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Control of ADAR1 editing of hepatitis delta virus RNAs

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Abstract

Hepatitis delta virus (HDV) uses ADAR1 editing of the viral antigenome RNA to switch from viral RNA replication to packaging. At early times in the replication cycle, the virus produces the protein HD Δ g-S, which is required for RNA synthesis; at later times, as result of editing at the amber/W site, the virus produces HD Δ g-L, which is required for packaging, but inhibits further RNA synthesis as levels increase. Control of editing during the replication cycle is essential for the virus and is multifaceted. Both the rate at which amber/W site editing occurs and the ultimate amount of editing are restricted; moreover, despite the nearly double stranded character of the viral RNA, efficient editing is restricted to the amber/W site. The mechanisms used by the virus for controlling editing operate at several levels, and range from molecular interactions to procedural. They include the placement of editing in the HDV replication cycle, RNA structural dynamics, and interactions of both ADAR1 and HD Δ g with specific structural features of the RNA. That HDV genotypes 1 and 3 use different RNA structural features for editing and control the process in ways related to these features underscores the critical roles of editing and its control in HDV replication. This review will cover the mechanisms of editing at the amber/W site and the means by which the virus controls it in these two genotypes.

1 Introduction

1.2 Hepatitis delta virus

Hepatitis delta virus (HDV) is an important human pathogen that causes potentially severe acute and chronic hepatitis. It requires simultaneous infection with hepatitis B virus (HBV). The helper function provided by HBV is the envelope protein, HBsAg, which is required for the assembly and release of HDV particles, as well as the ability of these particles to attach to and infect hepatocytes, the primary targets of infection. Compared with those infected with HBV alone, individuals infected with both HDV and HBV experience more severe liver disease, including cirrhosis, hepatocellular carcinoma and liver failure. Although HDV depends on HBV, current licensed anti-HBV pharmaceuticals are ineffective for treatment of this virus because HBsAg expression remains high enough to support continued propagation. Approximately 15 million individuals worldwide are chronically infected with HDV.

Eight clades (genotypes) of HDV have been identified (Deny 2006). Most molecular studies have been conducted using clones of genotype 1, which is the most geographically widespread and the predominant genotype in Europe and North America. Genotype 3 has also been of interest because it is the most distantly related genetically to other genotypes (~40% divergence at the nucleic acid level) and because it is associated with the most severe HDV disease in northern South America (Casey *et al.* 1993).

1.3 Genome structure of HDV

HDV is unique in that it uses a helper virus to provide its envelope protein, but the molecular virology of HDV is arguably even more unusual. The approximately 1700

nucleotide RNA genome is the smallest known to infect man. Yet, despite this small size, less than half the genome is devoted to encoding the sole viral protein, hepatitis delta antigen. To make up for the dearth of protein-coding information, the HDV replication cycle depends heavily on host factors and structural features of its RNA, including the unbranched rod-like structure of the entire genome, the double pseudoknotted ribozymes, and structures required for RNA editing. The genome and its replication intermediate, the antigenome, are circular RNAs that collapse into a characteristic unbranched rod-like structure in which about 70% of the nucleotides form base pairs (Fig. 1). This structure is essentially an elongated string of helices comprised of segments with 12 or fewer consecutive Watson-Crick base pairs interspersed with small internal bulges and loops, but no internal hairpins. In a two-dimensional representation of this structure (Fig. 1), the open reading frame for HDAg is on one side; sequences opposite the HDAg coding region in the circular RNA serve principally to form the unbranched rod structure. The unbranched structure plays several vital roles in the replication cycle. It is required for viral RNA replication (Casey 2002; Sato et al. 2004), binding to HDAg (Defenbaugh et al. 2009), the formation of viral particles (Chang et al. 1995; Lazinski and Taylor 1994) and for RNA editing and its regulation.

1.4 Hepatitis delta antigen

Hepatitis delta antigen (HDAg) is the sole protein encoded by HDV. This protein forms multimeric complexes and specifically binds HDV RNA in the unbranched rod-like structure. The two forms of HDAg produced during replication, HDAg-S and HDAg-L, differ structurally by the presence of an additional 19 or 20 amino acids at the C-terminus of HDAg-L. Functionally, they differ in two major respects. First, HDAg-S is required for RNA replication; HDAg-L not only does not support replication, but inhibits this process. Because of the multimeric nature of the protein, replication is highly sensitive to the relative levels of the two forms of HDAg: at an HDAg-L:HDAg-S ratio of 1:10 replication is reduced by as much as eight-fold (Chao *et al.* 1990; Xia and Lai 1992). The second functional difference between the two proteins is that HDAg-L is the limiting factor for the production of virus particles (Jayan and Casey 2005), which are formed via interactions between HBsAg and the C-terminal sequences unique to HDAg-L (Chang et al. 1991; Jenna and Bureau 1998).

1.5 The HDV Replication cycle

The current model of HDV replication involves a double rolling circle (Fig. 2). The circular genome serves as a template for the synthesis of two RNAs: the mRNA for HDAg, and multimeric (concatameric) RNAs of the opposite sense. The latter are cleaved by internal ribozymes and ligated to form circular antigenomes, which subsequently serve as templates for the production of circular genomes in the same manner. One of the more remarkable aspects of this replication scheme is that it is accomplished by redirection of Pol II for HDV RNA synthesis (Chang *et al.* 2008). The specific mechanisms involved are not fully understood, but appear to involve HDAg-S binding Pol II (Yamaguchi *et al.* 2001; Yamaguchi *et al.* 2007). Related to the use of the host RNA polymerase, HDV replication occurs in the nucleus.

