# Structural requirements for RNA editing in hepatitis δ virus: Evidence for a uridine-to-cytidine editing mechanism

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**ABSTRACT** Hepatitis δ virus (HDV) nucleotide 1012 is edited from uridine to cytidine in 10–40% of the RNA genomes during replication. This editing event is an important control point in the HDV life cycle because it results in both the packaging of viral RNA and the inhibition of HDV replication. We find that the editing event is highly specific for both the sequences neighboring nucleotide 1012 and the base-paired context of position 1012 within the unbranched rod structure of HDV RNA. Prior studies identified the base transition at nucleotide 1012 but were unable to distinguish between editing of the genomic versus the antigenomic strands [Luo, G. X., Chao, M., Hsieh, S. Y., Sureau, C., Nishikura, K. & Taylor, J. (1990) *J. Virol.* 64, 1021–1027]. In this study, comparisons of mutations that differentiate between base pairing in genomic and antigenomic RNAs indicate that the genomic strand of HDV is the actual editing substrate. We conclude that the virus uses a uridine to cytidine editing mechanism, which is provided by the host cell.

Hepatitis δ virus (HDV) is a subviral pathogen of humans. It requires concurrent infection with hepatitis B, which provides the viral coat protein (1, 2). Compared to infection with hepatitis B virus alone, coinfection or superinfection with HDV significantly increases the risk of more severe liver disease, including fulminant hepatitis (3). The HDV genome is an ≈1.7-kilobase, single-stranded, circular RNA molecule, which shares some unusual features with plant viroid RNAs (reviewed in ref. 4). The circular RNA molecule possesses significant intramolecular complementarity such that ≈70% of the nucleotides can form base pairs in an unbranched rod structure (5, 6). Consistent with a rolling circle replication mechanism similar to that of the plant viroid agents, monomers and multimers of both genomic and antigenomic RNAs are found in infected and transfected cells (7–9). Unlike the viroids, HDV produces a single protein, the hepatitis δ antigen (HDAg), which is translated from an antigenomic sense mRNA (9, 10). HDAg is an RNA binding protein (11–13) and is found as a mixture of 24- and 27-kDa species in both infected cells and virions (14, 15). The two forms of HDAg play central roles in the HDV replication cycle. p24 is necessary for HDV RNA replication in transfected cells in culture (8), while p27 both inhibits replication (16, 17) and enables packaging of the HDV RNA genome (18).

As shown by others (19), the heterogeneity of HDAg likely arises via an RNA editing mechanism wherein the antigenomically encoded stop codon for p24 is changed from UAG to UGG; the resultant translation product, p27, contains an additional 19 amino acids at the C terminus. Because of the circular replication cycle of HDV RNA, the base change was found in both the genomic and antigenomic RNAs (19); it was therefore unclear whether the genomic or the antigenomic RNA is the actual substrate for editing. An A-to-G transition in the antigenomic strand, possibly involving a host double-

stranded RNA modifying activity (20), has been suggested as a possible mechanism (19); however, other mechanisms, including a U-to-C transition in the genomic strand, could not be ruled out. In this report, we examine the sequence and structural requirements for editing and conclude that editing in HDV involves a highly specific U-to-C conversion in the genomic RNA.

## MATERIALS AND METHODS

**Plasmid Constructs and Mutations.** A full-length clone of HDV was obtained by joining the HDV fragments in clones pδ4 and pδ115 (5) at a common *Bst*XI site. A unit-length *Nhe* I fragment from the resulting clone was inserted into the *Nhe* I site of a derivative (J.L.C., unpublished data) of pGEM3Zf+ (Promega) to give the monomeric clone pGDC-1 and the head-to-tail dimeric clone pGDC-1×2. Partial digestions of pGDC-1×2 with *Sal* I (position 963) and *Sty* I (position 620) eventually yielded a clone, pGDC-1×1.2, which contains 1.2 genomes. The autocleavage domains, between nucleotides 620 and 963, are repeated in the 1.2-mer. Nucleotide numbering is according to ref. 5.

