A Specific Base Transition Occurs on Replicating Hepatitis Delta Virus RNA

GUANGXIANG LUO,¹ MEI CHAO,¹ SEN-YUNG HSIEH,¹ CAMILLE SUREAU,²
KAZUKO NISHIKURA,³ AND JOHN TAYLOR^{1*}

Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111¹; Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78284²; and The Wistar Institute, Philadelphia, Pennsylvania 19104³

Received 25 September 1989/Accepted 6 November 1989

Three independent lines of evidence showed that when an infectious clone of hepatitis delta virus of known sequence was used to initiate genome replication, up to 41% of the genomes were specifically mutated in the amber termination codon (UAG to UGG) for the open reading frame of the delta antigen, thereby increasing the length of the predicted protein from 195 to 214 amino acids. This change was detected only on molecules that participated in RNA-directed RNA synthesis.

Hepatitis delta virus (HDV) was discovered in humans in association with hepatitis B virus (HBV) (21). It has since been proved that HDV is a subviral satellite of HBV (11); it depends on HBV for packaging of the HDV genomic RNA and the delta antigen into virus particles (4, 5, 35). Within the infected cell, the HDV genome replicates independently of HBV (11), using a rolling-circle mechanism involving RNA-directed RNA synthesis of genomic RNA and its complement, the so-called antigenomic RNA (8). It is the antigenomic RNA that encodes the delta antigen, the only known protein of HDV (7, 19, 32).

In natural infections, the delta antigen appears as two species of similar electrophoretic mobility, which are referred to here as the 22- and 24-kilodalton (kDa) species (3, 4, 19, 32, 35). Three laboratories have published complete HDV sequences which between them predict delta antigens of 195 and 214 amino acids (12, 14, 30, 31). The sequences of Makino et al. (14) and Wang et al. (30) predict a protein of 214 amino acids. The prediction of Wang et al. (30), after correction for a single sequencing error (31), was actually more complicated; they sequenced seven partially overlapping cDNA clones and thus found 16 cases of microheterogeneity in the composite sequence. The variations were only single-base changes, but as shown in Fig. 1, one was of an adenosine to a guanosine in the termination codon for a predicted 195-amino-acid species, thereby increasing the predicted size to 214 amino acids. (Two more of the single-base changes are shown in Fig. 1; they cause changes only in the predicted amino acids.) The third published sequence, by Kuo et al. (12), predicts only the 195-amino-acid species. As related elsewhere, the sequence is otherwise very similar to those described by Wang et al. (30, 31). This is not too surprising, because the infectious material used in the two cDNA cloning studies was derived, albeit by different experimental animal transmissions, from the same initial human isolate.

Studies in several laboratories (7, 32) argue that the above-mentioned RNA sequence differences explain the two electrophoretic forms of the delta antigen that are normally observed in infected livers. Thus, when we expressed the cloned sequence of Kuo et al. (12) in the absence of RNA replication, we obtained only a single species, which we

designated as the 22-kDa species and expected to be the predicted 195-amino-acid species. However, as explained in this report, we made a puzzling observation when the full HDV sequence was used to transfect cells and genome replication occurred (11). There appeared along with the 22-kDa species a small but significant amount of the 24-kDa species. We found that this observation correlated with a specific base change being made on the replicating RNA genome.

This finding led us to consider the source of this RNA replication-associated mutagenesis. We already knew from the above-mentioned study of Wang et al. (30) that 16 examples of single-base substitutions had been found after the cDNA cloning of HDV from a single pool of virus. On closer examination, we noted that 15 of the 16 changes were base transitions, that is, between A and G or C and U. This could have been caused by transcription errors during HDV genome replication; viral RNA-directed RNA synthesis is frequently cited as being prone to errors by misincorporation (9, 24). However, recent studies suggest another mechanism that specifically causes specific base transitions. Normal cells contain an activity, inappropriately named "RNA duplex unwindase," that acts *in vitro* on double-stranded RNA substrates and deaminates adenosine, thus converting it to inosine (I) (1, 20, 27, 28). The *in vivo* function of this activity is not established, but if it acted on a replicating viral RNA, the I would first direct incorporation of C, which in turn would direct the incorporation of G. The net effect would be the replacement of A with G on one strand and of U with C on the complementary strand. There is circumstantial evidence that such unwindase action may have led to certain neurological variants of measles virus (2, 6) and also a variant of vesicular stomatitis virus (18). As presented here, we have direct evidence that during the replication of the HDV genome at least one specific A is replaced by G, leading to the translation of the larger delta antigen. Also, we evaluate the evidence that the change was via unwindase action.

