D. Rivers, A. Mondolfi, A. Bracho, D. Francis, M. A. Gerber, S. Thung, J. Gerin, J. E. Maynard, H. Popper, and R. Purcell.** 1984. Delta virus infection and severe hepatitis. An epidemic in the Yucpa Indians of Venezuela. Ann. Intern. Med. **100**:339–344.
16. **Hadziyannis, S. J., M. Sherman, H. M. Lieberman, and D. A. Shafritz.** 1985. Liver disease activity and hepatitis B virus replication in chronic delta antigen-positive hepatitis B virus carriers. Hepatology **5**:544–547.
17. **Junker, M. F., P. Galle, and H. Schaller.** 1987. Expression and replication of the hepatitis B virus genome under foreign promoter control. Nucleic Acids Res. **15**:10117–10132.
18. **Khudyakov, Y. E., and A. M. Markhov.** 1990. Amino acid sequence similarity between the terminal protein of hepatitis B virus and predicted hepatitis delta virus gene product. FEBS Lett. **262**:345–348.
19. **Kuo, M. Y. P., M. Chao, and J. 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., J. Goldberg, L. Coates, W. Mason, J. Gerin, and J. Taylor.** 1988. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: sequence, structure, and applications. J. Virol. **62**:1855–1861.
21. **Lo, S. J., Y. H. Lee Wu, J. S. Chiou, L. P. Ting, W. T. Liu, and K. B. Choo.** 1985. Characterization of restriction endonuclease maps of hepatitis B virus DNAs. Biochem. Biophys. Res. Commun. **129**:797–803.
22. **Luo, G., M. Chao, S. Y. Hsieh, C. Sureau, K. Nishikwa, and J. Taylor.** 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. J. Virol. **64**:1021–1027.
23. **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 human delta (δ) virus RNA. Nature (London) **329**:343–346.
24. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. **42**:3858–3863.
25. **Negro, F., K. F. Bergmann, B. M. Baroudy, C. Satterfield, H. Popper, R. H. Purcell, and J. L. Gerin.** 1988. Chronic hepatitis D virus (HDV) infection in hepatitis B virus carrier chimpanzees experimentally superinfected with HDV. J. Infect. Dis. **158**: 151–159.
26. **Ponzetto, A., P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin.** 1984. Transmission of the hepatitis B virus-associated δ agent to the eastern woodchuck. Proc. Natl. Acad. Sci. USA **81**:2208–2212.
27. **Rizzetto, M., M. G. Canese, J. L. Gerin, W. T. London, D. L. Sly, and R. H. Purcell.** 1980. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. J. Infect. Dis. **141**:590–602.
28. **Rizzetto, M., B. Hoyer, M. G. Canese, J. W. K. Shih, R. H. Purcell, and J. L. Gerin.** 1980. δ agent: association of antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees. Proc. Natl. Acad. Sci. USA **77**:6124–6128.
29. **Rizzetto, M., G. Verme, S. Recchia, F. Bonino, P. Farci, S. Arico, R. Calzia, A. Picciotto, M. Colombo, and H. Popper.** 1983. Chronic hepatitis in carriers of hepatitis B surface antigen with intrahepatic expression of the delta antigen. An active and progressive disease unresponsive to immunosuppressive treatment. Ann. Intern. Med. **98**:437–441.
30. **Sureau, C., J. Taylor, M. Chao, J. W. Eichberg, and R. E. Lanford.** 1989. Cloned hepatitis delta virus cDNA is infectious in the chimpanzees. J. Virol. **63**:4292–4297.
31. **Taylor, J., W. Mason, J. Summers, C. Aldrich, L. Coates, J. Gerin, and E. Gowans.** 1987. Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. J. Virol. **61**:2891–2895.
32. **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 molecular evolution of the gene. Nucleic Acids Res. **13**:2485–2502.
33. **Wang, K. S., Q. L. Choo, A. J. Weiner, J. H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Dennistion, J. L. Gerin, and M. Houghton.** 1986. Structure, sequence and expression of the hepatitis delta (δ) viral genome. Nature (London) **323**:508–514.
34. **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 and p27. J. Virol. **62**:594–599.
35. **Wu, J. C., S. D. Lee, S. Govindarajan, T. W. Kung, and L. P. Ting.** 1990. Correlation of serum delta RNA with clinical course of acute hepatitis delta virus superinfection in Taiwan: a longitudinal study. J. Infect. Dis. **161**:1116–1120.
36. **Wu, J. C., S. D. Lee, J. Y. Wang, L. P. Ting, Y. T. Tsai, K. J. Lo, B. N. Chiang, and M. J. Tong.** 1986. Analysis of the DNA of hepatitis B virus in the sera of Chinese patients infected with hepatitis B. J. Infect. Dis. **153**:974–977.