1.6 RNA editing in HDV replication

The HDV replication scheme, including the placement of editing, is shown in Fig. 2. Initially, the mRNA produced encodes HDAg-S. As replication proceeds, the antigenome RNA is edited such that the adenosine within the amber stop codon that terminates the HDAg-S ORF is deaminated to inosine. The codon is thus changed to tryptophan and the ORF is extended by an additional 19 or 20 amino acids to yield HDAg-L. In accord with the naming convention used for other editing sites that affect protein-coding regions, this editing

site is referred to as the amber/W site. As noted above, HDAG-S is required for viral RNA replication, whereas HDAG-L inhibits replication but is essential for virion morphogenesis. Thus, HDV uses RNA editing as a mechanism to switch from genome replication to packaging.

In cultured cells transfected with HDV cDNAs to initiate replication, editing, as well as HDAG-L production, increases from barely detectable levels 3 days post-transfection to around 25% by about 2 weeks (Casey *et al.* 1992; Chao *et al.* 1990; Sato *et al.* 2004; Wong and Lazinski 2002). In this manner, HDAG-L, which is the limiting component for production of HDV particles, accumulates and peaks after HDV RNA levels peak. The changes in editing levels are not due to changes in ADAR expression: no changes in ADAR1 levels were observed in cells transfected with an HDV replication expression construct or in HDV-infected liver (Jayan and Casey 2002b; Wong and Lazinski 2002). Rather, editing levels increase during virus replication due to the placement of editing in the virus RNA replication cycle. Unlike other editing substrates in which protein coding is affected, amber/W site editing does not occur on the HDV mRNA itself; instead, the substrate for editing is the antigenome, which is a replication intermediate (Fig 2). Edited antigenomes serve as templates for the synthesis of genome RNAs with C at the corresponding position; these genome RNAs then serve as templates for the production of mRNAs encoding HDAG-L as well as additional “edited” antigenomes (Fig. 2). This scheme has several important consequences. First, the percentage of edited RNAs increases as long as replication occurs because edited RNAs are sustained via replication; moreover, additional editing events continue to convert unedited antigenomes to edited versions. Second, because HDAG-L inhibits viral RNA replication, but is the limiting factor for virion formation, virus replication and packaging are sensitive to the rate at which editing occurs. Finally, because edited genomes are packaged into virions but are not expected to be infectious, virus propagation could be negatively affected by excessive editing. It is therefore not surprising that HDV uses several specific mechanisms to ensure editing levels that are maximally productive.

2 Mechanism of HDV amber/W site editing

2.1 Role of ADARs in amber/W site editing

The relative roles of ADAR1 and ADAR2 in HDV RNA editing have been examined by several approaches, including siRNA knockdowns and analysis of expression levels in relevant cells, and comparison of the effects of site-directed mutagenesis of the RNA structure on editing by these enzymes. Both ADAR1 and ADAR2 are capable of efficiently editing the amber/W site. Early analyses found that *Xenopus* and *Drosophila* ADARs, which are similar to human ADAR1 and ADAR2, respectively, efficiently edited the amber/W site in the HDV antigenome RNA *in vitro* (Casey and Gerin 1995; Polson *et al.* 1996). Furthermore, when overexpressed, both human ADAR1 and human ADAR2 were found to efficiently edit the amber/W site in transfected cells (Jayan and Casey 2002a; Sato *et al.* 2001). However, the relative abilities of these two enzymes to edit the site have not been directly addressed; only hADAR1 has been examined for its ability to edit the RNA *in vitro* (Linnstaedt *et al.* 2006; Wong *et al.* 2003). *In vitro*, editing occurred efficiently under conditions that included only HDV RNA and purified enzyme, indicating that no additional factors are required (Polson *et al.* 1996; Wong *et al.* 2003).

Based on relative expression levels and on the effects of siRNA knockdown in cultured liver-derived cells, it is most likely that ADAR1 is responsible for editing at the amber/W site during the course of HDV infection. ADAR1 expression is 10-20 fold higher than ADAR2 in HDV-infected liver and in the liver derived cell line Huh-7 (Jayan and Casey 2002b; Wong and Lazinski 2002). Knockdown of ADAR1 expression reduced levels of

editing in both reporter constructs and replicating genomes (Jayan and Casey 2002b; Wong and Lazinski 2002). Consistent with these results, in analyses of a series amber/W site mutations the patterns of editing activities by endogenous ADAR activity in Huh-7 cells better matched that obtained by co-transfected ADAR1 expression constructs (Casey and Gerin 1995; Polson *et al.* 1996; Sato *et al.* 2001). The ADAR1a splice form of ADAR1 is most abundant in infected liver and in Huh-7 cells and knockdown of this form in cultured cells reduced both editing and virus production (Jayan and Casey 2002b).