MATERIALS AND METHODS

Plasmids and transfections. HDV sequences cloned into the RNA expression vector pGem4B (pG4B; Promega Biotech, Madison, Wis.) were synthesized by standard procedures (23). Cloning into the eucaryotic expression vector

* Corresponding author.

FIG. 1. Microheterogeneity on antigenomic HDV RNA in the region spanning the C terminus of the coding region for the delta antigen. The two sequences are from Wang et al. (30) and contain single-base differences at three places (underlined), one of which, at position 1012, changes an amber termination codon into a tryptophan codon. The upper sequence is also identical to that of Kuo et al. (12).

pSVL (Pharmacia, Inc., Piscataway, N.J.) was as previously described (11). This construct was used to transfect both COS7 cells (11) and a chimpanzee (25). The animal was already a carrier of HBV. Six weeks after HDV transfection, the animal developed an apparently typical HDV infection. The serum and liver biopsy samples taken at this time contained HDV RNA and were used for the present study.

For the unwindase studies, we used the transcription vector pG4B with subgenomic inserts of HDV, as summarized below according to designation and location on the HDV genome: pG4B(PX), *Pst*I (1087) to *Xba*I (781); pG4B(XP), *Xba*I (781) to *Pst*I (655); pG4B(PP), *Pst*I (1087) to *Pst*I (655). For RNA transcription, the vectors were first linearized at a restriction enzyme site located downstream of the HDV insert and then copied with the RNA polymerases of phage T7 or SP6 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). When necessary, the RNAs were labeled with [α - 32 P]ATP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). The products were treated with DNase I (Promega) before gel purification and, as necessary, subjected to hybridization to make the appropriate intermolecular RNA-RNA structures shown in Fig. 5A. The electrophoretic mobility of the two intermolecular structures was tested by electrophoresis into polyacrylamide gels under nondenaturing conditions.

Oligonucleotide primers. The following HDV-specific oligonucleotides were chemically synthesized and are listed according to a designated letter code, position on HDV (12), and actual sequence, respectively: A, 894 to 919, CCGAC CCGAA GAGGAAAGAA GGACGC; B, 1152 to 1135, TGGGGGGTGT GAACTCGAAG GTGGATCGA; C, 1004 to 1022, GTATATCCCA TGGAAATCC; D, 1082 to 1069, GGAGTCCCGG AGTC; E, 999 to 1012, GAAGAGTATA TCCT; F, 999 to 1012, GAAGAGTATA TCCC. Note that at position 1012, C and F are mutated relative to the sequence of Kuo et al. (12).

Reverse transcription and polymerase chain reactions. RNA samples were extracted by treatment with pronase in the presence of sodium dodecyl sulfate. After one extraction with phenol and two with ether, the samples were precipitated with ethanol in the presence of salt and carrier dextran (Sigma Chemical Co., St. Louis, Mo.) (29). Some samples were additionally treated with DNase I (Promega) and repurified. Reverse transcription was done under standard conditions (23) with the enzyme from avian myeloblastosis virus (Life Sciences, Inc., St. Petersburg, Fla.). For polymerase chain reaction (PCR), the products were next treated with alkali to destroy the RNA and then aliquots were used as

templates for PCR. PCR reaction mixtures of 50 μ l with Taq polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) were as described previously (10), using 30 to 40 cycles on a Techne Tempblok set for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The primer combinations are described in the text and figure legends. With the primers mismatched at the 3' terminus, we followed the method of Wu et al. (34) and empirically determined that at an annealing temperature of 55°C there was optimum discrimination in the ability of primer E versus F to replicate wild-type versus mutant HDV.

