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Continuous expression and replication of the hepatitis δ virus genome in Hep G2 hepatoblastoma cells transfected with cloned viral DNA

(RNA virus)

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ABSTRACT **To establish stable cell clones allowing continuous replication of hepatitis δ virus (HDV), Hep G2, a hepatoblastoma cell line containing no hepatitis B virus (HBV) DNA sequences, was transfected with a recombinant plasmid containing a tandem trimer of HDV cDNA (driven by the simian virus 40 late promoter) and a neomycin-resistance gene. After selection with the neomycin analogue G418, at least two of the resistant clones were shown to have intact δ antigen by specific immunoblotting, and the δ antigen was located in the cell nucleus by immunofluorescence. Transfected cloned viral DNAs were found to be integrated into cell chromosomes. Replication of the HDV genome was demonstrated by the presence of not only genomic and antigenomic HDV RNAs but also HDV RNAs in multimeric and circular forms. In addition, a 0.8-kilobase antigenomic RNA containing a poly(A) tail and encoding the δ-antigen open reading frame was documented. Continuous replication and transcription of the HDV genome was thus achieved in these transfected cell lines. The results confirmed that replication of HDV was unassisted by HBV. Stable passage of such cell lines strongly suggests that HDV lacks direct cytopathicity in hepatocytes. These clones should be useful in studying the details of the HDV life cycle and the relationship between HDV and its helper virus, HBV.**

Hepatitis δ virus (HDV) is a defective infectious agent causing human hepatitis (1, 2). The virus contains an RNA genome and encodes its own nucleocapsid proteins (δ antigen) (3), but the envelope proteins utilized by HDV are supplied by hepatitis B virus (HBV) (3). Therefore HDV is a defective virus and is transmitted only in the presence of HBV (3, 4). Superinfection of HBV carriers by HDV frequently causes acute exacerbations and subsequent chronic hepatitis (1, 2). HDV clearly imposes a definite risk on the health of any population with a high prevalence of HBV carriers (1, 2). Thus it is important to study the HDV life cycle and its interaction with helper HBV. The successful cloning of the HDV genome has advanced the understanding of viral biology rapidly (5).

HDV is unique among animal viruses and has a small, circular RNA genome of about 1.7 kilobases (kb) (6–8). It is similar to viroids, the plant infectious pathogens with circular RNAs. Furthermore, HDV replication appears to go through rolling circles and generates multimeric intermediates (6, 9). Autocleavage and self-ligation activity of HDV RNAs have been demonstrated *in vitro* (10, 11). The HDV genome generally resembles viroids in structure, replication, and processing (12), but there are some differences: for example, the model of HDV replication consists of two rolling circles, whereas viroid replication is likely to go through one rolling circle (6, 13). Thus, the details of HDV replication have not yet been clarified.

Unlike the viroids, the HDV genome has an open reading frame (ORF) for viral nucleocapsid proteins (δ antigen) (7, 14). The δ antigen has been detected as a nuclear phosphoprotein (15) and is important in viral replication (16). Despite the importance of the δ antigen, its mRNA template has not been demonstrated conclusively (5). Most of the HDV antigenomic RNAs appear to be associated with genomic RNAs (6) and are unlikely to be mRNAs. One antigenomic RNA consisting of 0.8 kb and containing a poly(A) tail was detected in one infected chimpanzee (6). This RNA was postulated to be the translation template for δ antigen (5). However, this intriguing proposal has not been substantiated, probably because only a small amount of this RNA is present and the study utilizing infected liver tissue is inadequate in demonstrating it.

Therefore, an *in vitro* system providing the continuous replication and gene expression of HDV would be helpful in addressing these questions. Extending from successful transient viral replication by transfecting appropriate cell lines with HDV cDNAs (16, 17), we have established such stable clones by transfecting the human hepatoblastoma cell line Hep G2 with a recombinant plasmid containing a neomycin-resistance gene and three tandem cDNA copies of the HDV genome. These clones may be useful systems in studying the details of HDV life cycle and the interactions between HDV and HBV.

