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Initiation of Replication of the Human Hepatitis Delta Virus Genome from Cloned DNA: Role of Delta Antigen

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Beginning with three partial cDNA clones of the RNA genome of human hepatitis delta virus (HDV), we assembled the complete 1,679-base sequence on a single molecule and then inserted a trimer of this into plasmid pSVL, a simian virus 40-based eucaryotic expression vector. This construct was used to transfect both monkey kidney (COS7) and human hepatocellular carcinoma (HuH7) cell lines. In this way we obtained replication of the HDV RNA genome and the appearance, in the nucleoli, of the delta antigen, the only known virus-coded protein. This proved both that the HDV genome could replicate in nonliver as well as liver cells and that there was no requirement for the presence of hepatitis B virus sequences or proteins. When the pSVL construct was made with a dimer of an HDV sequence with a 2-base-pair deletion in the open reading frame, genome replication was reduced at least 40-fold. However, when we cotransfected with a plasmid that expressed the correct delta antigen, the mutated dimer achieved a level of genome replication comparable to that of the nonmutated sequence. We thus conclude that the delta antigen can act in *trans* and is essential for replication of the HDV genome.

Hepatitis delta virus (HDV) is a naturally occurring infectious agent of humans (20), and it has also been experimentally transmitted to both chimpanzees and woodchucks (18, 21). In all these cases, replication is apparently dependent on coinfection with a hepadnavirus. It is clear that the coat of the HDV particle, as found in the serum of infected animals, contains the surface antigen encoded by the hepadnavirus (2, 3, 18, 21), and so it would seem that the hepadnavirus provides functions for both HDV entry and release. Also inside the HDV particle is a single-stranded RNA species of about 1,700 nucleotides in length (21), which is unusual both because it is in a closed circular conformation and because it can fold on itself into an unbranched rod structure (5, 9, 29). This RNA, known as the genomic RNA, can also be found inside infected liver cells along with a relatively lower amount of a cRNA, the antigenomic RNA (5). Since no DNA intermediates have been detected, genomic replication is considered to procede via RNA-directed RNA synthesis, probably via a rolling-circle model (28). A major question has been whether the hepadnavirus provides functions needed for this replication of the HDV genome (28). In this study, we have resolved this question by transfection of cultured cells with molecularly cloned HDV sequences.

Another major question about HDV replication has been the function of the only known protein, the delta antigen. Rizzetto et al. (20) first detected this antigen in the nuclei of human liver biopsies and subsequently went on to prove the existence of HDV as a new infectious agent (21). It is now known that the antigen is also present inside the HDV particles (2, 3, 21). Multiple forms of the protein have been reported, usually in the range from 20 to 30 kilodaltons (kDa) (1, 2, 32). From molecular cloning studies, it would seem that a 24-kDa species is the most common species (4, 9, 12, 26, 29, 30) and the other species may arise from sequence variations in the HDV genome (26). The protein has recently been shown to be phosphorylated at serine residues and claimed to have RNA-binding activity (4). We have now used studies with molecularly cloned sequences to show that the antigen is essential for the replication of the genome and to obtain clues as to what one of its functions might be.

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MATERIALS AND METHODS

Cloning. Originally we used the RNA from the liver of an HDV-infected woodchuck to create a cDNA library in λgt11 (5, 9). For the present work, three of these clones were transferred to the plasmid pGem4Blue (Promega). Parts of these inserts were assembled onto a single plasmid, oriented relative to the *Eco*RI site at position 1427 (using the previously described notation for the 1,679-base [b] genome [9]). Then, by a strategy involving partial digestion and additional insertions, the total insert was raised to a trimer. This trimer was excised with *Bam*HI and *Pvu*II and force-cloned into the vector pSVL (Pharmacia) that had been cut with *Bam*HI and *Sma*I. Other pSVL constructs were made that contained the 1.1-kilobase-pair (kbp) fragment *Xba*I (781)-*Bgl*II (224), which spans the open reading frame for the delta antigen (1598 to 1014 [9]) and a dimer of the complete genome except for the deletion of 2 bp at 1434 to 1435.

Transfection. Basically we used the DEAE-dextran procedure as described by Cullen (6). The procedure was scaled down to 100,000 cells in a 16-mm-diameter well and only 120 ng of DNA in 50 μl per well. After transfection, the medium was changed every second day until harvest. The cells were then washed and removed by scraping. Half of the cells were used for RNA extraction (5) and Northern analysis (5), and the other half were examined by Western blotting analysis (7). In some experiments, the cells were grown on glass cover slips within the well and subsequently examined by immunofluorescence.