As explained in the text, we also used PCR as part of a cDNA cloning strategy. We converted HDV RNA into double-stranded DNA fragments spanning *Sal*I (962) to *Pst*I (1087). These were inserted into pG4B. Recombinants were screened (15) with an RNA probe [pG4B(PX)] as described above or with the oligonucleotide C that had been end labeled. Special conditions were needed for hybridization with the mutant oligonucleotide C to discriminate against the detection of unmutated sequences. We found empirically that optimum specificity was obtained with a washing solution at 45°C (17).

For nucleotide sequencing, recombinant plasmid DNAs were used in a dideoxy sequencing protocol (22) as modified to make use of the Taq polymerase (10).

In vitro modification of RNA and assay by thin-layer chromatography. RNA substrates labeled with [α - 32 P]ATP were prepared as described above. HeLa cell extracts were prepared by a modification of the method of Manley et al. (16) as described previously (28). Approximately 10 fmol of various RNA samples was incubated with 50 μ g of cell extract protein in 20 μ l of 50 mM Tris (pH 7.8)–0.15 M KCl–5 mM EDTA–25% glycerol–1 mM dithiothreitol. The reaction mixture was incubated at 37°C for 2 h, deproteinized with proteinase K, phenol extracted, and ethanol precipitated. The RNAs were then digested with nuclease P1 (Sigma) and subjected to thin-layer chromatography with solvent 2 of Wagner et al. (28) to assay the conversion of adenosine to inosine. Radioactivity was detected either by autoradiography or with a Radioanalytic Imaging system (AMBIS, San Diego, Calif.).

Protein analyses. HDV antigen was translated in vitro with a rabbit reticulocyte lysate (Bethesda Research Laboratories). Samples from DNA transfections along with the Western (immunoblot) analysis were all as previously described (11, 13).

FIG. 2. Western analysis of delta antigen species. After separation on a 12% acrylamide gel by the method of Laemmli (13), a Western analysis was done (11) with a patient antibody specific for delta antigen. The mobilities of prestained markers (Bethesda Research Laboratories) are shown at the left; lane 1 is a sample from the liver of an infected patient; lane 2 is an in vitro rabbit reticulocyte translation product of antigenomic HDV RNA which encodes the delta antigen; lanes 3 and 4 are from COS7 cells transfected with pSVL(Ag) and pSVL(D3), respectively; lane 5 is a plasma sample from a chimpanzee transfected with pSVL(D3).

RESULTS

Electrophoretic forms of delta antigen. As explained below, we made an unexpected finding from what we thought would be a routine Western analysis of the delta antigen in various samples. Liver samples from an infected patient (Fig. 2, lane 1) showed two species, designated the 22- and 24-kDa species, just as others have described (3, 4, 19, 32, 35). (Other species, such as one at about 20 kDa, were also present.) In vitro translation of the HDV sequence cloned by Kuo et al. (12) yielded only the 22-kDa species (lane 2), which was, as predicted from the nucleotide sequence, 195 amino acids. A band of the same electrophoretic mobility was also seen when a subgenome-size fragment of the HDV sequence of Kuo et al. (12) was expressed in transfected COS7 cells from the eucaryotic expression vector pSVL(Ag) (Fig. 2, lane 3) (11). However, a surprising finding was obtained when we studied the antigen made in cells transfected with the vector pSVL(D3), containing a trimer of the HDV genome (11). This construct allowed replication of the HDV genome. We still obtained the 22-kDa species, but we now obtained small amounts of additional protein species, including one that migrated the same as the 24-kDa species observed in infected liver (Fig. 2, lane 4). From densitometric analysis, the relative amount of this larger species was 5%. When we studied the plasma proteins of a chimpanzee that had been successfully transfected with the same recombinant HDV construct (25), we again obtained the 24-kDa band (Fig. 2, lane 5), but the relative amount now was 20%.