Given that interferon may be successful therapeutically for HDV-infected patients (Farci *et al.* 2004), and that HDV infection is capable of inducing production of cytokines (Jilbert *et al.* 1986), the relative activities of the constitutive and interferon-inducible forms of ADAR1 in amber/W site editing are of particular interest. Wong and Lazinski (2003) observed that the constitutive nuclear form of ADAR1 was more capable of editing the amber/W site than was the interferon-inducible form in the context of replication in cells transfected with ADAR expression constructs. Moreover, amber/W site editing was reduced by siRNA knockdown of both forms of ADAR1, whereas knockdown of the interferon-inducible form alone had no effect (Wong and Lazinski 2002). These observations are consistent with the higher expression level of the constitutive form of ADAR1 in the nucleus, where HDV RNA replication occurs. Wong and Lazinski (2002) did observe that the longer, inducible form of ADAR1 was responsible for the bulk of editing of an amber/W editing reporter RNA; however, this observation was probably a consequence of the use of a reporter mRNA with a long residence time in the cytoplasm. Thus, amber/W site editing in cultured cells is primarily due to the constitutive form ADAR1a, which is the most abundant form in the liver.

2.2 RNA structure requirements for editing

The specific RNA structural features of ADAR substrates and their interactions with ADAR double-stranded RNA binding motifs and deaminase domains are described in detail elsewhere in this volume. A common feature is base pairing that flanks the editing site and extends at least about ~25 bp in one direction. In most cases, base pairing extends 3' of sites and includes a limited number of mismatches, bulges and small internal loops that might help to properly position the deaminase domain (Ohman *et al.* 2000). As described below, the secondary structures required for amber/W site editing have been experimentally evaluated for two of the 8 HDV genotypes – genotype 1, which is the most common, and genotype 3, which is the most distantly related genetically. In both instances, the amber/W site is present in a base-paired context, but the structures differ considerably. Compared with other editing sites, the overall size of the base-paired structure required for HDV genotype 1 and genotype 3 amber/W site editing may be larger.

2.2.1 The HDV genotype 1 amber/W site—Site-directed mutagenesis has indicated that, for HDV genotype 1, amber/W site editing involves the unbranched rod structure (Casey *et al.* 1992; Sato *et al.* 2001), which is also required for HDV RNA replication (Casey 2002; Sato *et al.* 2004). This site occurs as an A-C mismatch pair in the midst of 8 canonical Watson-Crick base pairs (Fig. 1). Both the A-C mismatch and the base pairs immediately surrounding the site have been shown to be critical for editing in cells and in vitro (Casey *et al.* 1992; Casey and Gerin 1995; Polson *et al.* 1996). A-C mismatches, which are found in some (but not all) other editing sites, have been found to maximize editing efficiency (Casey *et al.* 1992; Herb *et al.* 1996; Lomeli *et al.* 1994; Polson *et al.* 1996; Wong *et al.* 2001).

The role of base-paired regions outside the 8bp immediately surrounding the genotype 1 amber/W site is not settled. Inspection of the RNA secondary structure downstream of the

site indicates that it contains base-paired segments but is more frequently disrupted by bulges, mismatches and small internal loops than the region 3' of other editing substrates (Fig. 1). The effects on amber/W site editing of site-directed mutations that either increase or decrease base pairing in this region raise the question of whether the quality of base pairing in this region is sufficient to play an important role in ADAR1 binding and activity (Jayan and Casey 2005; Sato *et al.* 2004). Mutations that improved base-pairing, particularly in the region 15-25 nt 3' of the editing site, increased editing significantly (Jayan and Casey 2005; Sato *et al.* 2004). Moreover, mutations that further disrupted base pairing had little detectable effect on editing (Jayan and Casey 2005). These results suggest that base pairing in the region up to 25nt 3' of the amber/W site might not be sufficient to recruit ADAR1 to the editing site *via* interactions with the DRBMs.

This conclusion appears to be consistent with the results of Sato *et al.* (2001), who analyzed amber/W site editing using reporter constructs transfected with ADAR expression constructs. These authors observed editing using a minimal construct that contained just 24 nucleotides - principally the A-C mismatch and only the surrounding 8 base pairs, and concluded, based on this and other data, that no additional secondary structure features were required. However, this interpretation is limited by the fact that ADAR1 was expressed at very high levels in the transfected cells. Several reports have indicated that such overexpression can alter the behavior of the enzyme. For example, Herbert and Rich (2001) found that overexpression of a form of ADAR1 lacking the double-stranded RNA binding domains exhibited levels of activity similar to that of the wild-type protein. We have observed a similar result for the genotype 1 amber/W site – overexpression of ADAR1 constructs lacking the DRBMs edited this site with efficiency approximately half that of wild type ADAR1 (Chen and Casey, unpublished). On the other hand, *in vitro* studies have shown that the DRBMs are essential for full enzymatic activity on both dsRNA substrates as well as substrates for site-specific editing (Liu *et al.* 1998; Liu and Samuel 1996). Overexpression of the protein also dramatically reduces editing specificity (Jayan and Casey 2002a). Overall, although the reasons for the discrepancy between the roles of the DRBMs in editing in cells and *in vitro* remains to be resolved, it is clear that results obtained in transfected cells expressing high levels of ADARs may need to be interpreted cautiously. Thus, the conclusions from Sato *et al.* (2001) are likely restricted by the high levels of ADAR1 expressed in the transfected cells and do not necessarily preclude a role for sequences and structures outside the immediate vicinity of the amber/W site for typical levels of endogenous ADAR1 expression.

