Replication of Human Hepatitis Delta Virus in Primary Cultures of Woodchuck Hepatocytes

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We obtained two lines of evidence that monolayer cultures of primary woodchuck hepatocytes support replication of the genome of human hepatitis delta virus (HDV). (i) From a Northern (RNA blot) analysis of the HDV-related RNA in infected cultures, both genomic and antigenomic 1.7-kilobase RNA species were detected at 11 days after infection. The ratio of genomic RNA to antigenomic RNA was 2:1 to 10:1, comparable to that previously reported in studies of experimentally infected chimpanzees and woodchucks. (ii) Replication in culture was also demonstrated by in situ hybridization with a strand-specific probe. Such studies showed that only a small fraction of the cultured cells supported replication and that in such cells the relative and absolute levels of the HDV RNAs were comparable to those in liver cells infected in vivo. Furthermore, as with the in vivo studies, the HDV RNAs were predominantly localized to the nucleus. In summary, we demonstrated that cultured cells supported both the early events of HDV adsorption and penetration and the intermediate events of genome replication.

The human hepatitis delta agent was discovered as a consequence of the initial finding by Rizzetto et al. (11) of a novel antigen, the delta antigen, in the nuclei of human liver biopsies. Subsequent experimental transmission studies with chimpanzees (12) and woodchucks (9) established that this antigen was encoded by an infectious agent, hepatitis delta virus (HDV). These studies also showed that HDV transmission was dependent upon the presence of a coinfecting hepadnavirus. Recently, recombinant probes of the single-stranded RNA genome of HDV have been made (5). The genome has been shown to be circular (4, 8, 16), and the complete 1,678-base sequence has been reported (16). Also, characterization of the HDV RNA replicative intermediates in the livers of experimentally infected chimpanzees and woodchucks has begun (4, 8). One particular problem of characterizing HDV replication in infected animals is that the peak of HDV replication in the liver is not reached for about 5 weeks. Around this time there is usually a dramatic increase in HDV replication, lasting only several days, and this is followed by an equally dramatic decrease in HDV replication (9). Consequently, it is often difficult to obtain liver samples at the optimal time. For this and other reasons, we undertook experiments to demonstrate the replication of HDV in cultured cells, and we achieved this objective by using primary cultures of woodchuck hepatocytes. Contrary to our expectations, HDV genome replication in this system did not seem to require coinfection with a hepadnavirus.

MATERIALS AND METHODS

Virus stocks. For these studies, one source of HDV was used: serum from a woodchuck (number 146) experimentally infected with both woodchuck hepatitis virus (WHV) and human HDV. The source of WHV was serum from another experimentally infected woodchuck.

Hepatocyte preparation. Viable woodchuck hepatocyte suspensions were obtained by an adaptation of the collagenase perfusion method of Berry and Friend (2). WHV-infected and noninfected woodchucks were anesthetized, and the livers were surgically exposed. The livers were cleared of blood by perfusion in situ with a single pass (2 liters at 150 ml/min) of Swimm 77 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.45) and 0.5 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], followed by 2 liters of the same medium containing 2.5 mM CaCl₂ and 0.5 mg of collagenase type I (Sigma Chemical Co., St. Louis, Mo.) per ml. Perfusion was carried out at 37°C through the inferior vena cava, and the perfusate was collected through the portal vein. A second pass of the collagenase-containing perfusate was used in some experiments to increase the yield of viable cells. After perfusion, the livers were removed and placed in 400 ml of L-15 medium (GIBCO) supplemented with 20 mM HEPES buffer (pH 7.45), 1 mg of glucose per ml, 10 μ g of insulin per ml (Sigma), 10 μ M hydrocortisone hemisuccinate (Sigma), 100 μ g of streptomycin per ml, 300 μ g of penicillin per ml, and 5% fetal bovine serum (complete medium). The livers were minced with scissors, and the liver fragments were gently stirred with a hand-held egg beater until viable cells were no longer dispersed. Undispersed tissue was removed by filtration through cheesecloth, and cells were recovered by centrifugation at 500 $\times g$ for 5 min. The cell pellet was washed twice in complete medium and suspended at 4°C in 4 volumes of Hanks balanced salt solution (lacking divalent cations) plus 1 volume of dimethyl sulfoxide. The cell suspension was frozen in 1-ml portions and stored under liquid nitrogen. The proportion of viable cells in the original suspension varied between approximately 20 and 80%, and recovery of viable hepatocytes was nearly complete after thawing.