Mutations were obtained by PCR using mutant primers. Primers >40 nucleotides long were first purified by acrylamide gel electrophoresis. The PCR conditions were as follows: 5 min at 95°C; 5 cycles of 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C; and then 20 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Mutations UGA and UAA were cloned as 125-base-pair *Sal* I/*Pst* I fragments into the monomeric HDV plasmid pGDC-1 and were subsequently cloned as dimers. Similarly, mutation 580A was cloned as a 185-base-pair *Bst*BI/*Nar* I fragment into pGDC-1 and was then cloned as a dimer. Mutations 578G, 580C, and 580U were cloned as *Bst*BI/*Nar* I fragments into pGDC-1×1.2; 580Δ and 583G were cloned as 205-base-pair *Nco* I/*Xba* I fragments into pGDC-1×1.2; 1014C and 1009C were cloned as *Sal* I/*Pst* I fragments into pGDC-1×1.2. Clones combining mutations were obtained by substituting the unique *Sal* I/*Nhe* I fragment of the mutation in the nucleotide 1012 region for the same region in the relevant clone carrying a mutation in the nucleotide 580 region. All clones were sequenced over the amplified regions to check for fidelity and for the presence of the desired mutation.

**Cell Culture and Transfection.** CV-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM glutamine, and penicillin/streptomycin. Cells were seeded at a density of $3 \times 10^3$ cells per cm² 1 day prior to transfection by the calcium phosphate method (21).

**RNA Analysis.** RNA was harvested by an SDS lysis procedure (22) 12–14 days posttransfection or as indicated. RNA was assayed for the conversion of 1012U to 1012C by a combination of reverse transcription/PCR amplification and restriction endonuclease digestion. RNA (0.5 μg) was treated with RQ1 DNase (Promega) for 20 min at 37°C. The DNase

was inactivated by incubation at 95°C for 5 min. cDNA was prepared by using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL), 1 mM dNTPs, and 12.5 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP (Amersham; 3000 Ci/mmol; 1 Ci = 37 GBq) and random hexamers (Pharmacia) as primers. A 340-base-pair cDNA fragment was amplified with *Taq* polymerase (Perkin–Elmer) in the PCR, using primers 5'-GACGCGAGACG-CAAACCTGT-3' and 5'-ATCGGCGGGAGGCAAGAACC-3', which correspond to positions 915–934 and 1254–1235, respectively; the amplification was performed with 25 cycles of 75 s at 94°C and 90 s at 60°C in a reaction vol of 50 $\mu$l. Five microliters of the amplification mixture was incubated with restriction endonucleases as indicated and electrophoresed on a 6% acrylamide (30:1) gel, fixed, dried, and exposed to x-ray film or scanned with an Ambis $\beta$ detector for quantitative results.

**Protein Analysis.** Cells were lysed in 50 mM Tris·HCl, pH 7.5/2% SDS/1 mM EDTA and incubated at 100°C for 10 min prior to analysis of HDAg by SDS/PAGE and immunoblotting (14).

## RESULTS

The important role of the edited base, nucleotide 1012, in the viral life cycle of HDV is indicated in Fig. 1A. Viral genomes with 1012U produce an antigenomic sense mRNA (9, 10), which contains a stop codon at position 196 to give the 195-amino acid HDAg protein p24. In comparison, genomes containing 1012C produce an mRNA with a tryptophan codon at position 196 and yield the 214-amino acid HDAg protein p27. Because p27 inhibits replication, only genomes with 1012U will initiate replication upon transfection of cells in culture (16, 17). Transfection of CV-1 cells with a dimeric HDV cDNA construct containing 1012U resulted in p24 production (Fig. 1B), as expected from the primary sequence. During the course of replication p27 appeared, accounting for ≈10% of the total viral protein 13 days posttransfection. The kinetics and extent of p27 production was similar to that obtained by others in HuH7 cells (18). The viral RNA can be analyzed by restriction enzyme digestion of amplified cDNA; 1012C genomes contain restriction sites (e.g., *Nco* I, *Nla* III, *Dsa* I), which 1012U genomes lack (23). Examination of the viral RNA by *Nco* I digestion of an amplified cDNA fragment showed that the identity of position 1012 changed from U to C with the same kinetics and to a similar degree as p27 production (Fig. 1C). This close correlation between the editing of position 1012 and the form of HDAg produced supports the conclusion (19) that the base change most likely accounts for all of the p27 produced; other possible mechanisms for p27 production (posttranslational modification, frameshifting, and use of suppressor tRNAs) need not be invoked.