More recent work indicates that the structure required for editing of the genotype I amber/W site may indeed be substantially larger than suggested by Sato *et al.* Inspection of the predicted secondary structure of the region 3' of the amber/W site indicates that the highest degree of base pairing is located from 64 to 100 nt downstream (Fig. 1). Truncation to within 77 nt 3' of the amber/W site diminished editing to less than one-third the level of full-length RNA and further reduction to 42nt 3' of the site led to nearly complete loss of activity (Chen and Casey, unpublished). This distal region includes 34 bp that are minimally disrupted by one mismatch pair and 4 asymmetric single nucleotide bulges. The role of sequences 5' of the amber/W site has not been examined in detail. Base paired segments more than about 45nt away from the 5' side of the amber/W site are not required, at least when the base paired region extending about 100nt 3' of the site is present (Chen and Casey, unpublished).

Editing of the well-characterized GluR-B R/G site by ADAR2 requires a 25 bp hairpin 3' of the site that contains 2 mismatch pairs (Jaikaran *et al.* 2002; Ohman *et al.* 2000); the predicted structures of other substrates for both ADAR1 and ADAR2 contain similar length contiguous base paired segments that contain few disruptions. The above results suggest that

HDV genotype 1 amber/W site may require a similar total amount of base pairing; however, unlike other substrates characterized thus far, the base pairing immediately flanking the amber/W site is not contiguous with other base paired segments that are required. A candidate model for the interaction of this structure with ADAR1 is that the DRBMs of ADAR1 bind within the base paired region between 64 to 100nt 3' of the amber/W site and position the deaminase domain at the editing site *via* a long range interaction that involves bending of the intervening partially dsRNA (Fig. 3). This model remains to be confirmed. No cellular substrates for site-specific adenosine deamination have yet been shown to use non-contiguous base paired segments. Perhaps such sites can be identified by expansion of current computational methods (see Chapter ? in this volume) to include structures that include non-contiguous base paired segments.

2.2.2 The HDV genotype 3 amber/W site—The secondary structure used by HDV genotype 3 for amber/W site editing differs from that used by genotype 1. Although genotype 3 also forms an unbranched rod structure that is required for replication, inspection of this structure indicated that the base pairing in the immediate vicinity of the amber/W site adenosine is much more disrupted than in genotype 1 (Fig 4). In fact, the genotype 3 amber/W adenosine is not edited when the RNA is in the characteristic unbranched rod conformation (Casey 2002; Linnstaedt *et al.* 2006). The inability of this structure to be edited is due solely to the local disruption in base pairing around the amber/W site, because RNAs harboring site-directed mutations that produce a structure similar to that of the genotype 1 amber/W site (an A-C mismatch pair flanked by 9 base pairs) are efficiently edited (Casey 2002). In addition to the unbranched rod structure, genotype 3 RNA is capable of forming an alternative branched structure in which the base pairing in the immediate vicinity of the editing site is increased (Fig 4). This structure is efficiently edited by ADAR1 both *in vitro* and in cells (Casey 2002; Linnstaedt *et al.* 2006, 2009). In this branched structure, ca. 220 nt of the unbranched rod structure are rearranged to form two ~25bp stem-loops (SL1 and SL2) flanking a central base paired region that includes the amber/W site, which is itself base paired (Fig. 4).

The elements of the branched structure play different roles in editing of the genotype 3 amber/W site. Editing requires the central base paired region in the immediate vicinity of the amber/W site and is sensitive to sequence variations in this region in different HDV isolates (Linnstaedt *et al.* 2009). Perhaps, because the 28 nt mostly base-paired region 3' of the genotype 3 amber/W site is disrupted by a 7 nt internal loop, and loops of more than 4 nt have been shown to affect editing of dsRNAs (Lehmann and Bass 1999), this region is not sufficient for editing *in vitro* (Linnstaedt and Casey, unpublished). Thus, like the genotype 1 amber/W site, the structural components of the genotype 3 site appear to extend over a larger segment of the RNA than for the GluR-B R/G site. Though SL1 stabilizes the branched structure required for editing, it does not participate in the editing reaction itself; removal of SL1 affects neither editing nor ADAR1 binding (Cheng *et al.* 2003). SL2, like SL1, stabilizes the branched structure, but may also play a direct role in editing. *In vitro*, SL2 is essential for ADAR1 binding and amber/W site editing of a miniaturized RNA that contains a limited amount of the unbranched rod structure beyond SL2 (Linnstaedt and Casey, unpublished). This result suggests a model for ADAR1 binding similar to that suggested for genotype 1 (Fig. 3). In this case, the binding of at least some of the DRBMs to SL2 may properly position the deaminase domain near the amber/W site. Conversely, in cells expressing longer RNAs that include more of the unbranched rod structure beyond SL2, removal of SL2 did not abolish editing (Cheng *et al.* 2003). Possibly, both SL2 and parts of the unbranched rod beyond SL2 can independently contribute to ADAR1 binding, perhaps *via* interactions with the DRBMs. Further analysis will be required to resolve this question.

3 Control of editing

The roles of HDAg-L in the HDV replication cycle, the multimeric nature of HDAg and the susceptibility of replication intermediates with extensive dsRNA character (including both the antigenome and the similarly structured genome) to editing by ADAR1 necessitate that editing be controlled. This control occurs at several levels: 1) minimizing editing at sites other than the amber/W site; 2) maintaining the optimal rate of amber/W site editing to fit the timing of the viral replication cycle; and 3) preventing over-accumulation of editing.