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Hepatocyte culture. The wells of Costar plates (24 16-mm-diameter wells), with or without an added glass cover slip (12-mm diameter), were coated with rat tail collagen before seeding of about 300,000 freshly thawed hepatocytes per well. Cultures were maintained in complete medium. Cells were infected 1 day after seeding, and the medium was then changed every second day.

Nucleic acid extraction. To extract a single well of cells, the medium was removed and the cells were solubilized with 2 ml of 10 mM Tris hydrochloride (pH 7.4)–10 mM EDTA–1 mg of pronase per ml–0.1% sodium dodecyl sulfate. This mixture was incubated at 37°C for 40 min, extracted once with 2 ml of phenol and twice with 5 ml of ether, and then precipitated with 2 volumes of ethanol in the presence of 0.2 N sodium acetate (pH 5) and 10 µg of dextran carrier (Sigma) (15).

Nucleic acid analysis. The total nucleic acid was subjected to glyoxalation and agarose gel electrophoresis as previously described (4). The nucleic acids were then electrotransferred to a charged nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) at 50 V for 2 h using an apparatus from International Biotechnologies, Inc., New Haven, Conn. With no baking step, the nucleic acids on the filter were deglyoxalated by the procedure of Thomas (13). The filter was then subjected to prehybridization and hybridization with M13 strand-specific probes as previously described (4). The only additional modifications were hybridizations at 50°C rather than 42°C and posthybridization washes with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 60°C rather than 55°C.

In situ hybridization. The in situ hybridization procedures used were as described elsewhere (E. Gowans, B. Baroudy, R. Purcell, and J. Gerin, *Science*, in press) and make use of RNA probes rather than the above-mentioned M13 probes. The probes were transcribed with SP6 and T7 polymerases from two recombinant plasmids, BMB-37 and BMB-104, respectively, which together represent the complete HDV sequence, and were radiolabeled with [¹²⁵I]CTP (a generous gift from New England Nuclear Corp., Boston, Mass.). Cell culture cover slips were washed in phosphate-buffered saline, air dried for 30 min, fixed in 4% paraformaldehyde for 5 min at room temperature, and then stored in 70% ethanol at 4°C for 24 to 48 h before hybridization. Hybridization was performed for 3 h at 50°C, and the cover slips were washed at high stringency (70°C in 0.1× SSC for 10 min) before autoradiography.

RESULTS

It has recently been reported that infection of woodchucks with human HDV leads to viremia in animals chronically infected with WHV but not in animals free of WHV (9). The progeny delta virus obtained from WHV carriers appears to be encapsidated in an envelope encoded by WHV, suggesting that it may be more highly tropic for woodchucks. This possibility led us to investigate whether the woodchuck-passaged virus could infect primary woodchuck hepatocyte cultures. Cells for this purpose were obtained from both uninfected and WHV-infected woodchucks and stored under liquid nitrogen.

When the frozen hepatocytes were thawed and plated on collagen gels, we found that only a small fraction were able to attach and spread to form a monolayer culture. Thus, after 1 day the monolayer was washed with medium to remove most of the nonviable cells. At this time, as shown by

phase-contrast microscopy (Fig. 1), the residual cells appeared healthy and were frequently formed into arrays reminiscent of liver plates (1); moreover, by nigrosin staining (6) we could deduce that >60% of these cells were viable (data not shown). Consistent with the fact that hepatocytes are largely terminally differentiated nondividing cells, it was noted that most of the cells were in fact binucleate. Two cultures were infected with serum from a viremic woodchuck that had been experimentally infected with both HDV and WHV. This HDV-positive serum thus contained both HDV and WHV. As a control for this experiment, we infected a separate culture with WHV alone. After 11 days, the nucleic acids were extracted and examined by electrophoresis. Both the genomic strand (Fig. 2A) and the antigenomic strand (Fig. 2B) of HDV could be detected in the HDV-infected cultures (Fig. 2, lanes 2 and 3). Most of both the genomic and antigenomic RNA species detected in the infected cells were of the same electrophoretic mobility as the 1.7-kilobase virion RNA. Small amounts of two bands of genomic strand RNA with mobilities of about 3.5 kilobases were sometimes detected on longer exposure (data not shown). These latter species were previously detected, but in relatively greater amounts, in HDV-infected livers (4) but are not found in virus particles.

