RNA Editing in Hepatitis Delta Virus

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Abstract Hepatitis delta virus (HDV) relies heavily on host functions and on structural features of the viral RNA. A good example of this reliance is found in the process known as HDV RNA editing, which requires particular structural features in the HDV antigenome, and a host RNA editing enzyme, ADAR1. During replication, the adenosine at the amber/W site in the HDV antigenome is edited to inosine. As a result, the amber stop codon in the hepatitis delta antigen (HDAg) open reading frame is changed to a tryptophan codon and the reading frame is extended by 19 or 20 codons. Because these extra amino acids alter the functional properties of HDAg, this change serves a critical purpose in the HDV replication cycle. Analysis of the RNA secondary

structures and regulation of editing in HDV genotypes I and III has indicated that although editing is essential for both genotypes, there are substantial differences. This review covers the mechanisms of RNA editing in the HDV replication cycle and the regulatory mechanisms by which HDV controls editing.

1
Introduction

1.1
HDV Produces Two Forms of HDAg from the Same Gene

Hepatitis delta virus (HDV) is often compared to viroids because of the characteristic unbranched rod secondary structure formed by its RNA and the relatively small size of its genome. However, unlike viroids, HDV does contain one gene that encodes the sole viral protein, HDAg. Early analyses showed two electrophoretic forms of HDAg in liver and viral particles isolated from serum (Bergmann and Gerin 1986; Bonino et al. 1981, 1984, 1986). (These forms were sometimes referred to by their apparent molecular weights, p-24 and p-27; they are denoted here as S-HDAg and L-HDAg for short and long, respectively.) Following the cloning of HDV cDNAs (Makino et al. 1987; Wang et al. 1986), a series of studies illuminated the functional roles of S-HDAg and L-HDAg in HDV replication: S-HDAg is required for replication of HDV RNA, and L-HDAg is required for the formation of HDV particles (Chang et al. 1991; Glenn et al. 1992; Hwang et al. 1992). Early studies found that L-HDAg also inhibits HDV RNA replication (Chao et al. 1990; Kuo et al. 1989), but more recent analyses suggest that this might not always be the case, particularly for antigenome RNA synthesis (Macnaughton and Lai 2002; Modahl and Lai 2000).

Cloning and sequencing of the genome in 1986 indicated heterogeneity at several positions in the 1679 nucleotide (nt) genome (Wang et al. 1986). This variability affected the predicted length of HDAg: some clones contained a UAG (amber) stop as the 196th codon and encoded a 195 amino acid protein, other clones had UGG at this location and encoded a protein 214 amino acids in length (Wang et al. 1986; Xia et al. 1990). Expression of protein from clones that contained either the UAG or UGG sequence showed that the former encoded S-HDAg and the latter L-HDAg (Weiner et al. 1988; Xia et al. 1990). Subsequently, a series of studies in cultured cells and in a chimpanzee infected by injection of an HDV cDNA clone led to the remarkable discovery that the heterogeneity at this position arose during the course of HDV replication. Although transfected cDNAs encoded only S-HDAg, both S-HDAg and L-HDAg were detected (Luo et al. 1990; Sureau et al. 1989). No L-HDAg was detected

when cells were transfected with an expression construct for S-HDAg that did not produce replicating HDV RNA. Thus, the appearance of L-HDAg was linked to HDV replication. Because of the different functions of S-HDAg and L-HDAg, the synthesis of L-HDAg late in the replication cycle is an example of a classic switch from viral RNA replication to genome packaging.

Analysis of HDV RNA isolated from the serum of the transfected chimpanzee and from transfected cultured cells showed that heterogeneity appeared at the position corresponding to the adenosine in the UAG stop codon for S-HDAg (Luo et al. 1990). Subsequent studies in transfected cells showed that the appearance of L-HDAg and sequence heterogeneity at this site are temporally correlated; moreover, mutations that abolished the appearance of heterogeneity also prevented L-HDAg production (Casey et al. 1992). These studies indicated that some genomes encoding S-HDAg are converted, or edited, to encode L-HDAg during the course of HDV replication. The site at which editing occurs has been termed amber/W in accord with the codon change that accompanies the sequence modification. Because of the essential functions of S-HDAg and L-HDAg editing plays a central role in the HDV replication cycle.