3.1 Restriction of editing to the amber/W site

ADAR1 and ADAR2 can extensively edit long (> 50 base-pairs) dsRNAs, in which up to 50% of adenosines may be deaminated. Clearly, promiscuous editing such as occurs on dsRNA would be deleterious to virus replication. Such highly promiscuous editing does not occur in HDV RNA in vitro (Polson *et al.* 1996), most likely because base-pairing in the HDV RNA unbranched rod structure is interrupted by frequent bulges, internal loops and mismatches, which have been shown to restrict editing on artificial dsRNA substrates (Lehmann and Bass 1999). However, because HDAg functions as a multimer, even moderate levels of non amber/W site editing could have deleterious effects by creating dominant negative inhibitors of replication. In fact, mutant forms of HDAg that arose as a result of spurious editing when ADAR1 or ADAR2 were overexpressed inhibited replication (Jayan and Casey 2002a). Thus, it is not surprising that promiscuous editing does not occur during typical HDV replication (Polson *et al.* 1998). In fact, the amber/W site is edited 600-fold more efficiently than the other 337 adenosines in the RNA (Polson *et al.* 1998).

There are three likely mechanisms by which HDV limits editing occurring at non-amber/W sites: 1) base pairing in the unbranched rod structure is frequently disrupted by internal bulges and loops that are likely to limit ADAR binding; 2) A-C mismatch pairs flanked by segments of canonical base pairing have been shown to be edited with the highest efficiency - the predicted secondary structure of genotype 1 RNA contains just 3 adenosines in such structures, one of which is the amber/W site; 3) the frequency of GA dinucleotides, which are strongly disfavored for editing (Polson and Bass 1994), is 60% higher than predicted based on a random distribution - indeed, G is the 5' neighbor of 48% of adenosines and both of the non amber/W adenosines predicted to be present as A-C mismatch pairs have G as the 5' neighbor.

It is important to note that, although the amount of editing that occurs at non-amber/W sites is very low relative to the amber/W site, such editing may occur at low levels during replication (Netter *et al.* 1995; Polson *et al.* 1998) and may contribute to the evolution of genetic changes in the virus that can affect the outcome of infection (Casey *et al.* 2006). Using a viroid-like model in which HDV RNA replication was decoupled from HDAg synthesis, Chang *et al.* (2005) observed A to G and U to C changes in the genome sequence that are consistent with editing by ADAR1 on the genome and antigenome, respectively. These changes accounted for 80% of all sequence changes that accumulated over the course of one year in this system. Similar results were found in experimentally infected woodchucks: more than two-thirds of sequence changes that accumulated during the course of HDV infection were consistent with ADAR activity (Casey *et al.* 2006; Netter *et al.* 1995). To be sure, none of the sequence modifications in these studies were specifically shown to be due to ADAR activity; however, the overwhelming bias in sequence changes towards transitions consistent with adenosine deamination strongly suggests that it plays an important role in the majority. Significantly, in one of the woodchuck studies (Casey *et al.* 2006), some of the potentially ADAR-initiated sequence changes were associated with the development of chronic HDV infection, a serious health complication in humans. Thus, in addition to playing a specific role in the HDV replication cycle by modifying the amber/W

site, ADAR activity may also provide a mechanism for virus evolution that can affect the course of infection.

3.2 Control of editing at the amber/W site

Considering the roles of HDAG-S and HDAG-L in virus replication, the amount of amber/W site editing that occurs is likely to be critical. Indeed, experimental manipulation of editing levels affected both RNA replication and virus production in ways consistent with the roles of HDAG-S and HDAG-L in these processes (Fig. 5). Thus, the rate of editing must be correlated with levels of viral RNA to maximize virus production. Furthermore, because edited genomes are packaged into virions, high levels of editing would compromise virus viability. Virus particles containing genomes encoding HDAG-L would not only be incapable of establishing replication on cell entry, but, in the event of co-infection with virions encoding HDAG-S, would be likely to inhibit replication of otherwise competent genomes. Thus, control of both the rate and ultimate level of editing is important for HDV to maximize the yield of infectious virus.

Comparison of HDV editing with other editing substrates indicates that amber/W site editing occurs much less efficiently. Cellular substrates for editing that are pre-mRNAs are not only frequently edited at high levels (~100% in some cases), but must be edited prior to processing in the nucleus. By contrast, amber/W site editing HDV is barely detectable 4 days following the initiation of HDV replication in transfected cells (Casey *et al.* 1992; Sato *et al.* 2004; Wong and Lazinski 2002), but increases gradually as replication proceeds.

In principle, the efficiency at which different adenosines are edited can be affected by a number of different mechanisms:

- ADAR expression levels
- Intrinsic activity of the editing substrate:
 - ADAR binding affinity
 - Accessibility of the targeted adenosine to the catalytic site after binding
- Substrate availability:
 - Alternative processing
 - Alternative folding
 - Binding by other factors

There is no evidence that editing rates, or efficiency, increase during the course of replication, nor do ADAR levels change (Sato *et al.* 2004; Wong and Lazinski 2002). The increased level of editing at later times during HDV replication is a consequence of the placement of editing in the replication cycle (Fig. 2). As discussed below, the intrinsic substrate activity of the amber/W site is sub-optimal for efficient editing in both genotype 1 and genotype 3 and this inefficiency contributes to the low editing levels observed. However, a corollary of reduced intrinsic activity of the amber/W site is decreased specificity for that site relative to other sites in the RNA. Thus, it is not surprising that both genotypes further decrease editing rates by limiting the availability of the RNA for editing by ADAR1. That the specific mechanisms used to limit both the intrinsic editing activity and substrate availability differ for these genotypes underscores the critical need for the virus to restrict editing rates.