One possible explanation for the appearance of the novel band was that there was readthrough of the amber termination codon and, as a result, synthesis of the 214-amino-acid species that contained at its C terminus an additional 19

amino acids (see Introduction and Fig. 1). Such readthrough can occur by tRNA suppression of the termination codon; Weiner et al. (32) documented this for delta RNA expressed in a suppressor-positive strain of *Escherichia coli*. However, in our studies, such a mechanism of suppression was apparently not involved because we found that in the absence of HDV genome replication (Fig. 2, lane 3) the large protein was not made. (There was the remote possibility that HDV replication induced a suppressor tRNA.) We thus tested the alternative hypothesis that HDV genome replication leads to single-base changes, some of which are in the termination codon and thus lead to the synthesis of the longer form of the delta antigen.

Sequence analysis of HDV RNAs from transfected cells. To test the above-described hypothesis, we sequenced the region of the genome that surrounds the termination codon for the smaller antigen (Fig. 1). Also, since the protein data indicated that only a small fraction (5 to 20%) of the RNAs would be changed, it was considered necessary to obtain cDNA clones and screen about 100 recombinant clones. The strategy we used is explained in Fig. 3. Antigenomic HDV RNA was reverse transcribed with an oligonucleotide primer, designated A in Fig. 3A, and then amplified by the PCR with primers A and B. The 258-base-pair product was gel purified and then digested with both *SaI* and *PstI*. The resulting internal 125-base-pair fragment was gel purified and ligated into the multiple cloning site of plasmid pG4B, which had been similarly digested and gel purified. The ligation mix was used to transform *E. coli* HB101, and the colonies obtained were screened (15) with a radiolabeled RNA probe specific for this region of the HDV genome. Sets of 100 such positive colonies were transferred and grown up on three identical agar plates; one was kept as a master plate, one was rescreened with the RNA probe to confirm the presence of the HDV insert (for example, Fig. 3B, left side), and one was screened with end-labeled oligonucleotide C (for example, Fig. 3B, right side). This oligonucleotide was designed to detect those recombinants in which the nucleotide at position 1012 on the antigenomic strand was changed from A to G; this converts the termination codon on the antigenomic RNA from UAG to UGG (Fig. 1). In the example shown, three positive clones were found and subsequently confirmed by dideoxy sequencing (10, 22) with end-labeled oligonucleotide D as the primer (Fig. 3C). The input RNA used in the example shown in Fig. 3 was from COS7 cells 2 weeks after transfection with the replication-inducing clone pSVL(D3).

When we used this approach with RNA from cells transfected with pSVL(Ag) or with pSVL(D2M), a mutant unable to replicate, and screened a total of 255 recombinant colonies, we found none that contained the changed base (data not shown). This demonstrated that the previously obtained changed sequences were not some quirk of using PCR (10). It also was consistent with the previously mentioned hypothesis that the change depended on the ability of the HDV RNA sequences to undergo RNA-directed RNA synthesis.

We also tested RNA samples from an HDV-transfected chimpanzee. Using liver RNA, we found 41 of 100 recombinant colonies to be mutant. We also wished to test the HDV RNA that was packaged and released into the blood; however, because virions contain only genomic RNA, we had to modify the strategy of Fig. 3A and carry out the reverse transcription step using primer B. The outcome was that we obtained 73 recombinant colonies from 200 that were positive for the change (data not shown). Thus, not only were the serum and liver results comparable, but apparently we

FIG. 3. Recombinant DNA cloning and sequencing of specific HDV RNA sequences. (A) The strategy for going from RNA to clonable double-stranded DNA spanning the region *Sal*I (962) to *Pst*I (1087) is explained in the text. (B) Recombinant clones were screened with an HDV RNA probe and then either reconfirmed as such (left side) or tested for hybridization to oligonucleotide C (right side). (C) Two recombinant clones from panel B were subjected to dideoxy sequencing. One clone was expected to be unchanged (wt) and the other to be modified (mutant) at position 1012. The sequencing confirmed this; the two clones differed only in A to G at 1012 (*).

obtained comparable results for both genomic RNA (serum) and antigenomic RNA (liver).

Thus, the approach of examining the HDV RNA gave results roughly comparable to the earlier protein studies (Fig. 2) in which we assayed the relative abundance of the longer form of delta antigen in the transfected chimpanzee and cell cultures, in which genome replication occurred.

