Efficient Hepatitis Delta Virus RNA Replication in Avian Cells Requires a Permissive Factor(s) from Mammalian Cells

YU-TSUENG LIU, ROB BRAZAS, AND DON GANEM*

*Howard Hughes Medical Institute and Departments of Microbiology & Immunology and Medicine,
University of California Medical Center, San Francisco, California 94143-0414*

Received 27 December 2000/Accepted 7 May 2001

Hepatitis delta virus (HDV) is a highly pathogenic human RNA virus whose genome is structurally related to those of plant viroids. Although its spread from cell to cell requires helper functions supplied by hepatitis B virus (HBV), intracellular HDV RNA replication can proceed in the absence of HBV proteins. As HDV encodes no RNA-dependent RNA polymerase, the identity of the (presumably cellular) enzyme responsible for this reaction remains unknown. Here we show that, in contrast to mammalian cells, avian cells do not support efficient HDV RNA replication and that this defect cannot be rescued by provision of HDV gene products in *trans*. Contrary to earlier assertions, this defect is not due to enhanced apoptosis triggered in avian cells by HDV. Fusion of avian cells to mammalian cells rescues HDV replication in avian nuclei, indicating that the nonpermissive phenotype of avian cells is not due to the presence of dominantly acting inhibitors of replication. Rather, avian cells lack one or more essential permissive factors present in mammalian cells. These results set the stage for the identification of such factors and also explain the failure of earlier efforts to transmit HDV infection to avian hosts harboring indigenous hepadnaviruses.

Hepatitis delta virus (HDV) is a small RNA virus that is transmitted from human to human only in conjunction with hepatitis B virus (HBV) infection (23). HDV is a clinically important pathogen, since dual infection with HBV and HDV is often associated with liver injury that is more severe than that due to HBV alone (13, 24, 25). The HDV genome is a 1.7-kb covalently closed, single-stranded, circular RNA that displays 70% self-complementarity, allowing it to base pair into a highly compact, rod-like structure (reviewed in references 16 and 18). These genomic features are found in no other animal virus but recall those of plant viroids. But unlike viroid RNAs, which are noncoding, HDV RNA harbors a single open reading frame that encodes a key RNA-binding protein known as hepatitis delta antigen (HDAg). HDAg specifically binds HDV RNA (7) and these HDAg-RNA complexes form the core of the HDV virion. As a result of an RNA editing event that occurs during replication (4, 19), HDAg occurs in two isoforms differing at their C termini. The small (S) isoform is absolutely required for RNA replication (14), while the larger (L) isoform inhibits RNA replication but promotes the envelopment of HDV RNPs by the envelope proteins of HBV (5, 8). This envelopment is the basis of the requirement for HBV coinfection, since only HDV genomes enveloped by HBV glycoproteins can exit the cell and propagate the infection.

No HBV functions are required for HDV RNA replication, which proceeds via RNA-based rolling circular intermediates (9). Unlike all other RNA viruses, HDV does not appear to encode its own RNA-dependent RNA polymerase. HDAg, its sole gene product, has no homology to known polymerases and no detectable polymerase activity. The responsible polymerase

is presumed to be of cellular origin, but its identity is unknown and little is known of host factors that affect viral replication.

Several years ago, we discovered that HDV RNA replication, which occurs efficiently in most cultured mammalian cells, proceeds poorly or not at all in certain avian cell lines (cited in reference 6), findings which have since been confirmed by others (6). Here we have further explored this observation, with the aim of assessing the generality of the block to replication in avian cells and whether it is due to the presence of inhibitory factors or to the absence of permissive ones. Our results strongly favor the latter hypothesis and provide a viable strategy for the identification of such factors.