MATERIALS AND METHODS

Construction of Plasmids. The plasmid pSVLD3 (kindly provided by J. M. Taylor) contains a trimer of the cloned HDV genome under the control of the simian virus 40 (SV40) late promoter and has been shown to allow transient expression and replication of the HDV genome after transfection into appropriate cell lines (16). The plasmid was linearized with *Sac* I and *Bam*HI at the polylinker site downstream from the HDV sequences and then ligated with a *Hin*dIII–*Bam*HI fragment containing the neomycin-resistance gene cassette derived from the plasmid betglodsneo (kindly supplied by Lynn Condrey of Fox Chase Cancer Center). The organization of the final construct, pSVLD3-neo, is shown in Fig. 1.

Transfection and Cell Selection. Hep G2 cells were plated at a density of 2×10^5 per 100-mm Petri dish and then transfected with 20 μg of pSVLD3-neo by the calcium phosphate method as described by Wigler *et al.* (18). After overnight

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Abbreviations: HDV, hepatitis δ virus; HBV, hepatitis B virus; ORF, open reading frame; SV40, simian virus 40.

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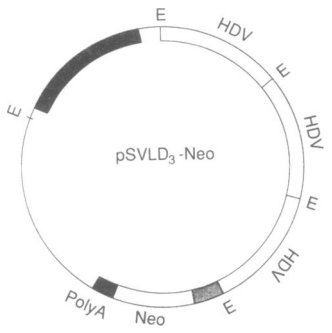

FIG. 1. The organization of plasmid pSVLD3-neo. The plasmid consists of three parts: the thin line represents the pBR322 sequences; the solid thick bar represents the SV40 late promoter cassette [including the polyadenylylation signal (PolyA)]; HDV and neomycin-resistance (Neo) sequences are designated. The cleavage sites for restriction enzyme *Eco*RI are shown (E). There is a single *Bam*HI site at the end of the neomycin-resistance gene. A truncated mouse β-globin gene promoter (gray bar) lies between the HDV sequences and the neomycin-resistance gene.

incubation with the DNA, the cells were washed with DMEM (Dulbecco's modified Eagle's medium; GIBCO) and incubated in DMEM with 10% fetal bovine serum. Selection was started 2 days later by adding 1 mg of G418 (GIBCO) per ml of medium. Twelve clones were obtained 1 month later and were transferred to a 24-well plate in medium containing 0.8 mg of G418 per ml for continuous selection. After becoming confluent, clones were screened by immunofluorescence staining as described below to detect expression of the δ antigen. Four clones were then passaged in the presence of G418 for 1½ months. At the time of characterization, they had been passaged eight times. Currently, they are maintained in DMEM with 10% fetal bovine serum.

Detection of δ Antigen by Immunofluorescence. Indirect immunofluorescence was used to screen clones for expression of the δ antigen. A human polyclonal antiserum with a high titer against δ antigen (by Abbott anti-delta assay, ≥1:5000) was used as the primary antibody. Cells were spread onto clean slides precoated with 0.05% polylysine and then were fixed in acetone. The fixed cells were washed once with phosphate-buffered saline containing 0.1% Triton X-100 and then incubated with diluted (1:12,800) primary antiserum overnight. After reaction with fluorescein isothiocyanate-labeled goat anti-human immunoglobulin antibody (1:40; Dako, Santa Barbara, CA) for 30 min and washing with phosphate-buffered saline, the cells were mounted and examined under a fluorescence microscope.

Western Blot Analysis for δ Antigen. Preparation of total cellular proteins and immunoblotting were performed as described by Harlow and Lane (19). The human polyclonal anti-δ antibodies were isolated by protein A-Sepharose chromatography according to the manufacturer's instructions (Pharmacia) and then diluted 1:200 for use as primary antibody in immunoblotting. A peroxidase-labeled mouse anti-human immunoglobulin antibody was used as secondary antibody (Amersham). 3,3'-Diaminobenzidine was used as substrate for color development.

Southern Blot Analysis. Total cellular DNA was obtained by digesting cells in sodium dodecyl sulfate/proteinase K solution and extracting the products with phenol/chloroform as described (20). For Southern blotting, 10 μg of DNA was digested with *Bam*HI or *Eco*RI in the recommended buffer (Bethesda Research Laboratories). The DNA was then electrophoresed overnight with undigested DNA and a positive control in a 0.7% agarose gel in 40 mM Tris acetate, pH 8.0/2 mM ethylenediaminetetraacetic acid. The fractionated DNA was electrophoretically transferred onto a nylon membrane in a Hoefer transfer unit (model TE50) and the transferred DNA was then hybridized with HDV cDNA probe, radiolabeled by nick-translation (21).