Immunofluorescence. The basic procedure used was a protocol from James Nelson (16), with modifications based on the study of Ou et al. (17). At 10 days after transfection of cells grown on a 12-mm-diameter glass cover slip, the cover slip was removed, rinsed at 4°C with phosphate-buffered saline (PBS), and then fixed and permeabilized with 100% methanol that had been cooled to −20°C. The cover slip was washed twice with PBS and blocked with PBS containing 1% bovine serum albumin prior to incubation with a 1:100

dilution in PBS of a fluorescein-conjugated patient antidelta antibody. The latter incubation was in a humidified incubator at 37°C for 30 min. The cover slip was washed three times with PBS and then incubated for 10 min with PBS containing ethidium bromide (1 μg/ml). The cover slip was again washed with PBS and then mounted onto a glass slide with Evanol (22) prior to examination with a Zeiss fluorescence microscope.

RESULTS

A trimer of the HDV genomic sequence was assembled in the simian virus 40 (SV40)-based vector pSVL. The construct was made so that the RNA transcription, controlled by the SV40 late promoter, should produce transcripts of genomic HDV RNA. This plasmid was transfected into two different cell types. The first was a human hepatoma cell line, HuH7, originally isolated by Nakabayaski et al. (15). Others have since shown that transfection of these cells by hepadnavirus sequences can lead to the assembly of infectious virus particles (19, 31). The second source of host cells was the monkey kidney cell line COS7, which has been transformed by Gluzman with the T antigen of SV40 (8). In these cells, unlike HuH7, we expected that the pSVL vector would be able to undergo significant DNA replication.

At various times after transfection we extracted the nucleic acids, removed the DNA with DNase, and then examined the RNA after glyoxalation and electrophoresis into gels of 1.5% agarose. As demonstrated in Fig. 1, 9 days after transfection we could detect significant amounts of HDV antigenomic RNA in both the COS7 cells (lane 1) and HuH cells (lane 2). The mobility of the major species of RNA was the same as that seen in the liver of an HDV-infected chimpanzee (lane 3). Relative to DNA markers, the species was equivalent to about 1.7 kb in length, as expected for the unit length of the HDV genome. It is important to realize that in the absence of HDV genome replication, the DNA used in transfection would be expected to produce not antigenomic RNA but only genomic RNA, and in addition, this RNA would not be 1.7 kb in length but about 5.1 kb, the size of the trimer.

In other gel analyses we also showed that the majority of the HDV RNA was not linear in conformation but circular (data not shown), just as it is in virions (5, 9, 12, 29) and naturally infected animals (5). Presumably the nascent HDV RNA transcripts undergo the specific cleavage and ligation reactions similar to those we have recently demonstrated with purified RNAs (10, 24, 28). We also found that in some cases the transfected cells demonstrated detectable amounts of antigenomic RNA as early as 2 to 3 days after transfection, although the results were generally clearer by 7 to 11 days.

The above studies all indicated that the HDV genome might be replicating in these cells. As a further marker, we examined the transfected cells for the appearance of the delta antigen. This was done in two ways. As a first approach we used a Western blotting analysis. As shown in Fig. 2, we detected in transfected COS7 cells a single band of 23.9 kDa (lane 1). (From our previous nucleotide sequencing data [9], we predicted the major band would be 195 amino acids or 22 kDa.) This pattern was similar to that obtained with an HDV-infected culture of primary woodchuck hepatocytes (lane 2) (27) or a liver sample from an HDV-infected patient (lane 3). Cell fractionation studies of transfected cells showed that about 85% of this activity was nuclear (data not shown).

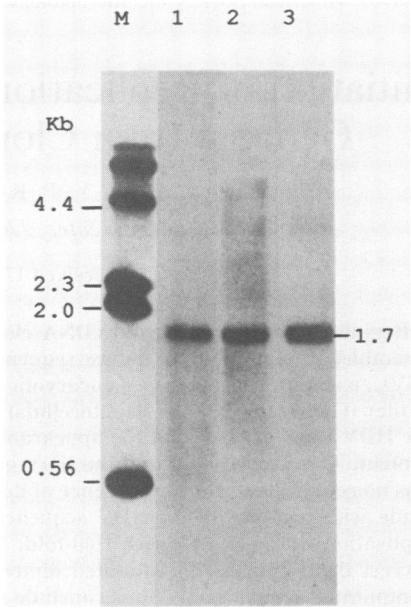

FIG. 1. Northern blot to detect antigenomic HDV RNA following transfection. RNA samples were denatured with dimethyl sulfoxide in the presence of glyoxal (13), subjected to electrophoresis in a gel of 1.5% agarose, and then transferred electrophoretically to a ZetaProbe nylon membrane (Bio-Rad) (5). Antigenomic HDV sequences were then detected by hybridization with an RNA probe to the entire antigenomic HDV RNA genome (5, 9). Lanes 1 and 2 contain samples of COS7 and HuH7 cells, respectively, at 9 days after transfection with the trimer of HDV in pSVL. Lane 3 contains an RNA sample from the liver of an infected chimpanzee. Lane M corresponds to end-labeled fragments of phage lambda DNA digested with *Hin*dIII.