There is indirect evidence that editing of position 1012 is highly specific. Cloning and sequencing of HDV genomes has revealed microheterogeneity within isolates; while position 1012 (or its equivalent) is heterogeneous in several isolates (5, 24, 25), other positions of microheterogeneity vary from one isolate to another. Furthermore, in transfection studies in which 1012U was modified to 1012C in 3–5% of the genomes, possible modification of other sites was estimated to occur at rates 500-fold lower (19). We addressed the sequence and structural specificity of the editing target by mutational analysis. Positions 1011 and 1012 were altered by site-directed mutagenesis such that the stop codon for p24 became UGA or UAA rather than the wild-type UAG. To observe p27 production in the UGA mutation would require editing of UGA to UGG, which is equivalent to the genomic conversion of 1011U to 1011C. Upon transfection with the UGA mutation, no p27 was detected (Fig. 2A), whereas p27 was readily found in cells transfected with wild-type cDNA. The failure to observe p27 in the UGA mutant indicates that 1011U was not edited. Thus,
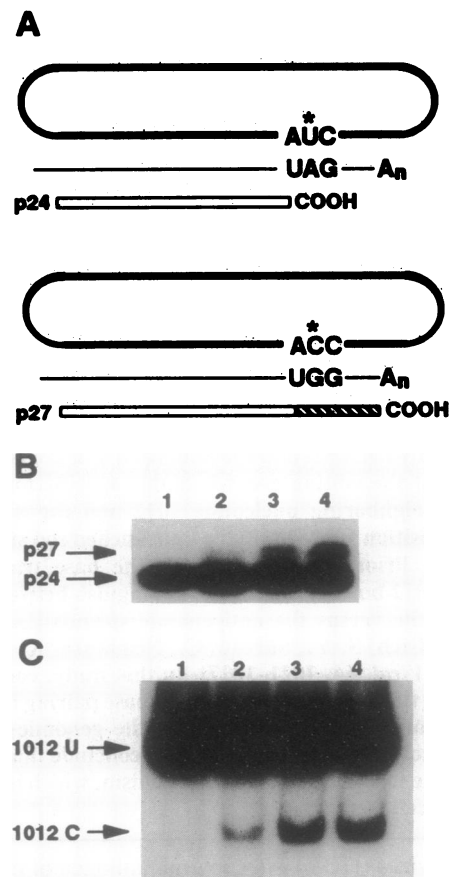
A



B



C

FIG. 1. The role of nucleotide 1012 in HDV replication and its modification from U to C after transfection of CV-1 cells with dimeric HDV cDNA. (*A*) HDV genomic RNA (circular heavy lines) with 1012U (*Upper*) or 1012C (*Lower*) are transcribed to give mRNAs (thin lines) with a UAG stop codon (*Upper*) or a UGG tryptophan codon (*Lower*), respectively. The 5' → 3' direction of the sequence is clockwise for the genomic circles and left to right for the antigenomic mRNA. Position 1012 is indicated by an asterisk. The mRNA with UAG is translated to give HDAg p24 (open rectangle). The mRNA with UGG yields HDAg p27, which contains an additional 19 amino acids (hatched rectangle) at the C terminus. The autocleavage domains of HDV RNA are at the right-hand end of the structures drawn. (*B* and *C*) CV-1 cells were transfected with a dimeric cDNA of HDV, pGDC-1×2, and harvested on days 3 (lanes 1), 6 (lanes 2), 9 (lanes 3), and 13 (lanes 4) posttransfection. (*B*) Immunoblot for HDAg. (*C*) RNA was analyzed for 1012U and 1012C by *Nco* I digestion of amplified cDNA followed by acrylamide gel electrophoresis. The amplified fragment is 340 base pairs long; *Nco* I digestion of fragments with 1012C yields a 240-base-pair product, while 1012U genomes yield uncut 340-base-pair products. Positions of fragments arising from the 1012U and 1012C genomes are indicated by arrows.

the editing at position 1012 is a highly specific process. This conclusion is supported by the results obtained with the UAA mutation. p27 production in the UAA mutant would require editing of U to C at both positions 1011 and 1012. Perhaps not surprisingly, p27 was undetectable in cells transfected with the UAA mutant (Fig. 2A) because two editing events would be required; changing either A alone would yield UGA or UAG, which are still stop codons. When we analyzed the RNA from cells transfected with this mutant, we found that editing did occur at position 1012 but at <30% of wild-type levels (Fig. 2B). It is clear from an analysis of both the UGA and UAA mutations that the editing that occurs during HDV replication is strongly dependent on the immediate sequence context of the target nucleotide.