Northern Blot Analysis of RNA. Total cellular RNA was isolated from clones by a single-step method using an acid/guanidinium thiocyanate/phenol/chloroform extraction (22). Poly(A)$^+$ RNA was purified by chromatography on an oligo (dT)-cellulose column. Glyoxal-treated RNA samples were subjected to Northern blot analysis as described (6). Strand-specific RNA probes was prepared by *in vitro* transcription (Riboprobe system; Promega) (16). Restriction fragments were separated by agarose gel electrophoresis and electroeluted to yield subgenomic probes.

Denaturing Polyacrylamide Gel Electrophoresis (PAGE). To separate circular forms of HDV RNAs from linear molecules, total cellular RNA from stable clones were electrophoresed at 55°C in 4% polyacrylamide gels containing 8 M urea (6). When the xylene cyanol marker dye had migrated 30 cm from the origin, the gel was dismantled and the RNAs were electrophoretically transferred to a nylon membrane. For detection of HDV RNAs, the nylon filter was hybridized with strand-specific RNA probes.

RESULTS

Transfection of Hep G2 Cells and Selection of G418-Resistant Clones. The recombinant pSVLD3-neo plasmids were purified by isopycnic banding and transfected into Hep G2 cells by the calcium phosphate method. From four transfected plates, 12 clones were successfully isolated and subjected to further subcloning. Immunofluorescence staining for intracellular δ antigen disclosed 4 clones that were strongly positive; 2 were weakly positive, and the remaining 6 were negative. Two strongly positive clones (designated SVLD3-N1 and -N3), a weakly positive clone (SVLD3-N6), and a negative clone (SVLD3-N8) were then continuously passaged for another 1½ months. All the clones were maintained in DMEM with 10% fetal bovine serum.

Localization of δ Antigen in the Nucleus of Transfected Cells. Indirect immunofluorescence demonstrated δ antigen in the cell nuclei of clones SVLD3-N1 and -N3 (Fig. 2 *B* and *C*). Hep G2 cells that were not transfected served as a control and were negative for fluorescence (Fig. 2*A*). Another clone, SVLD3-N6, was found to be weakly positive, and a fourth clone, SVLD3-N8, showed no immunofluorescence. The immunofluorescence in these stable cell lines was in the nuclei (Fig. 2 *B* and *C*), unlike previously reported transient expression systems in which δ antigen was located exclusively in the nucleoli (16). The immunofluorescent staining pattern in the cell lines described here resembled that in naturally infected hepatocytes (1, 23).

Identification of a Single, 24-kDa Species of δ Antigen in Positive Clones. The δ antigen was identified by immunoblot analysis of total protein obtained by disrupting 10^4 cloned cells. Clones SVLD3-N1 and -N3 revealed a discrete and distinct protein of ≈24 kDa (Fig. 2*D*, lanes 2 and 3). Clone SVLD3-N6 showed a faint signal at the same position (lane 4), and no signal was detected in untransfected Hep G2 cells (lane 1) or the negative clone SVLD3-N8 (lane 5). The δ antigens characterized in this system were approximately the same size as the 21- to 29-kDa δ antigens found in naturally infected hepatocytes. The δ-antigen ORF in our cDNA clone contains an amber stop codon and translation can only produce a 195-residue protein, about 22 kDa in size; therefore, the proteins expressed in SVLD3-N1 and -N3 could be tentatively considered full-sized. These proteins were functionally active in supporting HDV replication, as is shown below.