MATERIALS AND METHODS

Cell culture. QT6 (20), QT35 (a gift from M. Linial, Fred Hutchinson Cancer Center) (20), LMH, and chicken embryonic fibroblast cells were maintained in Dulbecco's modified Eagle's medium with 10% tryptose phosphate broth, 5% fetal bovine serum, and 1% chick serum. QM7 was obtained from the American Type Culture Collection (ATCC) (1). QCL-3 and 8C93 cells were kindly provided by B. Cullen (Howard Hughes Medical Institute and Duke University) (11) and H. Yin (University of Texas Southwestern Medical Center), respectively. All of the adherent mammalian cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, except for CHO-K1 cells, for which the medium also contained 40 μ g of proline per ml. These cells were transfected with either Superfect (Qiagen) or Eugene-6 (Roche). DT40 and BJAB cells were maintained and transfected as described previously (10, 15). Interspecific cell fusion between avian and mammalian cells was induced by PEG-1500 (Roche) for 1 min.

Plasmids. The expression plasmids for 1.1-mer HDV genomic (pSVL-D1.1 wild type [WT] and mutant) or antigenomic [pSVL-D1.1 (AG)] cDNA and SHDAg (pcDNA3-SHDAg) have been described (2, 3, 17, 26).

Immunofluorescence and in situ hybridization. To detect HDV replication at the single-cell level, short oligonucleotide DNA probes were used for in situ hybridization (12). A genome-specific 45-mer oligoprobe (2) 5' labeled with fluorescein isothiocyanate was synthesized by Life Technology. Cells were first fixed and permeabilized with methanol at -20°C for 15 min. Immunolocalization of β -galactosidase was performed with a mouse monoclonal antibody (Promega) and tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit secondary antibody. Subsequently, cells were postfixed with in situ fixation buffer (Ambion), stored in 70% alcohol overnight at 4°C , and rehydrated with 50%

* Corresponding author. Mailing address: Howard Hughes Medical Institute and Departments of Microbiology & Immunology and Medicine, University of California Medical Center, San Francisco, CA 94143-0414. Phone: (415) 476-2826. Fax: (415) 476-0939. E-mail: ganem@cgl.ucsf.edu.

TABLE 1. Host range of HDV RNA replication

Cell line	Species	Origin	Replication
293	Human	Kidney	Yes
BJAB	Human	B cell	Yes
HeLa	Human	Epithelial cell	Yes
Hep2	Human	Epithelial cell	Yes
HepG2	Human	Hepatoma	Yes
HuH7	Human	Hepatoma	Yes
SLK	Human	Endothelial cell	Yes
CV1	Monkey	Kidney	Yes
COS	Monkey	Kidney	Yes
3T3	Mouse	Fibroblast	Yes
L929	Mouse	Fibroblast	Yes
C2C12	Mouse	Myoblast	Yes
MDCK	Dog	Kidney	Yes
CHO-K1	Hamster	Ovary	Yes
QT6	Quail	Fibrosarcoma	No
QT35	Quail	Fibrosarcoma	No
QM7	Quail	Myoblast	No
QCL-3	Quail	Fibroblast	No
LMH	Chick	Hepatoma	No
DT40	Chick	B cell	No
CEF ^a	Chick	Fibroblast	No

^a CEF, chicken embryonic fibroblast.

formamide in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature. Cells were denatured at 65°C with Zip-Hyb buffer (Ambion) for 10 min, hybridized with probes overnight at 37°C, washed twice with 50% formamide in 2× SSC and in 0.1× SSC at 37°C, and finally mounted with Vectashield containing 4',6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Annexin V staining was applied to examine the effect of HDV on apoptosis. Cells were incubated with Cy3-conjugated annexin V (Biosource International) for 20 min before fixation in order to label apoptotic cells. Some QT6 cells were treated with 0.5 µg of staurosporine per ml overnight to induce apoptosis as a positive control.

Northern blotting and flow cytometry. These analyses were performed as previously described (3, 26).

RESULTS

Block to HDV replication in avian cells. To assay for HDV RNA replication, cells were transfected with 1.1-mer clones of HDV cDNA, the expression of whose genomic RNA strand was driven by a simian virus 40 (SV40) late promoter (pSVL-D1.1) or cytomegalovirus (CMV) immediate-early promoter (pCMV-D1.1). Expression of this RNA by conventional pol II transcription results in production of overlength genomic RNAs which undergo self-cleavage to unit-length linears by the intrinsic ribozyme activity of the RNA. Ligation of these RNAs yields monomer circles of genomic polarity; in the presence of S-HDAg, these structures are replicated to antigenomic RNA. Thus, the presence of antigenomic RNA in Northern blots of RNA from such cells provides proof of authentic HDV RNA replication. Table 1 shows a summary of the lines in which we have detected HDV replication following transfection of HDV cDNA. Not only can cells of many mammalian species support replication, but virtually all cell types tested appear competent for this activity. In fact, no mammalian cell line we have ever tested has failed to support HDV RNA synthesis (3, 26, 27; Y.-T. Liu, R. Brazas, and D. Ganem, unpublished observation).

