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Nucleotide Sequence Stability of the Genome of Hepatitis Delta Virus

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Cultured cells were cotransfected with a fully sequenced 1,679-base cDNA clone of human hepatitis delta virus (HDV) RNA genome and a cDNA for the genome of woodchuck hepatitis virus (WHV). The HDV particles released were able to infect a woodchuck that was chronically infected with WHV. The HDV so produced was passaged a total of six times in woodchucks in order to determine the stability of the HDV nucleotide sequence. During a final chronic infection with such virus, liver RNA was extracted, and the HDV nucleotide sequence for the 352-base region, positions 905 to 1256, was obtained. By means of PCR, we obtained double-stranded cDNA both for direct sequencing and also for molecular cloning followed by sequencing. By direct sequencing, we found that a consensus sequence existed and was identical to the original sequence. From the sequences of 31 clones, we found 32% (10 of 31) to be identical to the original single nucleotide sequence. For the remainder, there were neither insertions nor deletions but there was a small number of single-nucleotide changes. These changes were predominantly transitions rather than transversions. Furthermore, the transitions were largely of just two types, uridine to cytidine and adenosine to guanosine. Of the 40 changes detected on HDV, 35% (14 of 40) occurred within an eight-nucleotide region that included position 1012, previously shown to be a site of RNA editing. These findings may have significant implications regarding both the stability of the HDV RNA genome and the mechanism of RNA editing.

Many studies have considered the stability of the genomes of RNA viruses (13–15, 18, 23, 40). The genomes of RNA viruses have generally been found to be significantly less stable than those of DNA viruses. This instability is typically attributed to the virus-encoded RNA polymerases which lack a proofreading function and are thus considered to be error prone, a term that typically means an error rate of as much as 0.02 substitution per nucleotide per year. Human hepatitis delta virus (HDV) has a 1,679-base single-stranded RNA genome and has also been the focus of studies on genome stability (4, 12, 25, 26, 29, 34, 43, 48). However, HDV differs from all other animal RNA viruses in that its genome replication does not make use of a viral polymerase but rather redirects the host RNA polymerase II (16, 36). While it is generally thought that transcription by this enzyme lacks a proofreading function, recent data suggest that an associated transcription factor, elongation factor SII, might have 3′→5′ exonuclease activity (27). Other animal viruses, the retroviruses and the hepadnaviruses, also use polymerase II in the replication of their genomes (37). In the plant world, this is also true for the caulimoviruses and probably also the viroids (37, 44).

Previous studies of the stability of the HDV genome sequence can be classified into four groups. First, there are the comparisons between different natural isolates. A good example of this is a recent study of Casey et al. (4). They found not only small insertions and deletions but also a large number of single-nucleotide changes. As an extreme case, two isolates were found to differ by more than 37% in nucleotide sequence. Studies of a second class have looked at genome variations that occur over a year or more for an individual with a chronic HDV infection. Such studies have revealed changes estimated at up to 0.02 per nucleotide per year, just as for other animal RNA viruses (25, 34). A third class of studies has looked at variant HDV genomes as they exist at a single time in a single individual (35, 49). The original example of this is the study of Wang et al., which made clear that in the serum of an infected animal, there were several closely related HDV RNA species (49). From regions of the genome where more than one clone was obtained, they detected examples of what they called microheterogeneities. That is, there was more than one choice for a base at certain positions. They did not detect any insertions or deletions.

A fourth class of study, one that is probably more informative, involves examining sequence changes that arise when the infection is known to begin with a single sequence. Sureau et al. (47) were the first to study this for HDV. A chimpanzee was transfected with an infectious HDV cDNA clone (30). A limited examination of the HDV RNAs in the serum and liver of this animal led to the discovery of a specific and efficient site of sequence alteration (35), a change from uridine to cytidine at position 1012. Almost 40% of genomes were changed at this site (35). In retrospect, it was realized that this was one of the sites of microheterogeneity detected by Wang et al. (49). It was subsequently made clear that this change at position 1012 allowed a change in the termination codon for the only protein encoded by HDV, the delta antigen (50). This phenomenon of specific HDV RNA editing has been studied further. Distinct functional roles have since been ascribed to the two forms of the delta antigen (7, 8). The cDNA transfection study of Sureau et al. has also been repeated in woodchucks (41, 52). Also, with tissue culture, it has been demonstrated that the editing occurs in transfected cells and is detectable even when the HDV RNA is not undergoing replication (3, 9, 35, 51, 53).