The hepatocytes used in the experiment summarized in Fig. 2 were from an animal previously infected with WHV. Thus, when we rehybridized hepatocyte RNA with a probe that was strand specific to the WHV genome, we could detect related sequences in all of the samples, with sizes up to 3.2 kilobases (Fig. 2C). It should be noted that we were unable to detect any increase or decrease in such sequences for cultures with or without an additional WHV infection. Hepatocytes from two other animals were similarly tested and found to replicate the HDV RNA successfully. One such animal had not previously been infected with WHV, and although HDV RNA replicated in the cultured hepatocytes, there was no indication of WHV infection (data not shown).

Other experiments were undertaken to explore two experimental variables, namely, the multiplicity of infection and the time course of HDV RNA synthesis. Such experiments were performed, and the quantitations are summarized in Fig. 3A and B, respectively.

FIG. 1. Phase-contrast microscopy of cultured hepatocytes. Cells were cultured as described in Materials and Methods and are shown here at 1 day after plating. Bar, 100 µm.

The yield of genomic RNA, as detected at 14 days, showed an increase proportional to the amount of serum used as an inoculum (Fig. 3A). However, there was an indication of saturation in that 5 μ l of serum gave only a small increase relative to that seen when 1.5 μ l was used. The latter inoculum was calculated to correspond to about 5,000 molecules of HDV RNA per cell (data not shown). We quantitated the amount of HDV RNA in cultured hepatocytes for comparison with levels that we previously measured in liver samples from infected chimpanzees and woodchucks (4). The amount of antigenomic RNA was measured as an indicator of active infection since this RNA is not found in HDV virions.

The time course of infection usually showed that the amounts of both genomic and antigenomic RNAs were not significant until at least 6 days after infection (Fig. 3B). (In some experiments, genomic and antigenomic RNAs were detectable at day 6.) The ratio of genomic RNA to antigenomic RNA was in the range of 2 to 20, comparable to the range of 5 to 20 obtained previously with RNAs from livers of infected chimpanzees and woodchucks. The maximum amount of genomic RNA was only about 3,000 molecules per average cell. This value was only about 1 to 2% of what we had previously observed for infected chimpanzee and woodchuck livers.

In the experiments described above, HDV replication was monitored solely by detection of HDV-related nucleic acids in total infected cultures. Independent evidence was obtained for infection of single cells by using *in situ* hybridization. Figure 4 shows data obtained with a probe for genomic

FIG. 2. Gel analysis of RNA from infected hepatocytes. After 1 day in culture, cells were infected as follows: lanes 1 and 2, WHV serum; lanes 2 and 3, HDV-positive serum. After 11 days, the nucleic acids were extracted, glyoxalated, and examined by agarose gel electrophoresis and transfer to a nylon membrane as described in Materials and Methods. The membrane was successively hybridized with strand-specific M13 probes as follows: A, genomic HDV; B, antigenomic HDV; C, genomic WHV. Identical results were obtained when the first probe used was antigenomic HDV followed by genomic HDV (data not shown). The numbers on the right are molecular sizes in kilobases.

FIG. 3. Quantitation of HDV genomic RNA in infected hepatocytes as a function of multiplicity of infection and time since infection. Data were obtained as for Fig. 2, with autoradiograms being subjected to quantitation with an E-C densitometer and a Hewlett-Packard HP3380 integrator. Panel A shows the relative amount of genomic HDV RNA per culture after 14 days as a function of the volume of HDV-positive serum used as an inoculum. Panel B shows quantitation at various times after infection with a fixed volume of HDV-positive serum (1.25 μ l). The number of molecules of both genomic and antigenomic delta RNAs per average cell was deduced as previously described (4).

HDV RNA. By this assay, only about 2% of the cells were considered to be infected; this result was compatible with the quantitation of genomic RNA per average cell, as mentioned above. Both mono- and binucleate cells were susceptible to infection. In susceptible cells, the hybridization signal was very high. Quantitation of the *in situ* signals was therefore carried out. It was found that the ratio of genomic RNA to antigenomic RNA was about 5:1, in agreement with the total-nucleic-acid studies (data not shown). Relative to tissue sections of infected chimpanzee liver, the signals in individual cells were approximately comparable (data not shown; Gowans et al., *in press*). These findings, together with the previous quantitation of HDV in total cultures, were consistent with the interpretation that the HDV genome can replicate in at least some cultured cells to levels that appeared equivalent to those in hepatocytes of experimentally infected animals.