By contrast, we observed little or no replication of HDV in avian cells. As shown in Fig. 1 for the quail fibrosarcoma cell

FIG. 1. Northern and Western analysis for HDV replication in mammalian and avian cells. Monkey CV1 and quail QT6 cells were transfected with pSVL-D1.1 (lanes 1 and 6), mutant pSVL-D1.1 (lanes 2 and 7), pSVL-D1.1 + pcDNA3-SHDAg (lanes 3 and 8), mutant pSVL-D1.1 + pcDNA3-SHDAg (lanes 4 and 9) and pCMV-D1.1 (lanes 5 and 10) and were analyzed by Northern blotting for HDV antigenomic RNA synthesis (top panel). The total RNA of each sample is shown in the middle panel. Expression of S-HDAg was detected by Western blotting (bottom panel).

line QT6, transfection of QT6 with WT pSVL-D1.1 HDV genomes gave rise to only extremely low levels of antigenomic RNA (lanes 6 to 9), even with high efficiency transfection (routinely more than 20% [see Fig. 3]), deliberate overloading of the gel, and overexposure of the autoradiogram. This defect is also not due solely to inefficient expression of the SV40 late promoter in avian cells. Although the CMV promoter directed expression of reporter genes in QT6 at 5 to 10 times the level supported by the SV40 late promoter (not shown), transfection of QT6 by pCMV-D1.1 still resulted in extremely poor replication in avian cells, though a higher basal level was observable (Fig. 1A, lane 10).

This replication defect could not be ascribed to inefficient S-HDAg expression, since provision of abundant S-HDAg by cotransfecting an expression vector for this protein did not rescue the defect (Fig. 1, lane 8, top and bottom panels). (That the expression vector provides functionally competent S-HDAg in *trans* is shown by its ability to complement an HDV frameshift mutation in mammalian [CV1] cells [Fig. 1, lanes 2 and 4]; as expected, this complementation does not occur in QT6 cells [Fig. 1, lanes 7 and 9].) We note that the block to HDV RNA accumulation is not absolute—low levels of HDV antigenomic RNA can occasionally be detected following WT HDV transfection (Fig. 1, lane 10). Typically, however, levels of HDV antigenomic RNA are <1 to 2% of those achieved in mammalian cells transfected in parallel (Fig. 1, lanes 1 to 5).

To determine if the inability to support efficient HDV replication is unique to QT6 or is a general phenomenon of avian cells, we further tested other available avian cell lines by transfecting them with pSVL-D1.1 or pCMV-D1.1 and assaying for the accumulation of antigenomic RNA. Lines tested included

several other quail cell lines as well as chicken cell lines of fibroblastic, epithelial, and lymphoid origin (Table 1). All of these lines gave results identical to those observed in QT6, leading us to conclude that most or all avian cells are nonpermissive for HDV replication.