The present study represents an extension of the study of Sureau et al. (47). Our aim was to determine the stability of the HDV RNA genome, beginning with a single sequence, when the virus was multiply passaged in animals. Rather than starting with a transfected animal, we used cotransfected tissue

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TABLE 1. Assembly and passage of cloned HDV[a]

Step	Description
Assembly in cultured Huh7 cells	Cotransfection with HDV and WHV cDNA clones; particles released into growth medium at 4–12 days
Passage 1, in woodchuck chronically infected with WHV	Portal vein injection; particles released into serum at 63 days
Passage 2, in woodchuck repeated	Leg vein injection; particles released into serum at 34 days
Passage 3, in woodchuck repeated	Particles released into serum at 20 days
Passage 4, in woodchuck repeated	Particles released into serum at 28 days
Passage 5, in woodchuck repeated	Particles released into serum at 35 days
Passage 6, in woodchuck repeated	Liver sample taken at 73 days

[a] At the time of preparation of the final liver sample, the HDV had been in tissue culture for 12 days and in six consecutive woodchucks for a total of 253 days.

culture cells to assemble HDV. This virus was proven to be infectious in a woodchuck already chronically infected with the helper virus woodchuck hepatitis virus (WHV). After a total of six consecutive passages of this virus in woodchucks, we examined the RNA in the infected liver for HDV genome stability. We examined a 352-nucleotide sequence, using the known editing site, at nucleotide 1012, as a reference point for specific and efficient changes. Among our findings was the observation not only of uridine-to-cytidine changes but also of adenosine-to-guanosine changes. While the frequency of change at 1012 was significant (9.7%, 3 of 31 clones), there were also changes of at least comparable frequency nearby, at 1007 and 1005, as well as an almost random distribution of less frequent changes. These results have implications in terms of the mechanism of HDV RNA editing and concerning the fidelity of RNA-directed RNA synthesis.

MATERIALS AND METHODS

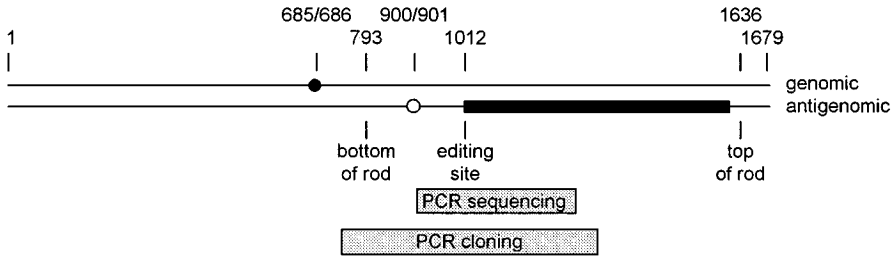
Assembly and transmission of HDV. Ten large plates (100-mm diameter) of human hepatoma Huh7 cells (38) were cotransfected with both HDV cDNA pSVL(D3) (30, 31) and a WHV construct, pCMW82 (45). Virus particles were harvested between 4 and 12 days after transfection as previously described (42). A pool of 40 ml was clarified, collected by centrifugation through sucrose, resuspended in 0.4 ml (4×10^9 RNA-containing particles), and used as the inoculum for injection into the portal vein of an anesthetized woodchuck. The passage history of the HDV thus obtained is summarized in Table 1. All animals were assayed weekly for HDV in the serum. In passage 1, the animal was sacrificed soon after it became positive for HDV. In passages 2 to 5, we identified that the animals had reached a high titer of acute HDV infection and then sacrificed them. In passage 6, the animal reached only a moderate level of HDV replication which persisted to day 73, at which time the animal was sacrificed and the liver RNA was extracted.

DNA primers. Figure 1 summarizes the various DNA primers that were used for reverse transcription, PCR amplification, and DNA sequencing. These are shown in relationship to the HDV RNA genome, with numbering according to the nucleotide sequence of Kuo et al. (31).