Integration of Transfected Cloned HDV DNA into Cell Chromosomes. Southern blot analysis was used to further characterize cloned viral DNAs that had been transfected

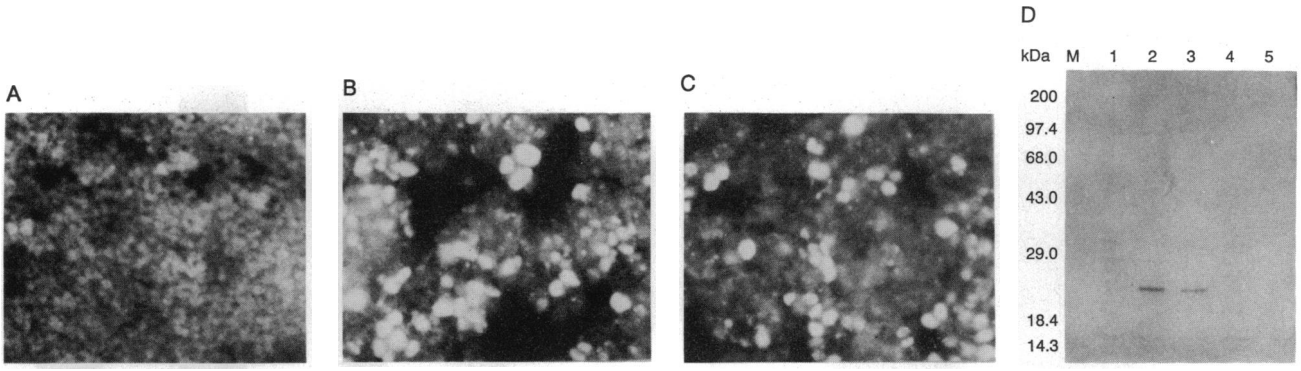

FIG. 2. Demonstration of δ antigen by immunological methods. (*A–C*) Cells were spread on glass slides and examined for δ-antigen expression by indirect immunofluorescence staining with specific anti-δ antiserum. Hep G2 cells that had not been transfected showed no fluorescence (*A*, ×40). Resistant clones SVLD3-N1 and N3 exhibited intense fluorescence in the cell nucleus (*B* and *C*, ×300). (*D*) The characteristics of expressed δ antigens were studied further by immunoblotting of total protein from untransfected Hep G2 cells (lanes 1) and from clones SVLD3-N1, -N3, -N6, -N8 (lanes 2–5, respectively). Lane M, protein size markers.

into these cell clones. No DNAs of episomal form could be detected by hybridization (Fig. 3, lanes 1U, 3U, 6U, and 8U). As the HDV cDNA has a single *Eco*RI site, digestion of chromosomal DNAs with *Eco*RI yielded a 1.7-kb fragment representing full-length cloned viral DNA in the cloned cell lines SVLD3-N1, -N3, and -N8 (lanes 1E, 3E, and 8E, respectively). Additional junctional fragments were also seen in the same lanes. Digestion with *Bam*HI, an enzyme that cleaves the whole pSVLD3-neo plasmid just once, released fragments larger than the unit length (lanes 1B, 3B, 6B, and 8B). We therefore concluded that the transfected plasmid DNAs were integrated into the cellular chromosomes of G418-resistant clones and that in SVLD3-N1, -N3, and -N8, the full-length cloned HDV cDNA was preserved.

Replication of HDV Genome in Selected Clones. In the plasmid pSVLD3-neo, transcription from the SV40 promoter can generate HDV RNA only of genomic polarity (16). Therefore, the presence of an antigenomic monomer is strong

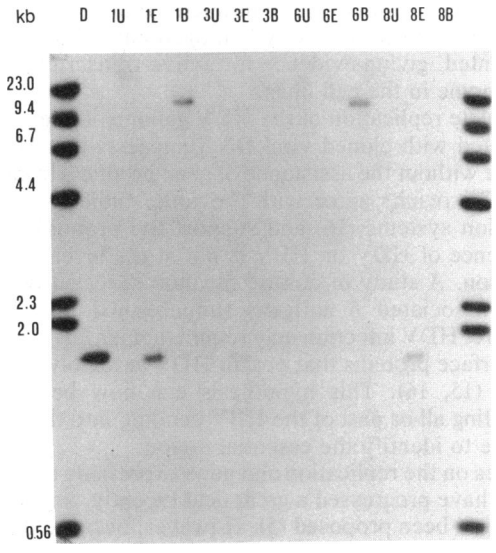

FIG. 3. Southern blot analysis for the status of transfected recombinant plasmid in cell lines. Total DNA samples (10 μg) from four cell lines were digested with restriction enzymes, subjected to agarose gel electrophoresis, transferred to a nylon filter, and then hybridized with a radiolabeled HDV cDNA clone. Lane D represents the unit-length cloned HDV DNA (10 pg). For the other lanes, numbers stand for clones (1 for SVLD3-N1, 3 for SVLD3-N3, etc.) and letters indicate undigested (U), *Eco*RI-digested (E), or *Bam*HI-digested (B) DNA. Outer lanes show size markers (*Hin*dIII fragments of bacteriophage λ DNA).

evidence for viral genome replication. Such claims can be further supported by the presence of circular viral RNAs, which cannot arise from transcription of integrated DNAs without replication.