The defect in avian cells is recessive. The above data (and those of reference 6) indicate that species-specific factors affect the replication of HDV RNA. Two general models can be considered for how such factors may operate: avian cells may lack permissive factor(s) that mammalian cells possess, or avian cells may harbor an inhibitor(s) of HDV replication missing from mammalian hosts. To distinguish between these models, we employed a somatic cell genetic approach involving interspecific cell fusion. First, pSVL-D1.1 was transfected into QM7 quail cells. One day later, CHO-K1 (hamster) cells were added to these cultures. Once the hamster cells were stably attached, half of the cocultures were fused with 50% polyethylene glycol (PEG), while the remainder were simply cocultured in the absence of fusogen. All cultures were incubated in 10 μ M 1- β -D-arabinofuranosylcytosine to prevent overgrowth of unfused cells, and 3 days later RNA from the cocultures was examined by Northern blotting for antigenomic RNA. As shown in Fig. 2A, products of HDV replication were readily detected when the cocultures were fused with PEG, but no HDV replication was observed in the absence of fusion. Similar results are observed in QT6 cells transfected with pCMV-D1.1 (Fig. 2C), although as expected, the basal level of HDV replication driven by this construct is higher (Fig. 1, lane 10). These results indicate that avian cells do not contain dominant inhibitors of replication. Rather, they suggest that avian cells lack permissive factors. Also, the fact that fusion is required for complementation to be observed indicates that the missing permissive factors are cell autonomous—they cannot be supplied in a paracrine fashion from nearby cells but must be directly introduced into the nonpermissive cell.

To examine this complementation at the single-cell level, we conducted the experiment whose results are shown in Fig. 2B. QT6 cells were transfected with pSVL-D1.1(AG), which expresses the antigenomic RNA of HDV from the plasmid DNA via an SV40 late promoter. One day later, C2C12 mouse myoblast clone 8C93, which is stably transfected with β -galactosidase (β -Gal), was added to the QT6 culture and the two populations were fused with PEG. Three days later, the cells were assayed for the accumulation of genomic RNA by in situ hybridization. (We chose to detect genomic rather than antigenomic RNA in this assay because of its greater abundance in infected cells.) In addition, β -Gal was detected by immunofluorescence using a specific monoclonal antibody. As shown in Fig. 2B, HDV RNA was detected only in multinucleate cells positive for β -Gal; we did not see any HDV RNA in any single cells negative for β -Gal (unfused QT6 cells) or in any single cells positive for this enzyme (unfused C2C12 cells).

Impaired HDV replication in avian cells is not due to cell toxicity or cell death. Recently, Chang et al. (6) have suggested that the inability of chicken LMH cells to support HDV replication is due to toxicity resulting from HDAG expression within these cells. This was based upon the observation that when HDAG expression vectors were cotransfected with green fluorescent protein (GFP) reporter plasmids, GFP expression was impaired in a dose-dependent fashion. These authors also

FIG. 2. HDV Replication in heterokaryons of mammalian and avian cells. (A) Quail cells (QM7) were transfected with HDV cDNA of genomic polarity in pSVL vector and cocultured with mammalian cells (CHO-K1). Cell fusion was induced by PEG. HDV antigenomic RNA synthesis was analyzed by Northern blotting for cocultures with (left lane) or without (right lane) fusogen. (B) Quail cells (QT6) were transfected with HDV cDNA of antigenomic polarity and cocultured with a C2C12 derived mouse cell line, which had been stably transfected with β -Gal plasmid. The β -Gal protein was labeled with TRITC (red, upper panel) by indirect immunofluorescence. HDV genomic RNA was detected by fluorescein isothiocyanate-labeled (green, upper panel) oligonucleotide probes. The nuclei of cells were shown by DAPI staining (blue, lower panel). (C) QT6 cells were transfected with pCMV-D1.1. Transfected QT6 cells were cultured either alone (lane 3) or with CHO-K1 cells (lanes 1 and 2); cells of lanes 1 and 3 were induced to fuse with PEG, and then RNA was extracted and analyzed as described in the legend to Fig. 2A.

claimed that the replication defect of HDV in LMH cells could be partially suppressed by the broad-spectrum caspase inhibitor ZVAD-fmk, and they suggested on this basis that induced host cell apoptosis was an important component of the nonpermissive phenotype. Here we show that neither of these assertions is generally true of avian cells. QT6 cells were cotransfected with an enhanced GFP (EGFP) expression vector and a fivefold excess of either pcDNA3, pcDNA3-SHDAg, pcDNA3-lacZ, or pSVL-D1.1; 3 days later, cells were examined by flow cytometry for GFP expression. As shown in Fig. 3, the percentages of transfected cells expressing GFP and the mean levels of GFP expression per cell were not significantly different in any of the groups. In particular, expression of HDAG (panel A) had no adverse impact on GFP expression.