As demonstrated in Fig. 3, our second approach for detecting delta antigen in transfected cells was by immunofluorescence of fixed cells. We could detect the appearance of delta antigen in about 1% of the transfected COS7 cells (Fig. 3C). By comparison of the same field studied by either phase-contrast (Fig. 3A) or staining with ethidium bromide (Fig. 3B), we could demonstrate that the delta antigen was predominantly located in the nucleoli of these COS7 cells. We obtained similar results with transfected HuH7 cells (data not shown). This location was somewhat puzzling, since in the livers of infected animals, the delta antigen has usually been reported as nucleoplasmic rather than nucleolar (25). Even in our own hands, with primary woodchuck hepatocytes infected with HDV (27), the fluorescence was found to be nucleoplasmic (Fig. 3D).

As an additional approach towards establishing that cloned HDV DNA could initiate RNA-directed genome replication in transfected cells, we assembled an HDV DNA with a 2-bp deletion at positions 1434 and 1435 so as to introduce a frame shift at amino acid 53 of the encoded delta antigen. This DNA was then inserted in the pSVL vector as a dimer and tested for its ability to transfect both COS7 and HuH7 cells. Quantitation of the data shown in Fig. 4, lane 2, indicated that the ability of this mutant to synthesize antigenomic RNA in COS7 cells was at least 40 times less than that obtained with the nonmutated DNA (lane 4). The amount of unit-length genomic RNA was similarly reduced (data not shown). We then considered the effect of cotransfection with a pSVL construct that contained only that part of the genome that encoded the delta antigen. In addition, the

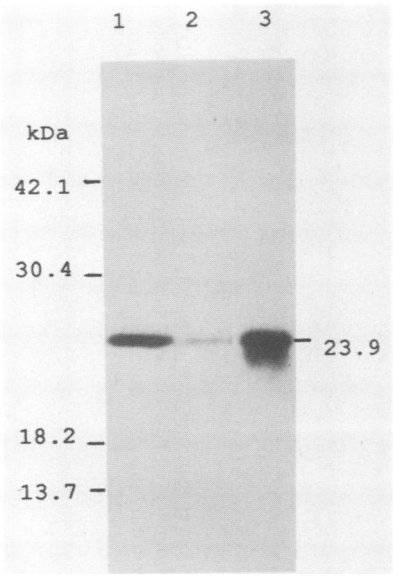

FIG. 2. Western blotting to detect delta antigen following transfection. Protein samples were subjected to electrophoresis on a sodium dodecyl sulfate gel of 15% polyacrylamide by the procedure of Laemmli (11) and then transferred electrophoretically to an Immobilon membrane (Millipore). Delta antigenicity was detected by using a patient antibody to HDV, followed by incubation with ^{125}I-labeled staphylococcal protein A (7). Lane 1 contains a sample of COS7 cells at 10 days after transfection with the trimer of HDV in pSVL. Lane 2 contains a protein sample from primary woodchuck hepatocytes infected with HDV (27), and lane 3 contains a sample from the liver of an infected patient. The band indicated as 23.9 kDa was deduced relative to the mobilities of prestained protein markers (Bethesda Research Laboratories): ovalbumin, 42.1 kDa; carbonic anhydrase, 30.4 kDa; α-lactoglobulin, 18.2 kDa; and lysozyme, 13.7 kDa.

orientation was such that the SV40 late promoter drove transcription not of genomic HDV RNA but of antigenomic RNA, so as to facilitate direct delta antigen translation. Quantitation of the data (Fig. 4, lane 3) indicated that we had now obtained apparent replication of the mutated genome in an amount approximately 90% of that in the unaided nonmutated genome (lane 4). As before, we obtained similar results with HuH7 cells (data not shown).