3.2.1 Sub-optimal RNA structures for editing at the amber/W site—The K12 transcript of human herpes virus 8 (HHV-8) yields both an mRNA and miRNA that are

edited at up to 76% under conditions of lytic virus replication (Gandy *et al.* 2007). Editing in vitro by ADAR1 of the genotype 1 amber/W site and the amber/W site in the genotype 3 RNA HDV_{PERU} is about 3-fold lower than that of the HHV-8 K12 editing site; editing of the genotype 3 HDV_{EC} amber/W site is even less efficient (Linnstaedt and Casey, unpublished). The features of the HDV amber/W sites responsible for the sub-optimal activity are different for genotype 1 and genotype 3. For genotype 1, the lack of sufficient base pairing within ~25nt 3' of the site limits activity. Increasing base pairing in this region increases both binding by ADAR1 (Chen and Casey, unpublished) and editing (Jayan and Casey 2005). In the branched structure used by HDV genotype 3, the amber/W site exists as an A-U base pair, which is edited less efficiently than the much more common A-C mismatch (Casey *et al.* 1992; Herb *et al.* 1996; Lomeli *et al.* 1994; Polson *et al.* 1996; Wong *et al.* 2001). This difference contributes to the sub-optimal activity of this site; changing to an A-C mismatch pair increases editing. Furthermore, additional sequence and structural variations within the region ~10nt 3' of the amber/W site contribute to differences in editing between the structures formed by Peruvian and Ecuadorian genotype 3 sequences (Linnstaedt *et al.* 2009).

3.2.2 Genotype 1 - control by binding of HDag to HDV RNA—While it is clear that the genotype 1 and genotype 3 amber/W sites are edited less efficiently than the HHV-8 K12 transcript in vitro, the differences in editing levels are not as dramatic as that seen in cells. Thus, the sub-optimal activity of the amber/W site cannot fully account for the lower efficiency of HDV editing. Although editing of HDV RNA 2-3 days following the initiation of replication is nearly undetectable, up to 40% of non-replicating genotype 1 RNA produced in transfected cells in the absence of HDag are edited (Casey and Gerin 1995; Polson *et al.* 1998). In the presence of HDag expression, editing of non-replicating RNA editing reporters is dramatically reduced (Polson *et al.* 1998; Sato *et al.* 2004). The levels of HDag-S required for this inhibition are similar to those seen in cells replicating HDV RNA (Polson *et al.* 1998). Thus, HDag prevents the rapid accumulation of editing early in the HDV genotype I replication cycle. HDag has been shown to specifically bind the HDV RNA unbranched rod structure (Defenbaugh *et al.* 2009). Thus, inhibition likely occurs by binding of HDag to HDV RNA and preventing access of ADAR1 (Polson *et al.* 1998; Sato *et al.* 2004). Inhibition is not due to interference with ADAR1 activity per se; as discussed below, HDag-S does not affect editing of genotype 3 RNA (Cheng *et al.* 2003). Moreover, recent work has shown that HDag inhibits editing of HDV RNA by ADAR1 *in vitro*; editing of the K12 RNA, which is not bound by HDag, is not affected. This mechanism of inhibition is advantageous with regard to the correlation between low editing activity and specificity. By binding the RNA, HDag is likely to interfere with editing at non-amber/W sites at least as effectively, if not more so, than it interferes with editing at the amber/W site. Consistent with this idea, sequence analysis of cDNA clones indicated that non amber/W editing events were highly correlated with amber/W changes on the same RNA (Polson *et al.* 1998). Both HDag-S and HDag-L inhibit editing equally well, indicating that this mechanism for controlling editing is not directly responsive to editing levels.

3.2.3 Genotype 3 - control by RNA folding dynamics—HDV genotype 3 restricts editing rates by a completely different mechanism. Editing of the genotype 3 amber/W site is not inhibited by HDag-S, either in transfected cells (Chen and Casey, unpublished) or *in vitro* (Cheng *et al.* 2003). The mechanistic explanation for this lack of inhibition is not yet clear, but may involve altered binding of HDag to the branched structure required for editing. Rather than HDag-binding, editing of the genotype 3 amber/W site is limited by the distribution of the RNA into several different folding conformations following synthesis. The branched structure required for editing of the genotype 3 amber/W site is less energetically stable than the unbranched rod structure and is therefore formed only

immediately following transcription of the RNA as a metastable structure (Linnstaedt *et al.* 2006, 2009). *In vitro*, only a fraction of the RNA folds into the branched structure and, with time, the RNA converts from the branched to the unbranched structure (Linnstaedt *et al.* 2006). Because only the branched structure can be edited by ADAR1, the fraction of RNA that folds into this structure determines the amount of editing that can occur. In support of this model, the predicted and observed *in vitro* folding tendencies of RNAs from two different HDV genotype 3 isolates correlated with the editing activities of these RNAs during replication (Linnstaedt *et al.* 2009).