To examine the effects of HDAG expression on apoptosis, we conducted the experiment whose results are shown in Fig. 4. QT6 cells were transfected with pEGFP either alone (panels A

FIG. 3. Flow cytometry analysis of the effect of S-HDAg on GFP coexpression. pEGFP was cotransfected with pcDNA3-SHDAg (A), pSVL-D1.1 (B), pcDNA3 vector (C), and pcDNA3-LacZ (D) into QT6 cells. A negative control without pEGFP transfection is shown (E). The GFP expression was analyzed 3 days after transfection by flow cytometry using a Becton Dickinson FACSCalibur.

and G) or together with pcDNA3-HDAg (panel B), pSVL-D1.1 (panel C), mutant pSVL-D1.1 (panel D), pcDNA3 (panel E), or pcDNA3-lacZ (panel F). Three days later, the cells were examined for GFP expression and, by staining with Cy3-labeled annexin V, for apoptosis. As a control for annexin V staining, we also treated QT6 cells with staurosporine, a potent inducer of apoptosis. Panel A shows that staurosporine indeed induced apoptosis, as judged by both enhanced annexin V staining and by the presence of apoptotic bodies seen by DAPI staining. However, neither HDAg expression nor transfection with WT HDV genomes either impaired GFP expression or enhanced apoptosis over the background level. Similar results have been obtained in DT40 chicken B cells (data not shown), indicating that these effects are not quail specific.

DISCUSSION

These experiments show that there is a profound (though not absolute) block to HDV RNA replication in cells of avian origin and define this block as being due to the absence of one or more permissive factors that are present in mammalian cells of many species and cell types. This block does not appear to be the trivial consequence of cytotoxicity of HDV products in avian cells, nor is it due to enhanced susceptibility to apoptosis. Although it is possible that some avian cell lines may be more sensitive to putative toxicities of this type, our results clearly show that such idiosyncratic toxicities, if they exist at all, cannot explain the general nonpermissiveness of avian cells. This interpretation is also consistent with the fact that, in recent experiments conducted by others (see Fig. 5 in reference 6), ZVAD-fmk treatment of HDV-transfected LMH cells did not appreciably restore viral antigenomic RNA levels, despite improved expression of cotransfected GFP reporters. Thus, even in that cell line, apoptosis was not the reason for impaired HDV replication.

We do not yet know the nature of the permissive factor(s) absent from avian cells. The fact that expression of HDAg *trans* does not reverse the block suggests that the defect is

likely to be downstream of HDAg expression. For example, if HDAg must interact with one or more host components to implement its replicative function, such components would be candidates for the missing factor(s), as would subunit(s) of the host-encoded polymerase required for HDV RNA synthesis. Other possibilities include (but are not limited to) host factors that might be involved in facilitating ribozyme-mediated RNA cleavage, the ligation of the RNA cleavage products, or the stabilization of newly replicated genomes.

Our results have two important implications for the study of HDV replication and pathogenesis. First, they suffice to explain earlier failures to transmit HDV to ducks bearing the duck hepatitis B virus (DHBV). Although HDV was readily transmitted by experimental inoculation of sera from HBV- and HDV-infected subjects into woodchucks bearing the woodchuck hepatitis virus (21), similar attempts at transmission to DHBV-infected ducks have generally been unsuccessful, despite early reports to the contrary (22). Second, the results of the complementation tests illustrated in Fig. 2 suggest that it may be possible to design gene transfer strategies to identify and clone the responsible missing factor(s). The identification of such factors should provide important clues to the nature of the host machinery required for HDV infection.

FIG. 4. Annexin V staining to analyze the effect of S-HDAg on cell death. QT6 cells were transfected with pEGFP either alone (A and G) or together with pcDNA3-SHDAg (B), pSVL-D1.1 (C), mutant pSVL-D1.1 (D), pcDNA3 vector (E), and pcDNA3-LacZ (F). Cells were stained with annexin V before fixation at day 3 after transfection. Some cells were treated with 0.5 μ g of staurosporine per ml (A) to induce apoptosis as a positive control for annexin V-Cy3 staining (middle panel). GFP expression is shown in the left panel and DAPI staining is shown in the right panel.