Independent PCR-based assay for detecting HDV sequence changes. The above strategy for the detection of nucleotide changes at position 1012 was quantitative, but the sensitivity was limited by the number of recombinant clones screened. In addition, it depended on cloning and sequencing. Thus, we exploited the alternative strategy of Wu et al. (34), which is able to specifically amplify only those molecules that contain the base change. This can be explained with Fig. 4. An RNA sample containing antigenomic HDV sequences was reverse transcribed with primer A, and then aliquots were subjected to PCR amplification with the primer pair E plus D or F plus D. The primers E and F were end labeled, and so the PCR products were assayed by a denaturing sequencing gel and screened for an 83-base species. The first primer pair detects the normal HDV sequence of Kuo et al. (12). Primer F, however, differs from E in its 3' nucleotide, which corresponds to position 1012. F will only pair precisely to antigenomic HDV RNA that has been modified from A to G at position 1012. As Wu et al. (34) have shown, the annealing temperature of the PCR reaction required optimization so that the first primer pair detected only unmodified HDV (Fig. 4B, left panel, lane 2 versus lane 1), while the second pair detected only modified HDV (Fig. 4B, right panel, lane 1 versus lane 2).

This strategy was applied to various RNA samples containing HDV sequences. For the COS7 cells transfected with pSVL(Ag), we detected only unmodified DNA (lane 4),

whereas in those transfected with pSVL(D3), we detected modified as well as unmodified DNA (lane 3).

A reconstruction to determine the specificity and sensitivity of this assay procedure was performed with unmodified DNA in the presence of decreasing amounts of modified DNA. We could readily detect 1 part of modified DNA in the presence of 100 parts of unmodified DNA (data not shown). This result also made clear the specificity of this PCR-based assay.

Action of unwindase on HDV RNA. In the previous sections, we showed that in the termination codon of the delta antigen, some RNA molecules are changed from A to G. As mentioned in the Introduction, we wished to test the hypothesis that this change was initiated by RNA duplex unwindase action converting A to I, followed by RNA-directed RNA synthesis perpetuating the I as G. We sought direct experimental evidence to support the role of unwindase. Also, since unwindase is known to have a specificity for double-stranded rather than single-stranded RNA, we were interested to know whether the rod structure of the delta RNA, with an average of 70% of bases paired, would also act as a substrate for unwindase.

Four different HDV RNA constructs were made (Fig. 5A). These RNA species were initially transcribed via bacteriophage RNA polymerases from recombinant transcription plasmids and were labeled with [α - 32 P]ATP. They were then hybridized, as necessary, to make the structures shown, before being incubated for 2 h at 37°C in an extract of HeLa cells that contained unwindase activity (16, 27, 28). After this, the RNAs were extracted, digested with nuclease P1,

and analyzed by thin-layer chromatography, followed by radioimaging. Since nuclease P1 releases 5' nucleotides, the conversion of A to I could be monitored simply as the transfer of label from pA to pI. This occurred to a significant extent only for the 100% double-stranded RNA (Fig. 5B, lane 2), and the extent of conversion was 30%. With the three other RNA substrates, the extent of conversion was much lower. It could, however, be detected with a 5-day exposure of an autoradiogram and was about 1%. Such modifications were proved to depend on treatment of the RNA with the cell extract that contained unwindase activity. Also, a heat-inactivated extract failed to induce the changes (data not shown). Figure 5B shows additional controls. Lane 5 showed that without P1 digestion the label remained exclusively at the origin. Lane 6 showed that with P1 digestion followed by phosphatase treatment, the label was released and migrated as free phosphate. The migrations of pA and pI were confirmed by using unlabeled standards that were located by UV light. From these studies, we deduced that if unwindase was involved in vivo in the modification of HDV RNA, then a fully double-stranded RNA would be a preferred substrate. Yet it remains possible that a lower extent of modification, but with some specificity, could also occur on HDV RNA that was either single stranded or folded into the rod structure. Another possible substrate could be created by an intermolecular interaction between HDV RNA and an RNA in the cellular extract. If unwindase caused the A-to-G change at position 1012 in the termination codon, the A would have to be in the antigenomic RNA. The modified base would then have to replicate to be perpetuated as a G.