Reverse transcription. For reverse transcription, we used a primer that bound to antigenomic RNA (primer 701-735 or 701-714). The RNA template (<1 μg) was copied by using avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) for 1.5 h at 37°C. The product was incubated with RNase A (160 μg/ml, 20 min, 37°C), treated with pronase and sodium dodecyl sulfate, extracted with phenol and then twice with ether, and precipitated with ethanol. As explained below, this product was then used for either PCR amplification and cDNA cloning or PCR amplification and direct dideoxy sequencing.

cDNA cloning. The *Taq* polymerase (Gibco BRL) is known to be able to add a single untemplated adenosine to the 3′ ends of the double-stranded DNA products of a PCR (10, 21). This is exploited in a kit (Promega) to facilitate ligation into a plasmid vector (pGEM-T) that has been linearized and then extended at its 3′ ends with thymidine. The RNA PCR was carried out as previously described, using *Taq* polymerase and a Techne dry-block thermal cycler (35). For the final PCR, the primers were 737-771 and 1301-1267. The PCR product was generated with 37 cycles of amplification and then gel purified. After ligation to the plasmid vector, this DNA was used to transform *Escherichia coli* JM109, and then positive colonies were initially selected by a blue-white screening system.

Dideoxy sequencing. Sequencing was applied to PCR products made directly from reverse transcripts or those made from the DNA released from recombinant clones. In the latter case, plasmid DNA was released by boiling the colonies in 100 μl of water for 2 min and then transferring them promptly to ice. In both cases, the DNA samples were next submitted to 37 cycles of PCR amplification using Vent polymerase (New England Biolabs) and primers 737-771, with a 5′-biotin, and 1301-1267. The PCR products were immobilized on avidin-coated superparamagnetic beads (Dynal) and then denatured with 0.1 M sodium hydroxide for 15 min at room temperature to separate antigenomic DNA from bead-bound genomic DNA (24). The eluate was neutralized with 4 M ammo-



genomic primers:	701 - 735	AGCCTCCTCGCTGGCGCCGGCTGGTCAACATTCCCG
	737 - 771	GGGGACCGGCCCCTCGGTAATGGCGAATGGGACCC
	859 - 880	CCCAGGTCGGACCGCGAGGAGG
	982 -1003	AAGGGCGGATCGGCTGGGAAGA
	1105 -1126	CCCCCGGGCGCTCCCCTCGATC
antigenomic primer:	1301 -1267	CAGGATCACCGACGAAGGAAGGCCCTCGAGAACAA

FIG. 1. Representation of the HDV genome as a double-stranded cDNA, with indication of primers used in PCR sequencing (both direct and postcloning) and PCR cloning. The shaded boxes indicate the regions used for PCR sequencing and PCR cloning. The nucleotide sequence and positions are from Kuo et al. (31). The self-cleavage sites on genomic (closed circle) and antigenomic (open circle) sequences are from Kuo et al. (32) and Sharmeen et al. (46), respectively. The predicted top and bottom of the HDV rod-like sequence are from Wang et al. (49) and Kuo et al. (31), as is the open reading frame on the antigenomic sequence for the delta antigen, indicated by the black box.

nium acetate (pH 4.8) and collected by precipitation with ethanol. Dideoxy sequencing of the released strand was carried out by using a kit (Sequenase) together with [^{35}S]dATP and primers 859-880, 982-1003, and 1105-1126, as summarized in Fig. 1.

RESULTS

Assembly and transmission of infectious HDV. For these studies, we started with a plasmid containing HDV cDNA (30) of known sequence (31). This plasmid and a plasmid which expresses the envelope proteins of WHV (45) were used to cotransfect cultured cells (42). Virus particles released from these cells proved infectious when injected into a woodchuck that was already chronically infected with WHV. As summarized in Table 1, particles from the serum of this animal were passaged consecutively into five more woodchucks. From the final animal, we examined the HDV nucleotide sequences in the infected liver.

Nucleotide sequence analyses. We chose to focus on only a part of the 1,679-base HDV genome. As indicated in Fig. 1, we selected the 352-nucleotide region from 905 to 1256 in the sequence of Kuo et al. (31). This corresponds to the region recently used by Casey et al. for an analysis of the sequence stability of as many as 14 different HDV isolates collected from around the world (4). The region is particularly informative for several reasons. (i) It includes the site of RNA editing at position 1012 (35), and it extends through (ii) part of the open reading frame for the delta antigen, (iii) the 19-amino acid extension unique to the large form of the delta antigen, and (iv) noncoding sequences which include the poly(A) signal and poly(A) site used for the mRNA of the delta antigen (19, 20).