REFERENCES

1. Antin, P. B., and C. P. Ordahl. 1991. Isolation and characterization of an avian myogenic cell line. *Dev. Biol.* **143**:111–121.
2. Bell, P., R. Brazas, D. Ganem, and G. G. Maul. 2000. Hepatitis delta virus replication generates complexes of large hepatitis delta antigen and antigenomic RNA that affiliate with and alter nuclear domain 10. *J. Virol.* **74**:5329–5336.
3. Brazas, R., and D. Ganem. 1996. A cellular homolog of hepatitis delta antigen: implications for viral replication and evolution. *Science* **274**:90–94.
4. Casey, J. L., K. F. Bergmann, T. L. Brown, and J. L. Gerin. 1992. Structural requirements for RNA editing in hepatitis delta virus: evidence for a uridine-to-cytidine editing mechanism. *Proc. Natl. Acad. Sci. USA* **89**:7149–7153.
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 assembly of hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* **88**:8490–8494.
6. Chang, J., G. Moraleda, and J. Taylor. 2000. Limitations to replication of hepatitis delta virus in avian cells. *J. Virol.* **74**:8861–8866.
7. Chang, M. F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. Lai. 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. *J. Virol.* **62**:2403–2410.
8. 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.
9. Chen, P. J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor. 1986. Structure and replication of the genome of the hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* **83**:8774–8778.
10. Coscoy, L., and D. Ganem. 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. USA* **97**:8051–8056.
11. Cullen, B. R., A. M. Skalka, and G. Ju. 1983. Endogenous avian retroviruses contain deficient promoter and leader sequences. *Proc. Natl. Acad. Sci. USA* **80**:2946–2950.
12. Femino, A. M., F. S. Fay, K. Fogarty, and R. H. Singer. 1998. Visualization of single RNA transcripts in situ. *Science* **280**:585–590.
13. Hadler, S. C., M. De Monzon, A. Ponzetto, E. Anzola, D. Rivero, A. Mondolfi, A. Bracho, D. P. Francis, M. A. Gerber, S. Thung, et al. 1984. Delta virus infection and severe hepatitis. An epidemic in the Yucpa Indians of Venezuela. *Ann. Intern. Med.* **100**:339–344.
14. Kuo, M. Y., 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.
15. Lagunoff, M., R. Majeti, A. Weiss, and D. Ganem. 1999. Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. USA* **96**:5704–5709.
16. Lai, M. M. 1995. The molecular biology of hepatitis delta virus. *Annu. Rev. Biochem.* **64**:259–286.
17. 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.
18. Lazinski, D. W., and J. M. Taylor. 1994. Recent developments in hepatitis delta virus research. *Adv. Virus Res.* **43**:187–231.
19. Luo, G. X., M. Chao, S. Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor. 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. *J. Virol.* **64**:1021–1027.
20. Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt. 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. *Cell* **11**:95–103.
21. 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 delta agent to the eastern woodchuck. *Proc. Natl. Acad. Sci. USA* **81**:2208–2212.
22. Ponzetto, A., M. Rapicetta, B. Forzani, A. Smedile, C. Hele, G. Morace, A. M. di Rienzo, P. Palladino, L. Avanzini, J. L. Gerin, et al. 1987. Hepatitis delta virus infection in Peking ducks chronically infected by the duck hepatitis B virus. *Prog. Clin. Biol. Res.* **234**:47–49.
23. 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.
24. 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.
25. Saracco, G., S. Macagno, F. Rosina, and M. Rizzetto. 1988. Serologic markers with fulminant hepatitis in persons positive for hepatitis B surface antigen. A worldwide epidemiologic and clinical survey. *Ann. Intern. Med.* **108**:380–383.
26. Wang, D., J. Pearlberg, Y. T. Liu, and D. Ganem. 2001. Deleterious effects of hepatitis delta virus replication on host cell proliferation. *J. Virol.* **75**:3600–3604.
27. Wei, Y., and D. Ganem. 1998. Activation of heterologous gene expression by the large isoform of hepatitis delta antigen. *J. Virol.* **72**:2089–2096.