Replication of the Human Hepatitis Delta Virus Genome Is Initiated in Mouse Hepatocytes following Intravenous Injection of Naked DNA or RNA Sequences

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As early as 5 days after DNA copies of the hepatitis delta virus (HDV) genome or even in vitro-transcribed HDV RNA sequences were injected into the mouse tail vein using the hydrodynamics-based transfection procedure of F. Liu et al. (Gene Ther. 6:1258–1266, 1999), it was possible to detect in the liver by Northern analyses of RNA, immunoblots of protein, and immunostaining of liver sections what were considered typical features of HDV genome replication. This transfection strategy should have valuable applications for in vivo studies of HDV replication and pathogenesis and may also be useful for studies of other hepatotropic viruses.

There are obvious advantages to the development of small animal models for studying the replication and associated pathogenesis of human hepatitis virus infections. To achieve this, several previous studies of human hepatitis delta virus (HDV) have considered the expression of HDV sequences in mice. Guilhot et al. constructed mice transgenic for expression of the delta proteins, in the absence of HDV genome replication, and found no obvious signs of toxicity (8). Polo et al. later made mice transgenic for HDV sequences (20) and in a separate study considered events following intramuscular injection of HDV DNA sequences (21). A limitation of both studies was that the observed replication was not liver specific. For the transgenic animals, replication in the liver was <1% relative to that detected in the skeletal muscle, and the immunopositive cells in the liver were bile duct cells rather than hepatocytes. While these studies made use of expression from HDV DNA sequences, a different strategy was used in an earlier study by Netter et al. in which HDV genome replication was initiated in mice following intravenous or intraperitoneal injection of HDV particles taken from the serum of an infected woodchuck (18). These infections were liver specific but involved no more than 0.6% of the mouse hepatocytes. More recently, Ohashi et al. used surgical procedures to implant human hepatocytes beneath the kidney capsule of a mouse, so as to subsequently be able to infect these cells with both HDV and hepatitis B virus (19).

With these previous studies in mind, we were intrigued by the recent publication of a hydrodynamics-based in vivo transfection procedure. Liu et al. first showed (14) and two subsequent studies have confirmed (25, 26) that following the rapid injection of cDNA sequences in a large volume into the tail vein of a mouse, there can be transient expression of these sequences in many tissues, the highest levels being in the liver. We therefore undertook to determine whether such transfection of an infectious HDV cDNA clone could lead to the initiation of genome replication in the mouse liver.

Previous studies have developed many HDV cDNA constructs which when transfected into cultured cells will induce HDV genome replication (10, 12, 24). Our initial experiment was to take one such construct, pDL456, and transfet it into mice by using the procedure of Liu et al. (14). Female BALB/cAnNcl mice at 3 weeks of age (about 16 g) were injected with 1.4 ml of saline containing the nucleic acid sequences (5 μg unless stated otherwise). Injection through the tail vein was carried out in a time of less than 5 s. Since pDL456 uses a simian virus 40 late promoter to direct the transcription from DNA of a greater-than-unit-length species of antigenomic HDV RNA, the accumulation of unit-length genomic RNAs can arise only via RNA synthesis that is RNA directed. We examined whether such genomic RNA could be detected in the livers of mice following injection (Fig. 1, lanes 2 to 8). No genomic HDV RNA was detected after 1 or 2 days but by day 5 notable accumulation had occurred. The amount increased by day 9 and then decreased from days 15 to 30. For comparison, the amount of genomic RNA detected at day 5 in transfected mouse liver was almost as much as that achieved at 5 days after transfection of cultured Huh7 cells with the same DNA construct (lane 9) (under conditions where the transfection efficiency was monitored, using a construct expressing green fluorescent protein, as about 40%). Not surprisingly, the amount was about five times less than for the liver of a woodchuck chronically infected with woodchuck hepatitis virus at the peak of a superinfection with HDV (lane 10). As in the original study of Liu et al., an increase in the amount of transfected DNA from 5 to 25 μg had very little effect on the accumulation of HDV genomic RNA at 5 days (lanes 12 and 13, respectively).

We also tested transfecting not with DNA but with 5 μg of antigenomic RNA, along with 0.3 μg of a capped subgenomic RNA, a combination of RNAs known to initiate HDV genome replication in transfected cultured mammalian cells (16). To our surprise, after 5 days HDV genome replication was detected in the liver of the transfected mouse (lane 14) and to a level equivalent to that initiated by transfection with the DNA construct (lane 12). Somehow the RNAses known to be present in the serum were unable to inactivate that naked input RNA
which initiated the HDV genome replication. As a control for these studies, we tested transfection with each RNA separately, and detected no initiation of genome replication (data not shown).

For the DNA transfection studies, we also examined at day 5 the RNA extracted from spleen, kidney, lung, heart, and hind leg skeletal muscle. Genome replication was not detected (that is, was <1% relative to that in the liver) (data not shown).