To demonstrate the presence of these HDV replication intermediates in stable cell lines, total cellular RNA of clones was analyzed by Northern blot hybridization. Antigenomic HDV RNA monomer was shown clearly in clones SVLD3-N1 and -N3 (Fig. 4*A*, lane 1 and 2) and weakly in SVLD3-N6 (lane 3). There was no signal in SVLD3-N8 (lane 4), which as noted earlier was negative for nuclear δ-antigen expression. Trace amounts of dimer were discerned in SVLD3-N1 and -N3 (Fig. 4*A*; see also Fig. 5*A*, lane 1). Hybridization with probe to detect RNAs of genomic polarity disclosed monomeric HDV RNA and trace quantities of dimer in SVLD3-N1 and -N3 (Fig. 4*B*, lane 1 and 2; see also Fig. 5*B*, lane 1).

The presence of circular HDV RNAs was detected by subjecting total cellular RNA from clones pSVLD3-N1 and -N8 to denaturing PAGE (6). Under these conditions, all linear RNAs and DNAs migrate to a cut-off point about 6 cm from the origin whereas the circular RNAs and DNAs migrate only 3 cm. This method demonstrated circular forms of HDV RNAs in SVLD3-N1 (Fig. 4*C*, lane 2; arrow c). No hybridization signal was detected with RNA from SVLD3-N8, the negative clone used as a control (lane 1). Hybridization with probes specific for the antigenomic strand also detected circular molecules (Fig. 4*D*, lane 2). In summary, all HDV replication intermediates ever identified in naturally infected liver were demonstrated in these transfected Hep G2 clones. This is compelling evidence that replication of the HDV genome occurs in these transfected cells.

Detection of an Antigenomic HDV RNA Containing a Poly(A) Tail and the δ-Antigen ORF. The viral nucleocapsid protein, δ antigen, is encoded by the antigenomic strand of the viral genome (7, 8). A previous study (6) suggested that a small proportion of antigenomic RNA was present as a poly(A)$^+$ RNA (6). This RNA was subsequently proposed to be mRNA for δ antigen (5). In order to examine this RNA species in these cell lines, poly(A)$^+$ RNAs were isolated by an oligo(dT)-cellulose chromatography and analyzed by Northern blotting. One species clearly hybridized to probes detecting the antigenomic strand (Fig. 5*A*, lane 2). Most of the other antigenomic RNAs were without poly(A) tails (lane 1). Furthermore, rehybridization of the same nylon filter with probes for genomic-strand RNAs disclosed no signal with the poly(A)$^+$ RNAs (Fig. 5*B*, lane 2). This is consistent with previous observation that the only poly(A)$^+$ HDV RNA was of antigenomic polarity and about 0.8 kb in size (6).

The region of this poly(A)$^+$ antigenomic RNA was further specified by hybridization with subgenomic probes. Full-