3.3 Control of final editing levels

As discussed above, genomes encoding HDAg-L accumulate during the course of HDV replication. Although these genomes are efficiently packaged into virions, they are not expected to be infectious because the encoded HDAg-L will not support RNA replication. There is no known mechanism employed by the virus to reverse the result of editing such that the genome could produce HDAg-S. Thus, the non-infectious genomes packaged into virions are, in a sense, a cost of editing to the virus. However, under conditions of high multiplicity of infection, the negative effects of packaging edited genomes may be far greater than the production of a population of viruses in which 25% are defective. During the acute stage of infection, viremia can approach 10^{12} particles per ml (Ponzetto *et al.* 1987); with a total of about 10^9 hepatocytes in the liver it is possible that cells will be infected by more than one virus particle. Under these conditions, genomes encoding HDAg-L could interfere with infection by HDAg-S-encoding genomes. Thus, it is likely important for the virus to not only prevent premature excessive editing by the mechanisms discussed above, but to also limit the total amount of editing that occurs, after sufficient HDAg-L has accumulated for producing virions.

Sato *et al.* (2004), demonstrated that HDAg-L controls its own synthesis by inhibiting HDV RNA replication and editing. This control appears to be intimately linked to the mechanisms by which editing occurs and by which editing rates are controlled for both genotypes 1 and 3. Because the antigenomic RNA is the substrate for editing, HDAg-L mRNA production from edited genomes requires two additional transcription reactions: synthesis of genome RNA, then synthesis of the mRNA. As levels of HDAg-L increase, these replication events are inhibited. But Sato *et al.* (2004) observed that editing levels were also controlled. To explain this result, it was suggested that newly synthesized RNAs are available to be edited by ADAR1 for a short time, but are rapidly bound by HDAg and become unavailable for editing thereafter (Sato *et al.* 2004). Thus, once RNA synthesis is stopped, amber/W editing also ceases.

Genotype 3 HDAg-L also inhibits replication of genotype 3 RNA (Casey and Gerin 1998). Although the study conducted by Sato *et al.* was performed using a genotype 1 clone, the model is also consistent with our understanding of how genotype 3 amber/W site editing occurs: editing of the genotype 3 amber/W site can only occur following folding of newly synthesized antigenome RNAs into the metastable branched structure (Linnstaedt *et al.* 2006, 2009). Once synthesis stops, editing will no longer occur because the RNA can no longer fold into the proper structure.

4 Relationship of HDV RNA editing to antiviral responses

Interferon alpha (IFN- α) induces expression of a form of ADAR1 that can efficiently edit the amber/W site (Patterson and Samuel 1995; Wong *et al.* 2003) and HDV replication is very sensitive to editing levels. It is therefore important to consider the relationships between IFN- α treatment, editing levels and HDV RNA replication, particularly in light of the clinical use of IFN- α for HDV therapy. Although some results have been reported, this

area is need of further study. Hartwig *et al.* (2004) showed that IFN- α treatment of Huh-7 cells led to an approximately two-fold increase in amber/W site editing and a marked decrease in total levels of HDAg expression 7 days post-transfection. These results appear to contradict an earlier study that found no effect of IFN- α on HDV replication (McNair *et al.* 1994). However, the lack of an effect in that work could be attributed to the use of a stably transfected cell line, in which HDV RNA levels could have been due to transcription from the integrated cDNA rather than viral RNA replication. In subsequent work, Hartwig *et al.* (2006) concluded that the increase in amber/W editing following IFN- α treatment is due to increased ADAR1-L expression. However, this conclusion needs further validation. The experimental system used did not fully knock down ADAR expression levels for the duration of HDV replication; thus, amber/W editing levels still rose nearly two-fold in cells treated with IFN- α even in the face of treatment with antisense oligonucleotides. Another unresolved question is how the extent of increased p150 expression observed by Hartwig *et al.* (2004) could account for the increase in amber/W editing. Even after (IFN- α) treatment, levels of ADAR1 p150 were lower than p110 (Hartwig *et al.* 2004), and Wong *et al.* (2003) previously showed that p110 is more effective editing the amber/W site during HDV replication. Thus, the extent to which the increase in amber/W site editing in IFN- α -treated cells is due to increased p150 expression, or to some other effect(s) of IFN- α treatment, remains to be determined.

Regardless of the specific process by which amber/W editing levels increase following IFN- α treatment, the results of Hartwig *et al.* (2004) suggest a potential mechanism of action of IFN- α therapy in infected patients that should be explored in greater detail. Do amber/W editing levels increase during therapy? Does sequence variability increase, as might be expected based on the effects of ADAR1 overexpression in transfected cells (Jayan and Casey 2002a)? How are HDV RNA replication levels affected? It is not clear whether to expect increased editing to produce decreases in HDV viremia, which is perhaps the simplest measure of antiviral success, because the connection between editing levels, HDAg-L expression and virus production might not be straightforward. In an analysis of genomes containing mutations that increased editing levels, it was found that virion production was not affected at early times, most likely because HDAg-L levels, which are limiting for virus production, remained constant because increased amber/W site editing counterbalanced decreased replication (Jayan and Casey 2005).