Many processes requiring interaction with specific RNAs recognize not only primary sequence, but also the secondary

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**A**

(wt)
UAG UGA UAA

p27 →

p24 →
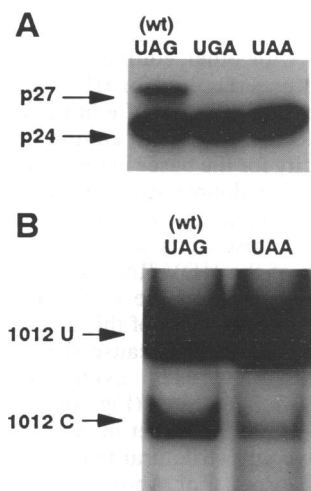
**B**

(wt)
UAG    UAA

1012 U →

1012 C →

FIG. 2. Mutations that change the stop codon of HDAg p24 abolish p27 production. CV-1 cells were transfected as described in Fig. 1 with dimeric cDNAs containing mutations of the p24 stop codon to UGA or UAA. Cells were harvested 13 days posttransfection for analysis of HDAg protein (*A*) and RNA (*B*), as described in Fig. 1, except that the restriction enzyme digestion was done with *Nla* III. wt, Wild type.

and higher-order structures of the target RNAs. One of the curious structural features of HDV RNA is that ≈70% of the nucleotides on one side of the circular molecule can pair with those on the opposing side to form an unbranched rod structure similar to that of the plant viroids (5, 6). This structure is essential for the replication of HDV RNA because extensive disruption of base pairing in the rod disables HDV RNA replication (J.L.C. and K.F.B., unpublished data). In the predicted rod structure generated by an RNA folding algorithm (26), nucleotides 1008–1016 are paired with nucleotides 576–584, as shown schematically in Fig. 3*A*. 1012U, the editing target, and 580G form a wobble pair in the midst of 8 Watson–Crick base pairs. For the antigenome, the bases corresponding to 1012U and 580G constitute an A·C
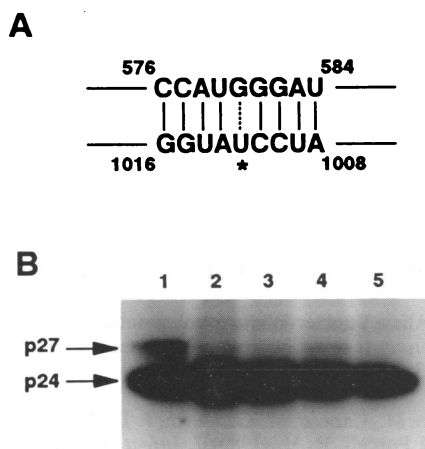
**A**

```
     576              584
── CCAUGGGAU ──
   │││││││││
── GGUAUCCUA ──
  1016      *      1008
```

**B**

```
              1   2   3   4   5
p27 →
p24 →
```

FIG. 3. Nucleotide 580G, which is paired with 1012U in the unbranched rod structure, is critical for editing of 1012U. (*A*) Schematic illustration of the base pairing surrounding nucleotide 1012 in the unbranched rod structure of genomic HDV RNA. Watson–Crick pairs are indicated by solid lines, the G·U wobble pair is indicated by a dotted line. Nucleotide 1012 is indicated by an asterisk. (*B*) 580G was mutated to 580A (lane 2), 580C (lane 3), or 580U (lane 4), or it was deleted (lane 5). HDV cDNAs containing these mutations or the wild-type sequence (lane 1) were transfected into CV-1 cells as described in Fig. 1. Cells were harvested 13 days posttransfection and analyzed for HDAg by immunoblot analysis.

mismatch pair. To assess the possible contribution of elements of the structure depicted in Fig. 3*A* to the editing of 1012U, we mutated nucleotide 580G such that it was changed to A, C, or U, or it was deleted. Transfection of the resulting constructs showed that any alteration of position 580 greatly diminished editing of 1012U, as detected by p27 production (Fig. 3*B*), as well as restriction enzyme analysis of amplified cDNA (data not shown). The level of HDAg produced by the mutant constructs indicates that the level of HDV replication was not affected by the mutations. As 580G is 432 nucleotides away from 1012U in the linear sequence, the strong dependence of editing activity on the identity of this position indicates that the specific context of position 1012 within the rod structure contributes to forming the proper substrate for editing.