For our sequencing, we made use of two PCR-based procedures. The first, called direct PCR sequencing (4), basically involves RNA PCR of viral sequences followed by dideoxy sequencing of the PCR product. This approach is of limited value. It can reveal the consensus nucleotide sequence for the region, if such a consensus exists. In addition, against such a consensus, it is possible to detect those sequence variants that arise via single-nucleotide changes if such variants are at least present at >20% relative to the consensus sequence. Sequence variants involving insertions or deletions are difficult to detect by this method. In the second method, we made a double-stranded DNA PCR product through the specific region and then ligated this fragment into a plasmid vector. The ligated DNA was used to transform *E. coli*, and recombinant clones were selected and the plasmid DNA amplified via PCR prior to dideoxy sequencing.

As a first step to determine nucleotide changes on the HDV genome during the passage in animals, we directly sequenced the reverse transcription-PCR product made from antigenomic HDV RNA in infected liver. We studied the antigenomic rather than the genomic RNA in the liver. The primary reason for this choice was to avoid genomic RNA that might have been already assembled into particles and released from the infected cells. A second reason was that any antigenomic RNA in the cell must have undergone at least one round of RNA-directed RNA synthesis in that cell.

Such direct sequencing demonstrated that there was a predominant nucleotide sequence. This consensus sequence not only existed but was indistinguishable from the original nucleotide sequence (39). We did detect a minor component relative to this consensus, which corresponded to about 20% of the uridine at 1007 being changed to cytidine (39). However, to confirm this and to reliably detect less abundant species, we also used RNA PCR with cloning, prior to PCR sequencing. Since PCR procedures can introduce sequence changes, we carried out, in parallel, the following negative control. Anti-genomic cRNA species, as synthesized in vitro with phage SP6 RNA polymerase, were submitted to cloning and sequencing. Ten clones were sequenced through the 352-base region. Not a single nucleotide change relative to the known sequence was detected (39). As will now be explained, this result was different from that obtained for many of the 31 clones of antigenomic RNA from the infected liver.

TABLE 2. Sequence variations detected on HDV liver RNA between nucleotides 905 and 1256[a]

Clone	Position (change)
1	1005 (U-C), 1007 (U-C)
2	1007 (U-C), 1012 (U-C), 1192 (U-C)
3	942 (A-G), 1005 (U-C), 1007 (U-C)
4	1090 (U-C)
5	942 (A-G), 1005 (U-C), 1007 (U-C)
6	1229 (U-C)
7	NC[b]
8	NC
9	NC
10	1209 (G-A)
11	NC
12	NC
13	1007 (U-C)
14	1001 (A-G), 1007 (U-C), 1018 (A-G), 1019 (A-G), 1219 (U-A)
15	1182 (U-C)
16	NC
17	NC
18	994 (G-C), 1172 (U-C)
19	NC
20	912 (A-C), 941 (A-G)
21	966 (A-G), 990 (A-G)
22	946 (C-G)
23	NC
24	1007 (U-C)
25	1051 (C-U)
26	950 (U-C), 951 (U-C), 953 (U-C), 963 (U-C), 1012 (U-C)
27	1012 (U-C)
28	963 (U-A), 1077 (G-A)
29	NC
30	1034 (U-C)
31	1007 (U-C)

[a] After the passage history summarized in Table 1, the HDV antigenomic RNA sequences were amplified by RNA PCR and then cloned and sequenced as described in the text.
[b] NC, no changes.

Table 2 summarizes the data for 352-nucleotide region for each of the 31 clones sequenced. Ten of the 31 sequences (32%) were identical to the original sequence. The other 21 sequences had changes, but there were no insertions or deletions. All of the detected changes were single-nucleotide substitutions. The frequency of such changes per 352-nucleotide region is summarized in Fig. 2. The average number of changes per region was 1.35.

The nature of these single-nucleotide changes is summarized in Fig. 3. It was not surprising that most of the changes were base transitions rather than transversions (14). However, of the four possible transitions, two predominated; 60% (24 of 40) were uridine to cytidine, and 20% (8 of 40) adenosine to guanosine. This is the nature of the change as expressed for the genomic RNA, even though the liver RNA was reverse transcribed so as to copy the HDV antigenomic RNA. Now antigenomic RNA is not present in virions but is produced during genome replication. Therefore, because of this replication, we do not know on which strand of the RNA, genomic or antigenomic, a particular change actually took place. Also, we