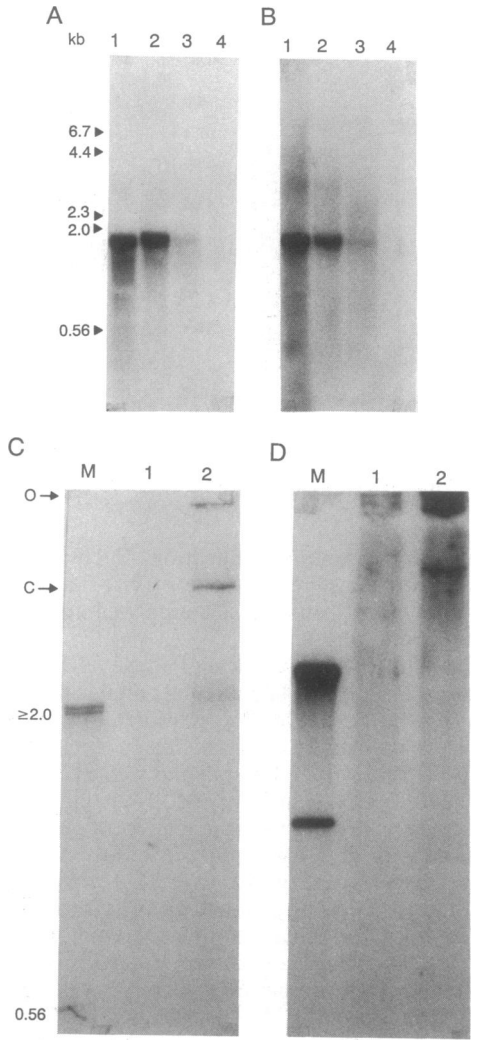

FIG. 4. Characterization of HDV replication intermediates in transfected cell lines. (*A* and *B*) Total RNA samples (10 μg) from four cell lines underwent Northern blot analysis for antigenomic-stranded (*A*) and genomic-stranded (*B*) RNAs. Lane 1, SVLD3-N1; lane 2, SVLD3-N3; lane 3, SVLD3-N6; lane 4, SVLD3-N8. (*C* and *D*) Analysis of circular-form HDV RNAs was performed by separation in denaturing PAGE, electroblotting to a nylon filter, and hybridization. *C* demonstrates genomic-stranded HDV RNA (autoradiographic exposure time, 6 hr). Lane M contained *Hin*dIII fragments of λ DNA; the cut-off point for linear molecules is shown by the mark ≥2.0 kb. The origin is indicated by arrow o and the position of the circular-form RNA is designated by arrow c. Lane 1, negative clone (SVLD3-N8); lane 2, positive clone (SVLD3-N1). *D* represents the detection of antigenomic RNAs (exposure time, 48 hr).

length cloned HDV DNA was isolated from the plasmid by *Sal* I digestion. Further digestion with *Rsa* I created two fragments. The smaller fragment contained the whole δ ORF, and the larger fragment comprised the rest of the HDV genome (Fig. 5*E*). The poly(A)$^+$ HDV RNA hybridized strongly with the smaller fragment (Fig. 5*C*, lane 2) but only weakly with the larger one, even after a prolonged exposure (Fig. 5*D*, lane 2). It was concluded that the poly(A)$^+$ HDV RNA contained predominantly the δ-antigen ORF sequences.

DISCUSSION

By transfecting Hep G2 cells with cloned viral DNAs and a selection marker, we succeeded in establishing stable cell lines capable of continuous HDV replication and gene

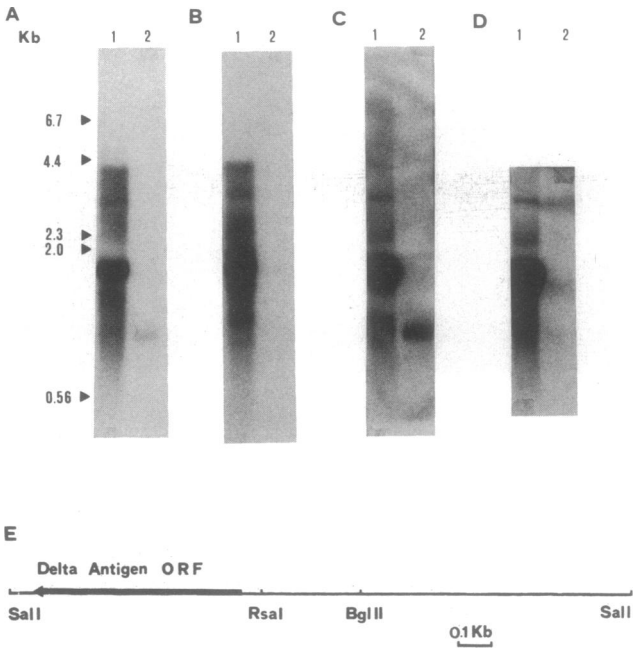

FIG. 5. Identification of poly(A)$^+$ viral RNA by hybridization. Cellular poly(A)$^-$ (5 μg; lanes 1) and poly(A)$^+$ (3 μg; lanes 2) RNAs were subjected to Northern blot analysis. (*A*) RNA from cell clone SVLD3-N1 hybridized with RNA probe specific for the antigenomic strand. (*B*) The same filter was rehybridized with RNA probe specific for the genomic strand. (*C*) RNA from cell line SVLD3-N3 hybridized with a subgenomic probe including the δ ORF (short *Sal* I–*Rsa* I fragment; exposure time, 16 hr). (*D*) RNA from SVLD3-N3 hybridized with larger fragment (exposure time, 72 hr). (*E*) Positions of relevant restriction enzyme sites and the δ-antigen ORF in the viral genome.