We used immunoblots to detect the delta antigen in the transfected livers (Fig. 2). As for the genomic RNA, there was a similar appearance and subsequent disappearance of the small delta protein. We also looked for the large delta protein, which has not previously been detected in mouse cells undergoing HDV genome replication (20, 21) but is a constant occurrence for replication in most other settings (15, 22). This large delta protein was not obvious (Fig. 2, lanes 3 to 6). However, from examination of a five-times-greater amount of total protein at day 9, the large species could be clearly detected (lane 11). This identification is based on comparison with the proteins from an infected woodchuck liver (lane 8) and with the migration of protein from Huh7 cells transfected with constructs that express either small delta protein (lane 9) or both small and large delta proteins (lane 10). From a quantitation of the data in lane 11, we deduced that 3% of the accumulated delta protein in the liver of the mouse at 9 days after transfection with HDV DNA was of the large form, consistent with posttranscriptional RNA editing. Nevertheless, this value is lower than what we have detected for transfected cells (23). It is also low in comparison to the value of 27% for the infected woodchuck liver (lane 8). However, one must remember that the woodchuck tissue was taken at the peak of HDV replication, and in the transfected mouse there is no helper virus and no spread of the HDV.

The above studies showed that in the liver of a transfected mouse there was both the accumulation of HDV RNA-directed RNA transcripts and the appearance of the delta antigens. To determine if this replication was taking place in hepatocytes, we used immunostaining to detect the delta proteins in liver sections. At 5 days after transfection with 5 μg of HDV DNA, we observed that most of the staining was in the nuclei of hepatocytes (Fig. 3A). As expected for normal hepatocytes, many of these positive cells were binucleate. Overall, about 2.9% (92/3,155) of hepatocytes were positively stained for delta protein. (Similar results were obtained after the two-RNA transfection [data not shown].)

When the same procedure was applied to liver samples taken at day 9 after transfection, we observed two differences. First, the number of positively staining hepatocytes increased to 3.8% (158/4,115). (We saw similar increases with the Northern analyses [Fig. 1] and the immunoblot assays [Fig. 2].) Second, and possibly of more significance, the intracellular distribution of this staining was in most cases different. Now, for the majority of positive cells the staining was spread throughout both the nucleus and the cytoplasm. In only 16% of the positive cells did we observe staining that was predominantly nuclear.

At day 15, the positive staining cells were reduced to 1.4% (44/3,048). As can be seen in Fig. 3C, the intracellular distribution of staining was in most cases throughout the cells. For <10% the staining was predominantly nuclear, and in some
cases the staining was mostly cytoplasmic. Further studies will be needed to determine the basis for these time-dependent changes in delta antigen distribution. For example, as with previous studies of transfected cultured cells, is there a role of the large delta protein and possibly other variants of the delta protein in provoking such changes (1, 2)?

We also noted that the cells that stained positive for delta antigen were not uniformly distributed throughout the liver sections. This is more clearly shown in Fig. 3D, which is as for Fig. 3B but at a lower magnification. There was a preponderance of delta antigen localization near blood vessels, which can be identified as the central vein but not the portal vein. This preference may be a consequence of the hydrodynamics of the transfection procedure and is consistent with the original explanation offered by Liu et al. (14) that as a consequence of the speed and volume of the injection, the DNA solution will arrive at the liver against the normal direction of circulation, via the hepatic vein and the associated central vein system.

In two previous studies on HDV replication in mouse tissues, there was a specific claim that the relatively rare subgenomic polyadenylated RNA, considered to act as the mRNA for the delta protein, was not detectable (20, 21). For this reason, we made additional efforts to detect this species in the liver. To enrich for the polyadenylated species, total liver RNA from mice at 5 days after HDV DNA transfection was subjected to two rounds of selection by binding to oligo(dT)-cellulose. As a positive control, the RNA from the liver of an infected woodchuck was similarly fractionated. The RNAs were then examined by Northern analysis to detect antigenomic RNA species.

As shown in Fig. 4, we initially could not detect the subgenomic-sized mRNA for either the total RNA (lane 3), the nonpolyadenylated RNA (lane 4), or the polyadenylated RNA (lane 5) from the transfected mouse liver. The same was true for the corresponding RNA samples from the infected woodchuck liver (lanes 7 to 9). However, this lack of detection reflected only on the fact that the species was of relatively low abundance, because when we examined a 100-times-larger sample of polyadenylated RNA, this species became obvious for both the transfected mouse (lane 6) and the infected woodchuck (lane 10).

While these studies show that the transfected mouse expresses a polyadenylated RNA with apparently the same size as the mRNA detected in infected woodchucks, we thought it necessary to test whether the 5' ends were in fact the same. Previous studies have shown that the 5' end for the mRNA detected in infected woodchucks and transfected cultured cells is located predominantly at nucleotide (nt) 1630 (7). This was done by a 5'-RACE (rapid amplification of 5' cDNA ends) procedure employing a Smart PCR cDNA library construction procedure.
FIG. 4. Northern detection of HDV sequences following selection of polyadenylated RNA species. RNA was extracted from transfected mouse liver (day 5) and infected woodchuck liver (day 25). Two rounds of selection on oligo(dT)-cellulose (9) were used to prepare polyadenylated (A+) and nonpolyadenylated (A−) RNA. Lanes 3 to 6 represent RNAs from the transfected mouse. Lane 3, 1.5 μg of total RNA; lane 4, A− RNA from 1.5 μg; lane 5, A+ RNA from 1.5 μg; lane 6, A+ RNA from 150 μg. Similarly, lanes 7 to 10 represent samples from infected woodchuck liver, except that all samples were reduced by 50%. Lane 1 is an end-labeled 1-kb DNA ladder as size marker, and lane 2 is 500 pg of unit-length HDV cDNA as a quantitation standard. The positions of unit-length antigenomic RNA and of mRNA are indicated at the right.

help determine the role of the host in the observed clearance of virus replication in hepatocytes. Also, extracts made from transfected liver might help in studying aspects of genome replication, including RNA transcription and the role of host proteins.

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