In situ analysis revealed a feature of delta replication not yet studied with extracts of cultured hepatocytes, namely, that most genomic RNA and antigenomic RNA accumulates in the nucleus. Quantitation of grains showed more than 90% in the nucleus (Fig. 4).

FIG. 4. HDV RNA detected by in situ hybridization in the nuclei of infected hepatocytes. After 1 day in culture on collagen-coated cover slips, the hepatocytes were infected with HDV-positive serum and incubated for 11 days. In situ hybridization was carried out as described by Gowans et al. (in press), with a genome-specific RNA probe. The autoradiographic exposure time was 3 days, and the cells were stained by hematoxylin and eosin. Bar, 50 μ m.

DISCUSSION

The present study showed that primary cultures of woodchuck hepatocytes were able to support at least part of the replication cycle of HDV. At 10 to 14 days after infection, significant amounts of both genomic and antigenomic delta RNAs were detected. In cells that were infected, the amounts of delta RNA were comparable to those obtained for infected animals. Furthermore, as with the studies of RNA in infected livers (4), we were able to detect circular forms of both genomic and antigenomic RNAs and also small amounts of species greater than 1.7 kilobases long (data not shown).

There were, however, several limitations to this experimental system. (i) Only a few percent of the cells supported genome replication. This value is actually comparable to those of HDV infections of woodchucks in which the fraction of hepatocytes expressing HDV antigen was reported to be less than 10% during the first passage (9). (ii) The cycle of replication in the cultured cells was probably incomplete. Actually, the release of progeny virions was not demonstrated. (iii) The studies were with only one source of virus (from the serum of an HDV-infected woodchuck). To date, infections with serum from an HDV-infected human, with or without coinfection with WHV, have been unsuccessful. (iv) The host cells required elaborate perfusion and culture conditions, and even then they were terminally differentiated, undergoing no further replication. We have so far been unable to achieve comparable infection of primary duck hepatocytes (14) or of continuous liver cell lines, such as human HepG2 and Hep3B (7). Nevertheless, the hepatocyte system as it exists is sufficient to help answer some of the important questions regarding the molecular biology of replication of this novel infectious agent.

Probably the prime question of HDV replication is to explain the role(s) of the hepadnavirus. It is clear from the work of others that animal transmission of HDV depends upon prior infection or coinfection with a hepadnavirus (9,

12). However, is this true for those aspects of HDV replication that take place in cultured cells? Consider the role of the hepadnavirus at three stages in HDV replication. (i) It is known that the HDV envelope contains the surface antigen of the hepadnavirus helper. Does this mean that the envelope of the hepadnavirus specifies the host range of HDV, at least at the level of virus uptake? This idea is plausible, but it remains possible that HDV encodes an as yet undetected protein (if it is correct that the delta antigen must be considered strictly internal [3]) which is present, with hepadnavirus surface antigen, on the surface of the HDV particle. (ii) As far as the middle events of the HDV replication cycle are concerned, we have shown that the culture system allows what appears to be normal replication of the genome. Does the hepadnavirus play a role in this? One argument against this possibility is that, in the presence or absence of HDV, we have not been able to infect cultured cells with WHV and subsequently detect WHV genome replication. Also relevant is the fact that the standard HDV inoculum probably has many more infectious units of HDV relative to those of hepadnavirus; in the serum of an HDV-positive chimpanzee, the ratio was reported to be as much as 100,000:1 (10). (iii) Finally, to complete the HDV replication cycle, the newly replicated HDV genomic RNA has to be assembled into virus particles and released from the cell. At least in infected animals, this step seems to be dependent upon concomitant hepadnavirus replication because the envelope of the released HDV particles contains hepadnavirus surface antigen. Clearly, many of the details of the HDV replication cycle remain to be elucidated, especially the reason why the genomic HDV is specifically packaged, how the delta antigen insinuates itself in the packaging, and whether or not there is an additional HDV-coded protein(s) involved.

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