From these studies we made two conclusions. First, since the mutation in the HDV sequence led to a major reduction in the amount of genomic and antigenomic HDV RNA produced, the HDV RNA production observed following transfection with the nonmutated DNA was more likely to be due to RNA-directed genome replication than to some accumulation of DNA-directed RNA transcripts that were not only of both genomic and antigenomic polarities, but were apparently correctly processed to unit-length circles. Second, since the mutation was in the coding region for the delta antigen, it was likely that the antigen itself was necessary for genome replication. This second conclusion was independently supported by the result that provision of the delta antigen from another plasmid was sufficient to rescue genome replication to a level comparable to that of the nonmutated plasmid. Further studies were needed to test the alternative interpretation, that rescue of the mutated genome was achieved by homologous recombination with the rescuing plasmid (23) rather than by rescue in *trans*, via the delta antigen. We used the polymerase chain reaction (14) together with hybridization with an 18-b oligonucleotide probe specific for the mutated genome and found that the rescued HDV genome still contained the 2-bp deletion (S.-Y. Hsieh and J. Taylor, data not shown).

Our interpretation that the delta antigen could be necessary for RNA-directed HDV genome replication raised an important question. If transfection of cloned HDV DNA could initiate such genome replication, what was the initial source of that antigen? Our DNA construct was such that the SV40 late promoter directed transcription of HDV RNA, but it was of genomic RNA and not of the antigenomic RNA, which we knew was the RNA that encoded the delta antigen (4, 9, 29, 30). One possible explanation is that transfection might lead to at least a small amount of DNA-directed transcription of antigenomic RNA, which could become the initial source of mRNA for the obligatory delta antigen. Another explanation is that even though the delta antigen is necessary for the overall replication of the HDV genome, it is not necessary for certain early RNA-directed RNA transcription; in particular, that transcription which leads to the antigenomic RNA that acts as the cytoplasmic mRNA for delta antigen.

DISCUSSION

We have observed that transfection of both liver (HuH7) and nonliver (COS7) cells with a DNA trimer of the HDV genome led to an apparent replication of the HDV RNA genome. There was neither a specific need for liver tissue nor a need for hepadnavirus proteins or sequences, at least for this part of the HDV replication cycle. Thus, the role of hepadnaviruses in the total cycle of HDV genome replication in animals is limited to virus entry or release.

We found that in transfected cells, HDV genome replication was dependent on expression of the HDV-encoded delta antigen. This antigen, when expressed in *trans* from a second plasmid, facilitated the replication of a mutated HDV genome. This may mean that the delta antigen is somehow involved in facilitating RNA-directed RNA synthesis. How could it do this? At 22 kDa, it may be too small to act alone as a polymerase, but it could act to modify the template specificity of a host-encoded polymerase. As mentioned earlier, an alternative possibility is that the delta antigen is not directly involved in RNA-directed RNA transcription. Either way, there must be some specific features of HDV RNA that allow it to act as a template for such transcription. We note that the HDV RNA (5, 9, 29) can fold into an unbranched rod structure and speculate that this may be an essential template feature.

In the context of such speculations about the mechanism of action of the delta antigen, two further observations can be made. The first concerns the intracellular localization of the antigen. In our transfection studies, the majority of the delta antigen was found by immunofluorescence to be located in the nucleolus. In agreement with Chang et al. (4), we obtained the same localization even when cells were transfected with a plasmid that expressed only the sequences for the antigen itself (data not shown). However, the location was not nucleolar but rather nucleoplasmic in cells that were naturally infected. We currently have no explanation for this discrepancy. The second comment concerns the presence of delta antigen in virions. Others have shown that both the delta antigen and the RNA genome are present inside the virus particle (3, 21). Furthermore, Bonino et al. (3) have reported, and we have confirmed (M. Chao, unpublished observations), that when the particles are disrupted with a nonionic detergent, there is no detectable association be-