DISCUSSION

Three lines of evidence showed that during the replication of the HDV genome there was a nucleotide change in the amber termination codon of the delta antigen. In the three clones that were partially sequenced and did have the A-to-G change, we were unable to find any additional changes in the approximately 100 bases of flanking sequence. To search for other changes on the HDV genome, we modified the strategy used in Fig. 3 to obtain more sensitivity. We thus found eight changes at other sites, but the frequency was about 500-fold lower than in the termination codon. Also, the changes were independent of genome replication (data not shown).

We found that the fraction of molecules changed at the termination codon was as much as 41% for the HDV RNAs from the liver and serum of the infected chimpanzee. This relatively high level could be related to the fact that the infection had been established more than 6 weeks in the animal, in contrast to the transient transfection studies with the cultured cells. However, in a separate series of transient transfections, we observed that the extent of change (as judged by Western analysis) depended on the extent of genome replication and could be at least as high as in the infected chimpanzee (data not shown). Thus, the appearance of the change was independent of cell-to-cell spread.

The change only occurred when there was RNA-directed RNA replication of HDV. Presumably the change occurred either after or during rounds of RNA-directed RNA synthesis. What is the evidence that the change might have occurred via the action of the recently found cellular activity, RNA duplex unwindase (1, 20, 27, 28)? (i) Examples exist with other RNA viruses (2, 6, 18). (ii) The change of A to G was consistent with the known properties of unwindase action (1, 28). (iii) We could reconstruct A-to-I changes on HDV RNA in vitro. (iv) The 15 of 16 examples from the

FIG. 4. PCR-based approach to detect specific single-base changes in HDV RNAs. (A) As explained in the text, the strategy was to reverse transcribe antigenomic HDV RNA sequences with primer A and then use samples of this product as a template for PCR with either primers E and D or F and D. Primer F, unlike E, will give perfect base pairing only to antigenomic sequences that have been modified by an A-to-G change at position 1012. (B) Primers E and F were end labeled so that the PCR product could be assayed by using a denaturing acrylamide gel and screening for an 83-base species. The left panel shows the detection of wild-type (wt) sequence; the right panel shows the mutant sequence. Lanes 3 and 4 made use of RNA from COS7 cells transfected with pSVL(D3) and pSVL(Ag), respectively. As controls for specificity of amplification, we used PCR templates that were pure wild-type DNA (lane 2) or pure mutated DNA (lane 1). The nucleotide sequence of these two controls was obtained in Fig. 3C. Lanes M and the associated numbers refer to a size marker of bacteriophage ϕ X174 DNA digested with *HinfI* and end labeled.

HDV sequencing of Wang et al. (30) were all consistent with unwindase action. At best, these four lines of evidence are circumstantial. For item iv, the directionality of the base transitions is not known. For item iii, our reconstructions with double-stranded RNA could be argued to be irrelevant because they allow multiple changes, whereas HDV replication has so far revealed only the single change in the termination codon. (Of course, molecules with multiple changes could have been selected against, for example, by means of competence for replication.) It remains possible that unwindase action was on a specific region of intermolecular or even intramolecular double-stranded RNA. And it is also possible that unwindase was not involved at all.

If unwindase action was definitely the cause of change on HDV and certain other RNA viruses (2, 6, 18), then there would be important consequences for our understanding of the replicative structures of these viruses. Since unwindase, at least in vitro, has a clear preference for RNA substrates that are double stranded, we would have to conclude that the RNA species that are modified must, at least transiently, exist in a double-stranded conformation. In this respect, we have previously isolated double-stranded HDV species from infected cells and tissues (8, 26). However, there is the dogma, as recently restated by Weissmann (33), that double-stranded viral RNA structures are either the nonproductive conse-

FIG. 5. Ability of HDV RNA structures to act as substrates for RNA duplex unwindase. (A) The four constructs shown contained different amounts of intermolecular and intramolecular base pairing. They were assembled by using RNA species synthesized *in vitro* in the presence of [α - 32 P]ATP. (B) They were then subjected to treatment with unwindase activity, followed by extraction and digestion with nuclease P1 before chromatography with solvent 2 of Wagner et al. (28). Radioactivity was quantitated *in situ* with a Radioanalytical Imaging system. The indicated positions of pA and pI were established by cochromatography of unlabeled standards (Sigma), as localized with UV light. Sample 5 was a control of undigested RNA, which remained at the origin. Sample 6 corresponds to a digest as in sample 1 but followed by an additional treatment with bacterial alkaline phosphatase to verify the release of free phosphate (p).

quences of "collapsed" transcription or artifacts of extraction.