To further address the structural requirements for editing, positions on either side of the 580·1012 pair were first mutated individually to disrupt the Watson–Crick base pairing; pairs of mutants were then combined to reestablish the canonical pairing (Fig. 4*A*). As judged by RNA levels (Fig. 4 *B* and *C*) and HDAg levels (data not shown), these mutant constructs replicated as well as the wild type. The substitution of 578A·1014U with 578G·1014C is a naturally occurring variation found in an isolate of HDV from the South Pacific island of Nauru (25). The effects of changing the 578A·1014U pair are shown in Fig. 4*B*. Disruption of the A·U pair in the mutants 1014C and 578G dramatically reduced editing of
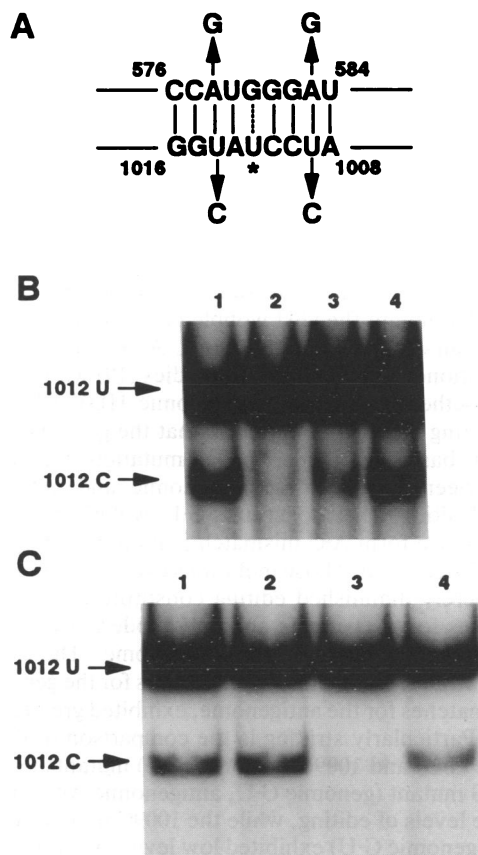
**A**

```
              G         G
              ↑         ↑
     576     |         |     584
── CCAUGGGAU ──
   ││││┊│││││
── GGUAUCCUA ──
  1016  |    *    |    1008
        ↓         ↓
        C         C
```

**B**

```
              1   2   3   4
1012 U →

1012 C →
```

**C**

```
              1   2   3   4
1012 U →

1012 C →
```

FIG. 4. Inhibition of editing by disruption of base pairing surrounding 1012U in the genomic strand rod structure. (*A*) As described in Fig. 3*A*. Selected positions were individually mutated as indicated to disrupt base pairing. (*B*) CV-1 cells were transfected with wild-type or mutant cDNAs and harvested on day 13 posttransfection for RNA analysis as described in Fig. 1 except that the restriction endonuclease *Dsa* I was used; wild-type (lane 1), 1014C (lane 2), 578G (lane 3), and 578G·1014C (lane 4). (*C*) As described in *B*, with the mutations 583G (lane 2), 1009C (lane 3), and 583G·1009C (lane 4).

1012U, while restoration of the structure with the naturally occurring G·C pair (578G·1014C) restored editing to ≈75% of wild-type levels. Similar to the observations for the 578·1014 pair, the 583A·1009U base pair was found to be important for maximal editing activity. Editing of 1012U was diminished by replacement of the 583A·1009U pair with an A·C mismatch in the mutant 1009C (Fig. 4C, lane 3) and was restored to ≈65% of wild-type levels by repairing with 583G·1009C (lane 4). In all cases, constructs that exhibited reduced editing at position 1012 produced low or undetectable levels of p27 (data not shown).

The mutational analysis indicates that nucleotides 578A, 583A, 1009U, and 1014U contribute primarily structural rather than sequence specificity to the editing target by forming base pairs that flank the editing site. Disruption of these base pairs inhibits editing, while changing the nucleotides to form a different pair has only a modest effect on the extent of conversion. Of the 8 Watson–Crick base pairs surrounding the 580G·1012U pair, we find that 4 (the two mentioned above plus 584·1008 and 582·1010) can be changed to other canonical pairs without significantly reducing the extent of editing at position 1012 (data not shown). The lower degree of editing observed for the double mutants 578G·1012C and 583G·1009C, which restore base pairing, suggests that some positions contribute sequence specificity, which may modulate editing levels. This observation could be important for the naturally occurring variation found in the Nauru isolate (25).