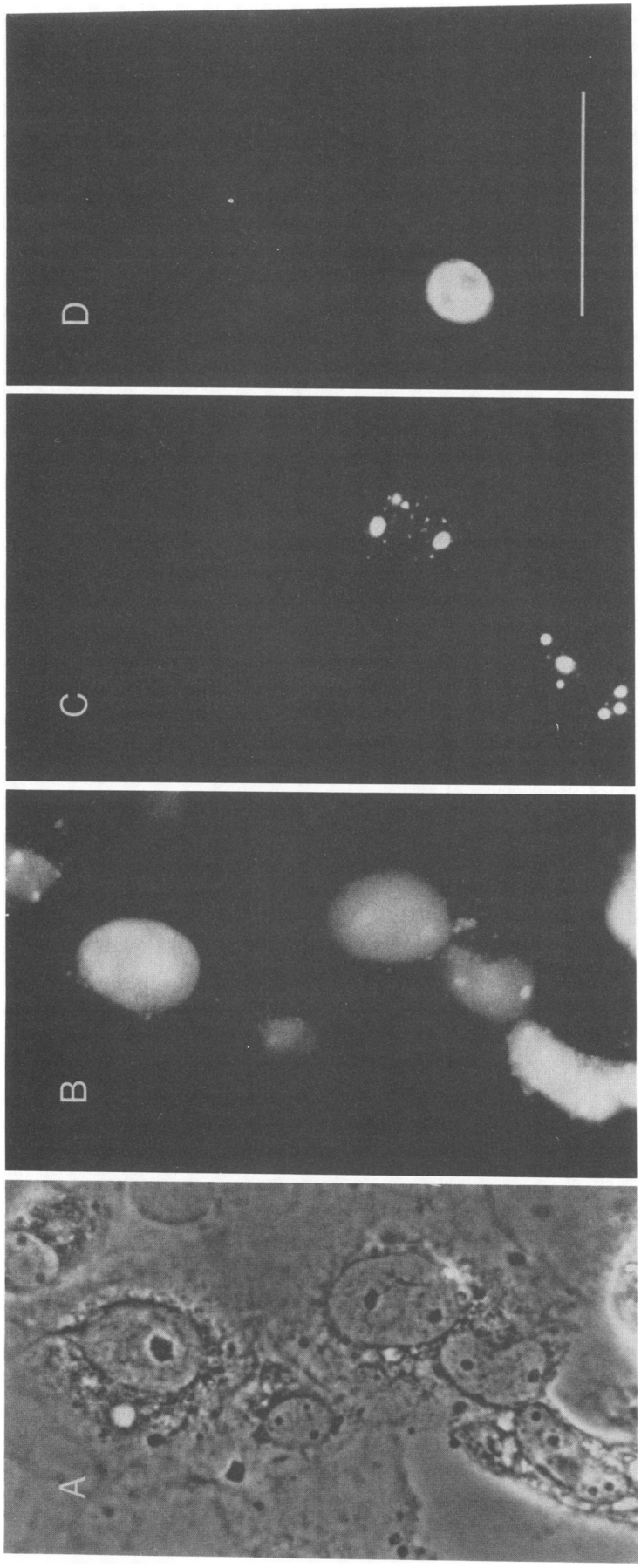

FIG. 3. Immunofluorescence to detect delta antigen in transfected cells. COS7 cells were grown on 12-mm-diameter glass cover slips and then transfected with the trimer of HDV in pSVL. After 10 days, the cells were fixed and examined as follows. (A) Phase-contrast; (B) fluorescence with ethidium bromide; (C) labeling with fluorescein-conjugated antibody specific for HDV. As a control, panel D shows a sample of primary woodchuck hepatocytes at 10 days after infection, examined by the same procedure used in panel C. Bar, 50 μm.

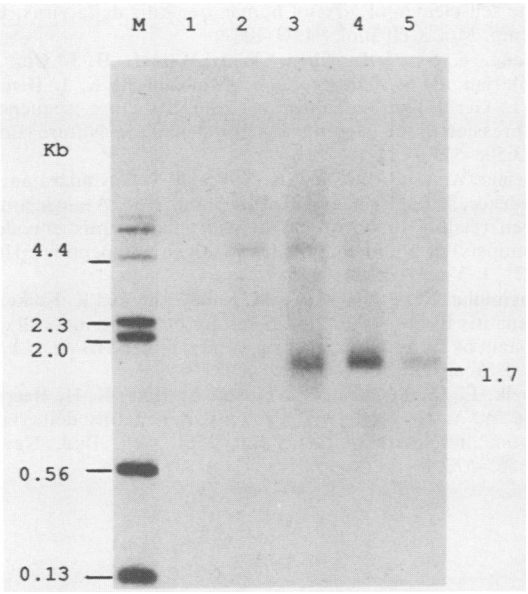

FIG. 4. Northern blot to detect antigenomic HDV RNA following transfection with mutated HDV sequences. The examination procedure was largely the same as described in the legend to Fig. 1. The RNA samples were isolated from COS7 cells at 10 days after the following transfections: lane 1, no DNA; lane 2, HDV genome mutant; lane 3, HDV genome mutant plus subgenomic plasmid encoding HDV antigen; lane 4, nonmutant HDV DNA. Lane 5 is a control of RNA from the liver of a chimpanzee infected with HDV. It is important to note that the RNA probe used here could detect only that part of the HDV genome that was not present in the HDV antigen clone.

tween this delta antigen and RNA. These results are in apparent conflict with those of Chang et al. (4), who reported that the delta antigen can act as an RNA-binding protein. Either way, since we have shown here that the delta antigen is necessary for HDV genome replication, it may be that in natural infections the delta antigen present within the infecting particle may facilitate the earliest events of RNA-directed RNA transcription.

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