While our studies offer an explanation of the long-standing puzzle of HDV encoding two different electrophoretic forms of delta antigen, they also raise new questions. Previously, we have shown that the smaller antigen when provided in *trans* allows a mutant genome to replicate (11); in this experiment, no large antigen was detected (M. Chao, unpub-

lished data), implying that the small antigen is sufficient for genome replication. Also, recent experiments have shown that in the absence of the small antigen, the large antigen is not sufficient for genome replication (M. Chao, S.-Y. Hsieh, and J. Taylor, unpublished data). Experiments are needed to address whether the changes occur in every infected cell. Maybe the two forms have different functions or even a cooperative function, such as in packaging. We are tempted

to speculate that the replicating HDV exploits the ability of its genome to be modified by the host, so as to allow the synthesis of the additional larger form of the delta antigen.

ACKNOWLEDGMENTS

J.T. was supported by grant MV-7M from the American Cancer Society, by Public Health Service grants CA-06927, RR-05539, and AI-26522 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania, and K.N. was supported by grant CA-46676 from the National Cancer Institute.

We thank William Mason, Wang-Shick Ryu, and Richard Katz for critical reading of the manuscript, Laura Coates for technical assistance, and Tony Yeung for chemical synthesis of the oligonucleotides used in the study. The sample of HDV-infected human tissue was provided by Eric Gowans, and the human antidelata serum was provided by John Gerin.