There are positions within the structure depicted that do contribute greater sequence specificity. Alteration of 1011G to 1011A in the UAA mutation, which exhibited reduced editing of 1012U (Fig. 2B), disrupts the pair 581G·1011C; however, restoration of base pairing with 581A·1011U did not boost editing levels at all (data not shown). The lack of editing in the UGA mutation (Fig. 2A) suggests additional sequence specificity. This mutation, in effect, switches the positions of the 580G·1012U wobble pair and the 581G·1011C pair, with no disruption of base pairing overall; it could be important that in the UGA mutant the G·U wobble pair is situated between two G·C pairs rather than between U·A and G·C.

As mentioned above, previous studies (19) were unable to define whether genomic or antigenomic HDV RNA is the actual editing target. We conclude that the genomic RNA is the target, based on comparisons of mutations that differentiate between base pairing in genomic and antigenomic RNAs. Positions that constitute G·U wobble pairs in the genomic sense form A·C mismatch pairs in the antigenomic sense and vice versa. Those mutations examined in Fig. 4 that most severely diminished editing constitute disruptive A·C mismatches for the genome and only moderately destabilizing G·U wobble pairs for the antigenome. The converse mutants, which result in G·U wobble pairs for the genome and A·C mismatches for the antigenome, exhibited greater editing activity. Particularly striking is the comparison of the 583G (genomic G·U) and 1009C (genomic A·C) mutants (Fig. 4C). The 583G mutant (genomic G·U, antigenomic A·C) exhibited wild-type levels of editing, while the 1009C mutant (genomic A·C, antigenomic G·U) exhibited low levels of editing, which could be rescued by the restoration of base pairing. Similar results were obtained for the 578A·1014U pair. The 578G mutant, with a genomic G·U wobble pair, was impaired ≈70% for editing of 1012U, but it was at least 3 times more effective than the 1014C mutant with an A·C mismatch pair (Fig. 4B). Because the editing target appears to be the genomic RNA, we conclude that the actual editing event is a uridine-to-cytidine transition, which has not previously been reported in mammalian cells.

## DISCUSSION

When compared with other editing systems, the sequence and structural features required for HDV editing appear to be unique. We have found that HDV editing requires a double-stranded region centered on the editing target, with some sequence specificity for the bases in the immediate vicinity of the target base. The double-stranded nature of the HDV target evokes a comparison with editing that uses guide RNAs for specific insertions and deletions (27). In this analogy, one side of the HDV RNA would serve as a guide for editing the sequence on the opposing side of the rod structure. The appropriateness of this analogy remains to be more thoroughly examined, because there appears to be a low level of editing of 1012U even when the opposing "guide" base, 580G, is mutated (Fig. 3B). In another editing system, the C-to-U editing target in ApoB-100 mRNA does not appear to have any structural features in common with the HDV editing target. For ApoB-100, nucleotides neighboring the edited base have little or no effect on the degree of editing (28), but a stretch of 11 nucleotides beginning 5 nucleotides away is important (29); the role of RNA secondary structure in ApoB-100 editing is unclear. Little is known about the role of specific sequences and secondary structures in C-to-U and U-to-C editing processes that have been described in plant mitochondrial mRNAs (30–33).

The editing process for HDV is likely to be regulated directly or indirectly by HDV replication. First, editing requires replication of the HDV RNA; nonreplicating defective genomes do not exhibit editing (19) (J.L.C., unpublished data). Second, there is reason to believe that the timing and extent of editing would be controlled. If the base change occurred too soon or too efficiently, viral RNA levels would be lower due to inhibition of replication by p27, and a higher percentage of the packaged viral genomes would be not only defective but inhibitory. Inefficient editing would result in low p27 levels and presumably less-efficient packaging. From the time course of the appearance of p27 and 1012C, it appears that the editing process is regulated such that it does not occur until sufficiently high levels of HDV RNA and p24 have accumulated. It is reasonable to hope that understanding the editing process and its regulation will aid our understanding of HDV pathogenesis and contribute to antiviral therapies.

As HDV produces just the HDAg protein, which functions in replication and packaging of the viral RNA, it is most likely that the HDV editing activity is provided by the host cell. The simplest mechanism for such a U-to-C transition would be a transamination reaction similar to that used in the cytidine biosynthetic pathway. Presumably, there exists a cellular substrate for this editing system. We expect that the study of this editing system and its cellular and viral targets will be yet another door to discovery opened by this fascinating virus.

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