expression. The evidence can be summarized as follows: (*i*) the δ antigen important for viral replication was detected by both immunoblotting and immunofluorescence; (*ii*) RNA analysis demonstrated not only abundant monomeric HDV RNA and small amounts of multimeric HDV RNAs but also circular forms; (*iii*) a poly(A)$^+$ antigenomic viral RNA was documented, giving evidence for active transcription of the viral genome in the cell lines.

Complete replication of the HDV genome in Hep G2 cells transfected with cloned viral DNA suggests that HDV can replicate without the assistance of gene products from helper HBV. The results agree with previous studies of transient expression systems (16) and support the proposal that the dependence of HDV on HBV is not at the level of genome replication. A study of culture medium revealed no free or particle-associated δ antigens (unpublished observation). Therefore, HDV infection may require helper HBV solely for HBV surface proteins that permit HDV assembly and transmission (15, 16). This hypothesis can now be tested by transfecting all or part of the HBV genome into the SVLD3-N1 clone to identify the essential region.

Studies on the replication and gene expression of the HDV genome have progressed a great deal recently, and a general scheme has been proposed (5). However, because there was no cell line capable of stable replication of the HDV genome, all studies had to rely upon either naturally infected liver tissues or transient expression systems (6, 9, 16, 17). The limitations of such approaches include the paucity and variable level of molecules important for the viral life cycle. The stable cell lines established in this study may solve the problem and will greatly facilitate investigation of HDV replication. For example, preliminary characterization of replication intermediates in our cell lines revealed circular HDV RNAs of both genomic and antigenomic polarities. The

double-rolling-circle model for HDV replication proposed from previous observations in infected chimpanzees was thus confirmed (6, 25). Other replication intermediates should also be available in amounts sufficient to unravel the details of HDV replication.

The demonstration of an antigenomic, poly(A)$^+$ RNA covering the δ-antigen ORF provided a solid starting point to examine the mRNA template for δ antigen. The inconsistency of previous reports on the search the poly(A)$^+$ antigenomic viral RNA is now clarified (9, 16, 17). Successful demonstration of this RNA again proves the usefulness of our cell lines. Results from studying the possible protein product of this RNA species may help determine whether the HDV is actually a negative-stranded virus (2, 5). Determining the ends of the RNA species may identify the elements important for viral transcription, and even the origin of replication (10). In addition, a similar strategy for gene expression may be relevant to plant infectious agents with circular genomes.

The histologic observations that δ hepatitis lacks a prominent inflammatory cell infiltrate in the presence of microvesicular steatosis and eosinophilic necrosis have led to the suggestion that HDV has a direct cytopathic effect (26). However, continuous passage of the SVLD3-N1 cell line with active HDV replication exhibited no adverse effect on cell viability. Such findings dispute the previous conjecture, and underscore the importance of host immune responses and the coexistent HBV infections in the pathogenesis of HDV-related hepatitis.

Although these cell lines will be useful, there are differences between the viral properties of the transfected cell lines and those of naturally infected hepatocytes. For example, in natural infections, δ antigens appear to be of more than one species (23), though the cause of this heterogeneity is not known. It could be due to posttranslational modification (15) and subsequent processing; alternatively, it could be the result of the presence of more than one viral strain in natural infections (27). Consistent with the latter possibility, *in vitro* transfections experiments using cloned DNA produce just one species of δ antigen (15, 16).

In conclusion, cell lines capable of supporting continuous HDV replication and gene expression were established from transfected Hep G2 cells. This system was shown to be useful in studying the details of the viral replication scheme and will probably be valuable in examining viral dependence on helper HBV. Finally, these cell lines could be employed to examine *in vitro* the effects of antiviral regimens on HDV replication and translation.

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