LITERATURE CITED

- Bass, B. L., and H. Weintraub. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* 55:1089-1098.
- Bass, B. L., H. Weintraub, R. Cattaneo, and M. A. Billeter. 1989. Biased hypermutation of viral RNA genomes could be due to unwinding/modification of double-stranded RNA. *Cell* 56:331.
- Bergmann, K., and J. L. Gerin. 1986. Antigens of hepatitis delta virus in the liver and serum of humans and animals. *J. Infect. Dis.* 154:702-706.
- Bonino, F., K. H. Heermann, M. Rizzetto, and W. H. Gerlich. 1986. Hepatitis delta virus: protein composition of delta antigen and its hepatitis B virus-derived envelope. *J. Virol.* 58:945-950.
- Bonino, F., B. Hoyer, J. W.-K. Shih, M. Rizzetto, R. H. Purcell, and J. L. Gerin. 1984. Delta hepatitis agent: structural and antigenic properties of the delta-associated particles. *Infect. Immun.* 43:1000-1005.
- Cattaneo, R., A. Schmid, D. Eschle, K. Bacsko, V. ter Meulen, and M. A. Billeter. 1988. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. *Cell* 55:255-265.
- Chang, M.-F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. C. Lai. 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. *J. Virol.* 62:2403-2410.
- Chen, P.-J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor. 1986. The structure and replication of the genome of the hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* 83:8774-8778.
- Domingo, E., E. Martinez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortin, C. López-Galindez, P. Pérez-Brena, N. Villanueva, R. Nájera, S. VandePol, D. Steinhauer, N. DePolo, and J. Holland. 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance—a review. *Gene* 40:1-8.
- Higuchi, R. 1989. Using PCR to engineer DNA, p. 61-70. *In* H. A. Ehrlich (ed.), *PCR technology*. Stockton Press, New York.
- Kuo, M. Y.-P., 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.
- Kuo, M. Y.-P., J. Goldberg, L. Coates, W. Mason, J. Gerin, and J. Taylor. 1988. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: sequence, structure, and applications. *J. Virol.* 62:1855-1861.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Makino, S., M.-F. Chang, C.-K. Shieh, T. Kamahora, D. M. Vannier, S. Govindarajan, and M. M. C. Lai. 1987. Molecular cloning and sequencing of a human hepatitis delta virus RNA. *Nature (London)* 329:343-346.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gelfer. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proc. Natl. Acad. Sci. USA* 77:3855-3859.
- Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273.
- O'Hara, P. J., S. T. Nichol, F. M. Horodyski, and J. J. Holland. 1984. Vesicular stomatitis virus defective interfering particles can contain extensive genomic sequence rearrangements and base substitutions. *Cell* 36:915-924.
- Pohl, C., B. M. Baroudy, K. E. Bergmann, P. J. Cote, R. H. Purcell, J. Hoofnagle, and J. L. Gerin. 1987. A human monoclonal antibody that recognizes viral polypeptides and in vitro translation products of the genome of hepatitis D virus. *J. Infect. Dis.* 156:622-629.
- Rebagliatti, M. R., and D. A. Melton. 1987. Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. *Cell* 48:599-605.
- Rizzetto, M., M. G. Canese, J. Arico, O. Crivelli, F. Bonino, C. G. Trepo, and G. Verme. 1977. Immunofluorescence detection of a new antigen-antibody system (delta-antidelta) associated to the hepatitis B virus in the liver and in the serum of HBsAg carriers. *Gut* 18:997-1003.
- Sanger, F., S. Nicklen, and R. A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Sharmeen, L., M. Y.-P. Kuo, G. Dinter-Gottlieb, and J. Taylor. 1988. The antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J. Virol.* 62:2674-2679.
- Steinhauer, D. A., and J. J. Holland. 1986. Rapid evolution of RNA viruses. *Annu. Rev. Microbiol.* 41:409-433.
- Sureau, C., J. Taylor, M. Chao, J. E. Eichberg, and R. E. Lanford. 1989. Cloned hepatitis delta virus cDNA is infectious in the chimpanzee. *J. Virol.* 63:4292-4297.
- Taylor, J., M. Kuo, P.-J. Chen, G. Kalpana, J. Goldberg, C. Aldrich, L. Coates, W. Mason, and J. Summers. 1987. Replication of hepatitis delta virus, p. 541-548. *In* W. Robinson, K. Koike, and H. Will (ed.), *Hepadna viruses*. Alan R. Liss, Inc., New York.
- Wagner, R. W., and K. Nishikura. 1988. Cell cycle expression of RNA duplex unwinding activity in mammalian cells. *Mol. Cell. Biol.* 8:770-777.
- Wagner, R. W., J. E. Smith, B. R. Cooperman, and K. Nishikura. 1989. A double-strand RNA unwinding activity introduces structural alterations by means of adenosine to inosine conversions in mammalian cells and *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* 86:2647-2651.
- Wain-Hobson, W., P. Sonigo, O. Danos, S. Cole, and M. Alizon. 1985. Nucleotide sequence of the AIDS virus LAV. *Cell* 40:9-17.
- Wang, K.-S., Q.-L. Choo, A. J. Weiner, H.-J. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton. 1986. Structures, sequence and expression of the hepatitis delta viral genome. *Nature (London)* 323:508-513.
- Wang, K.-S., Q.-L. Choo, A. J. Weiner, H.-J. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton. 1987. Structure, sequence and expression of the hepatitis delta viral genome. *Nature (London)* 328:456.
- Weiner, A. J., Q.-L. Choo, K.-S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton. 1988. A single antigenic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24^s and p27^s. *J. Virol.* 62:594-599.
- Weissmann, C. 1989. Single-stranded RNA. *Nature (London)* 337:415.
- Wu, D. Y., L. Ugozzi, B. K. Pal, and R. B. Wallace. 1989. Allele-specific enzymatic amplification of β -globin specific genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl. Acad. Sci. USA* 86:2757-2760.
- Zyzik, E., A. Ponzetto, B. Forzani, C. Hele, K.-H. Heermann, and W. A. Gerlich. 1987. Proteins of hepatitis delta virus in serum and liver. *UCLA Symp. Mol. Cell. Biol.* 70:565-577.