1.2
What Is RNA Editing?

The term RNA editing was first used in the late 1980s to describe an unusual process in which multiple Us are inserted and deleted in trypanosome mitochondrial mRNAs (Benne et al. 1986). The usage of the term was subsequently expanded as it was applied to other, less drastic, examples of nucleotide changes in RNAs, including deamination of C to U in apoB100 mRNA in small intestine (Scott 1989), deamination of glutamate receptor subunit B (gluRB) pre-mRNA in brain (Higuchi et al. 1993), and insertion of nontemplated Gs in the P gene of paramyxoviruses (Curran and Kolakofsky 1990). Thus, broadly defined, the term RNA editing describes processes other than splicing that result in the modification of an RNA sequence from that of its template. While collectively referred to as RNA editing, these sequence revisions involve a wide range of mechanisms. In the two types of editing used by mammalian cells, C to U and A to I, the modified base within the RNA molecule is deaminated; there is no evidence that the phosphate backbone is broken during the editing process.

1.3
Adenosine Deamination at the Amber/W Site in the HDV Antigenome

One difficulty encountered in establishing the mechanism of editing at the amber/W site was identifying the RNA substrate: assays performed on repli-

cating RNAs could not definitively determine whether the substrate for editing was the genome or the antigenome, or even whether editing was the result of co-transcriptional misincorporation. Although initial attempts led to the erroneous suggestion that the genomic RNA might be the substrate, in which case editing would occur as a U to C transition (Casey et al. 1992; Zheng et al. 1992), the use of nonreplicating RNA expression constructs that could exclusively produce either genomic or antigenomic RNA in transfected cells led to the unambiguous conclusion that editing occurs on the antigenome RNA (Casey and Gerin 1995). This result was further supported by analysis of editing on in vitro transcribed RNAs mixed with nuclear extracts: only antigenomic RNA was edited at the amber/W site (Casey and Gerin 1995). This observation indicated that HDV editing occurs post-transcriptionally, and is not the result of transcriptional misincorporation. Subsequently, it was shown that RNA adenosine deaminase (ADAR) from *Xenopus laevis* can edit the amber/W site in the HDV antigenome with considerable specificity in vitro (Polson et al. 1996).

Accordingly, the type of RNA editing used by HDV is adenosine deamination. In this process, the amino group of adenosine is removed and replaced with a keto oxygen. Because this position of the base is changed from a hydrogen bond donor to an acceptor, the Watson–Crick base-pairing preference of this nucleotide is changed from pairing with U to pairing with C. Therefore, in any subsequent functions that involve base pairing (such as translation, RNA-templated transcription, and splice site identification) the edited position will behave as G rather than the original A. Adenosine deamination has the potential to produce as many as 15 different recodings of an RNA transcript, including the creation of a methionine start codon and the abolition of stop codons. Thus, for example, when the adenosine at the amber/W site in the HDV RNA is edited, a UAG amber stop codon is changed to UIG, which behaves like UGG, and encodes tryptophan. As indicated by this example, sites in RNAs that undergo adenosine deamination have been named according to the coding change brought about by editing.

1.4
The Role of RNA Editing in the HDV Replication Cycle

In the HDV replication scheme editing occurs at the amber/W site on the antigenomic RNA (see Fig. 1). The cycle begins with genomes encoding S-HDAg, the form required for RNA synthesis. Three RNA species are produced: the mRNA encoding S-HDAg, the antigenome, which serves as replication intermediate, and the genome. During replication, in some antigenome RNAs the adenosine at the amber/W site is deaminated to inosine. Because inosine forms base pairs with C rather than U, subsequent genome RNA synthe-
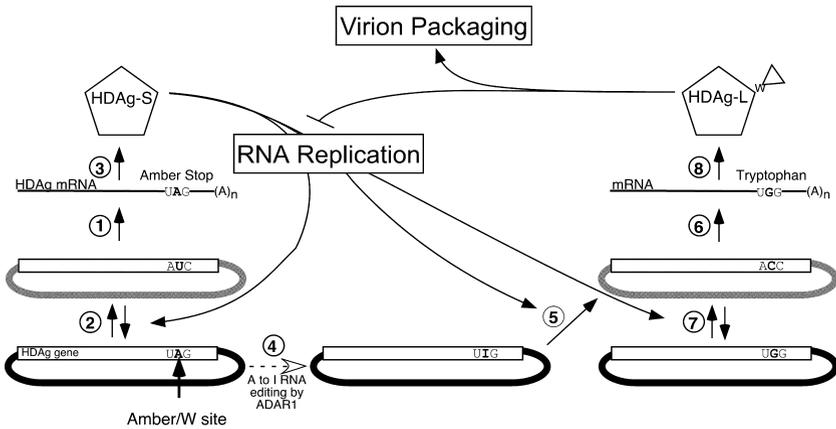
Fig. 1 The role of RNA editing in the HDV replication cycle. *Dark heavy lines* represent antigenomic sense RNAs; *gray lines* indicate genome RNAs. *1*, Synthesis of mRNA encoding S-HDAg; *2*, replication of full-length antigenomic and genomic RNA; *3*, translation of S-HDAg, which is required for RNA replication; *4*, during replication some of the antigenomic RNA is edited at the amber/W site by the host RNA adenosine deaminase ADAR1; *5*, antigenomic RNA containing I at the editing site serves as template for the synthesis of genomic RNA containing C at the complementary position; *6*, synthesis of mRNA encoding L-HDAg; *7*, replication of genomic and antigenomic RNA encoding L-HDAg; *8*, translation of L-HDAg, which inhibits RNA replication, and is required for virion packaging