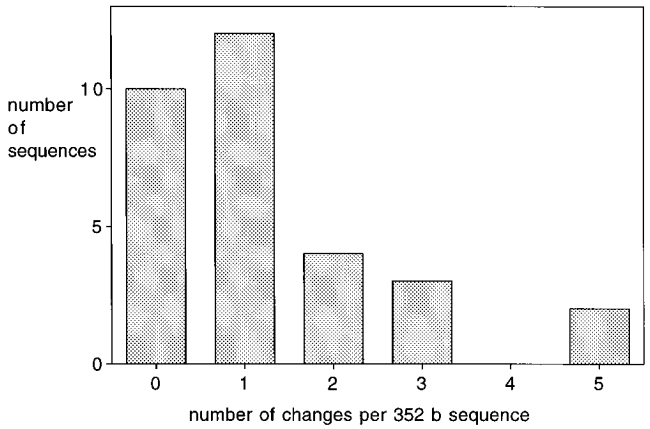


FIG. 2. Frequency of nucleotide changes per 352-base (b) sequence. Data are from Table 2.

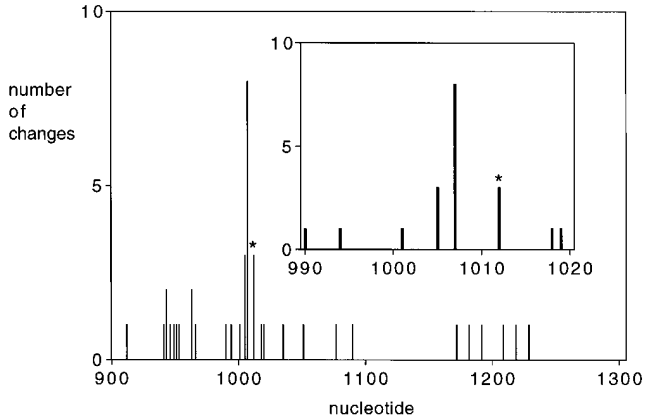


FIG. 4. Locations of single-nucleotide changes detected. Data are from Table 2. The change at 1012 is indicated by an asterisk. The inset shows in greater detail the region with the highest level of changes.

cannot tell whether the region sequenced was part of a replication-competent genome.

The distribution of the changes across the sequenced region is summarized in Fig. 4. Some of the changes occurred more than once. Of special interest is the change of uridine to cytidine at 1012, which corresponds to the previously described RNA-editing reaction that allows the synthesis of the large form of the delta antigen (35). We observed 9.7% (3 of 31) of the clones to be changed at this site. This value was independently confirmed by an RNA-editing assay (53) as 9% (39). However, as can be seen in the inset of Fig. 4, there were just as many changes at position 1005 and even more at 1007 (26%, 8 of 31).

DISCUSSION

At the outset of this study, we used cDNA clones cotransfected into cultured cells to both initiate HDV genome replication and achieve assembly of virus-like particles. These particles were able to initiate infection of a woodchuck.

Because this study was initiated with a single HDV sequence (31), we had the opportunity to study the subsequent conservation of this sequence. After six consecutive passages in woodchucks (Table 1), the HDV nucleotide sequences in the infected liver were examined through a 352-nucleotide region (Fig. 1). This region corresponds to that previously studied by Casey et al. (4) for 14 different HDV isolates taken from patients at different locations around the world. These isolates differed from each other by more than 30%.

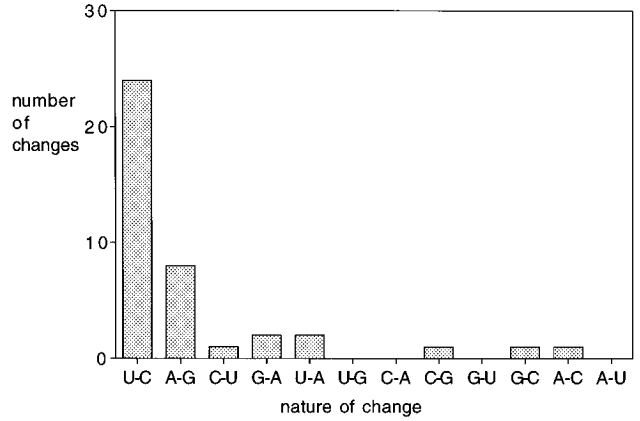


FIG. 3. Nature of single-nucleotide changes detected. Data are from Table 2.

By direct PCR sequencing, a consensus sequence was observed (39), and it was the same as our original sequence (31) used to transfect the cultured cells. We also sequenced 31 cDNA clones through the same region and found that 32% of our sequences (10 of 31 clones) were identical to the original cDNA sequence (Table 2). If it can be assumed that this rate of 32% unchanged per 352 nucleotides applies to the remainder of the 1,679-nucleotide genome, then we deduce that only around 0.3% of the genomes would have no changes.