Related to the effects of IFN- α treatment are the questions of whether virus infection itself affects ADAR1 expression (and editing levels) by either inducing IFN- α expression (Jilbert *et al.* 1986) or by inhibiting IFN- α signaling pathways (Pugnale *et al.* 2009). Although levels of the interferon-inducible form of ADAR1 were reported to be the same in normal and HDV-infected liver (Jayan and Casey 2002b), it is possible that the analysis of bulk tissue samples would not have been sensitive to localized IFN- α expression near foci of infection. A recent report indicated that HDV may inhibit IFN- α signaling pathways (Pugnale *et al.* 2009). The mechanism by which this inhibition occurs has not been determined. Although this result appears to contradict the observed IFN- α induction of ADAR1 p150 in cells transfected with HDV (Hartwig *et al.* 2004), there are a number of differences in the experimental approaches (timing and dose of IFN- α treatment, timing of HDV replication, accounting for untransfected cells); thus it remains unclear whether there is a controversy. Undoubtedly, there is much to be done to understand the interactions between HDV and IFN- α and the extent to which these interactions involve editing of the HDV RNA by ADAR1.

5 Perspective

Compared with cellular substrates for RNA editing, the HDV amber/W site displays both significant similarities and instructive differences. Although the base pairing in the immediate vicinity of sites is similar, ongoing analysis of the HDV amber/W site suggests that base paired segments further removed from the site (at least in a linear configuration) may be important, perhaps by binding the DRBMs of ADAR1. Whether such long-range interactions occur in cellular substrates is currently unknown. Determination of the specific interactions between ADAR1 elements and structural features in HDV RNA, including both the amber/W site itself and segments further removed, remains a critical goal. Possibly, understanding the interactions employed by HDV could expand current concepts of what constitutes a structure in cellular RNAs likely to be targeted by ADAR1. The mechanisms by which editing is controlled in different cellular environments and on different cellular RNAs has not been widely examined. However, it seems likely that control of editing will be just as important for cellular targets as for HDV. As control of editing of cellular substrates is analyzed in more depth, the mechanisms employed by HDV to control editing (including competition between ADARs and other proteins for the site, and RNA folding variability) will more than likely be among the mechanisms found to be used for these targets. Finally, the connections between ADAR1, IFN- α , and HDV editing are in need of further study, particularly in light of the therapeutic effects of IFN- α in HDV patients.

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Fig. 1.

The structure of the HDV genome. Upper. The dark line indicates the circular HDV genome, which forms an unbranched rod-like structure. Dashed vertical lines indicate base pairing between opposite sides of the circular RNA. The rectangle indicates the HDAg coding region; HDAg-S is encoded by the open portion of the rectangle; additional sequences added as a result of editing at the amber/W site and which form the C-terminus of HDAg-L are indicated by the striped portion. Lower. The predicted base pairing of a section of the antigenomic RNA that includes the amber/W site.

Fig. 2.

The role of RNA editing in the HDV replication cycle. ① Genome RNA (gray rounded rectangle) serves as the template for synthesis of mRNA that is translated to produce HDAg-S, which is required for further RNA synthesis. ② Via rolling circle replication, the genome serves as the template for synthesis of the antigenome (black rounded rectangle) which also serves as the template for synthesis of additional genome RNA. ③ As replication proceeds, a fraction of antigenome RNAs is edited at the amber/W site by ADAR1 (wavy dashed arrow). ④ Edited antigenomes serve as the template for synthesis of "edited" genomes (gray rounded rectangle with ACC). ⑤ "Edited" genomes serve as templates for synthesis of mRNAs encoding HDAg-L, which is the limiting factor in virus packaging but also inhibits replication. ⑥ Simultaneously, "edited" antigenomes and additional "edited" genomes are synthesized via rolling circle replication. Thus, editing levels accumulate as replication proceeds. Note that the numbered scheme is intended to indicate an expanding repertoire of activities that persist as long as replication occurs rather than a stepwise progression in which earlier processes are terminated.

Fig. 3.

Schematics showing hypothetical interactions between amber/W sites and ADAR1. In both cases, the DRBMs of ADAR1 (indicated by the open rectangles) may interact with base paired segments further removed from the editing site than in the GluR-B R/G site. For genotype 1, sequences more than 42 nt distal from the amber/W site, along the partially double stranded structure, appear to be involved. In genotype 3, sequences more than 25 nt away in the ~25bp SL2 structure are required for editing *in vitro*.

Fig. 4.

Editing of the HDV genotype 3 amber/W site requires a branched structure. Schematics show the unbranched rod (upper) and branched structures (lower). The ~25 base pair stem loops SL1 and SL2 stabilize the branched structure. RNA secondary structures in the vicinity of the amber adenosine are shown for both the unbranched rod and branched structures. The unbranched rod structure is not a substrate for editing by ADAR1 due to the disrupted base pairing around the adenosine, which is indicated by the 5-point star.

Fig. 5.

Effects of experimental variations in amber/W editing activity on HDV replication and virion formation.

Fig. 6.

Models for control of editing rates of the HDV genotype 1 and genotype 3 amber/W sites. Editing of the genotype 1 site (upper) involves the unbranched rod structure and is inhibited by HDAg, which binds this structure following synthesis. In this model, HDAg and ADAR1 compete for binding to the RNA. Editing of the HDV genotype 3 site (lower) requires a metastable branched structure that is formed only following transcription and eventually rearranges to the unbranched rod. Editing is limited by the fraction of RNA that folds into the branched conformation during transcription.