In other RNA virus systems, multiple passages of the virus, especially at a high multiplicity of infection such as was used in our study, frequently lead to the production of defective interfering genomes. Such genomes typically contain significant deletions and can replicate only in the presence of unmodified genomes (22). In contrast to this, we detected no insertions or deletions on HDV. Our interpretation is that if they occurred for HDV, they were not tolerated. This interpretation is consistent with other studies in which deleted forms of the HDV genome could not be made to replicate (33).

In a negative control study in which in vitro-transcribed RNA was PCR amplified, cloned, and sequenced, we were unable to detect any changes in 10 clones. This corresponds to an error rate of <0.0003 changes per nucleotide. This is at least 10 times less than the average rate that we observed for the HDV RNA from the infected liver. Nevertheless, it still cannot be rigorously excluded that the procedures that we used for studying HDV RNA might have been responsible for the creation of some of the detected changes, or even for the removal or inappropriate "correction" of other changes.

The 40 single-base changes that we detected were predominantly transitions rather than transversions (Table 2). Of particular interest was that two kinds of transition predominated: 80% of the changes in the genomic RNA sequence were either uridine to cytidine or adenosine to guanosine (Fig. 3). Now the RNA editing that changes the termination codon of the small form of the delta antigen is equivalent to a uridine-to-cytidine change on the genomic RNA at position 1012 (35). It is interesting to consider the possibility that the other uridine-to-cytidine changes were created by the same mechanism and that even the adenosine-to-guanosine changes were also made by the same mechanism, but acting on the antigenome rather than the genome. Although the RNA editing at 1012 has been

reported as occurring on genomic RNA (3, 53), a recent reinvestigation by Casey and Gerin (2) has presented evidence to the contrary. It seems that the change at 1012 is actually on the antigenomic RNA and that the change is equivalent to changing adenosine to guanosine. If this is the case, HDV RNA editing may be via the double-stranded RNA adenosine deaminase. This ubiquitous enzyme, previously referred to as unwindase, acts on double-stranded RNA species and deaminates adenosine to inosine (1, 28). There is now evidence that the double-stranded RNA adenosine deaminase acts on cellular RNAs (6) and even other viral RNAs (5). At one point, it was even speculated as a candidate for HDV RNA editing (35).

It is maybe informative to consider the locations and the possible consequences of the changes that were detected. Of the 40 changes detected on HDV, 35% (14 of 40) occurred within an eight-nucleotide region that included position 1012, the previously studied site of RNA editing (Fig. 4). We note that several clones demonstrated closely spaced clusters of nucleotide changes either in this or other regions (Table 2). In addition to the RNA-editing site at position 1012, the 352-nucleotide region of the HDV genome was studied by us and by Casey et al. (4) contains three sequence elements of distinct function: (i) part of the open reading frame for the delta antigen (1256 to 1014), (ii) a region which, because of editing at position 1012, is converted from noncoding to coding (1013 to 954), and (iii) a noncoding region (953 to 905) with important *cis*-acting regulatory elements such as the poly(A) signal and poly(A) site (19). Using the data of Table 4, we deduce rates of change per nucleotide for these three regions to be 0.0016, 0.0108, and 0.0053, respectively. Even though nucleotide changes in the coding region were less frequent, of those that might alter amino acids in a functional open reading frame, 9 of 14 corresponded to nonconservative amino acid substitutions (11). One clone showed changes that would inactivate the poly(A) signal (19) and could thus interfere with replication competence. However, for the other changes, in the absence of additional studies, we really do not know whether the altered molecules retained replication competence.

In an examination of 14 independent HDV isolates by Casey et al. (4), there was as much as 37% divergence in the nucleotide sequence. In our study of the same region on the HDV genome, we found no evidence of progressive evolutionary changes in HDV sequence. Kos et al. (29) reached a similar conclusion on the basis of HDV passaged in chimpanzees. Likewise, we did not detect any major evolutionary steps, the so-called episodic evolution (17), or punctuated equilibrium (40). Of course it is likely that 253 days of in vivo passage in woodchucks is too short a period of time in which to detect such changes. The observed stability of our HDV sequence also has to be considered in the light of an extensive passage history of the HDV before its subjection to cDNA cloning. As related by Kos et al. (29) and Dény et al. (12), an initial human isolate was experimentally passaged twice through chimpanzees and then five times through woodchucks before we made the cDNA with which to assemble an infectious clone (30, 31).

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