sis results in the appearance of C at the corresponding position in the genome. Transcription from such genomes leads to the production of mRNA encoding L-HDAg, which can limit further RNA synthesis and initiates the packaging process. It is important to note that, unlike cellular mRNA substrates for RNA adenosine deamination, HDV mRNA is not edited directly. Rather, editing occurs on the full-length antigenome, which is a replication intermediate. Consistent with this model, some of the sequences that form the structure required for editing (see below) are more than 300 nt downstream of the polyadenylation and ribozyme sites, and are not included in the mRNA sequence. Furthermore, analysis of RNA in viral particles indicates that genome RNAs contain the expected C at the position complementary to the amber/W site.

From the scheme depicted in Fig. 1 it is clear that editing plays a central role in the HDV replication cycle. Because L-HDAg is a limiting factor for virus production, insufficient editing reduces virus output and is likely to limit propagation in the host (Jayan and Casey 2002b, 2005). Conversely, excessive editing strongly diminishes viral RNA accumulation (Jayan and Casey 2002a, 2005; Sato et al. 2004). Moreover, because edited genomes are

also packaged into virions, excessive editing reduces the infectivity of viral progeny. The remainder of this chapter will cover recent efforts to identify the specific host enzymes involved in HDV RNA editing, the RNA secondary structures involved, and the factors that determine where and how much editing occurs.

2
Host Enzymes Required for HDV RNA Editing

ADAR edits adenosines in double-stranded RNA (dsRNA); this activity is present in nuclear extracts from numerous metazoan species (Bass). As mentioned above, it was shown that ADAR from *Xenopus laevis* can edit the amber/W site in the HDV antigenome with considerable specificity in vitro (Polson et al. 1996). While only one ADAR has been identified in *Xenopus*, mammalian cells contain two related genes, ADAR1 and ADAR2, that encode proteins capable of editing adenosine in dsRNA (Melcher et al. 1996; O'Connell et al. 1995; Patterson and Samuel 1995; Yang et al. 1997). These proteins contain a catalytic deaminase domain along with three or two, respectively, copies of dsRNA binding motifs (DRBMs). Both genes are essential for viability in mice (Brusa et al. 1995; Wang et al. 2000). Both ADAR1 and ADAR2 can edit HDV RNA at the amber/W site in transfected cultured cells (Jayan and Casey 2002a; Sato et al. 2001; Wong et al. 2001). However, because the level of ADAR1 mRNA expression is considerably higher than ADAR2 in liver, it seems likely that ADAR1 is responsible for editing during HDV infection of hepatocytes. Consistent with this idea, knockdown experiments using small interfering RNA (siRNA) have shown that the short form of ADAR1, which is localized in the nucleus, is responsible for amber/W site editing during HDV replication in Huh-7 cells (Jayan and Casey 2002b; Wong and Lazinski 2002). Because the HDV amber/W site was edited with high specificity in vitro using just purified *Xenopus* ADAR, no additional factors aside from HDV RNA and ADAR are required for amber/W site editing to occur (Polson et al. 1996). Nevertheless, it is possible that additional factors, such as HDAg, can contribute to the efficiency and specificity of editing (see Sect. 3).

3
Factors Affecting Substrate Selection

ADAR activity was first identified due to its ability to extensively modify adenosines in dsRNA. Indeed, the deamination of up to 50% of adenosines

in dsRNA destabilized base pairing to such an extent that the activity was initially described as an 'RNA unwindase' (Bass and Weintraub 1987, 1988; Wagner et al. 1989). In such dsRNAs, the likelihood of editing at individual adenosines is determined largely by: (1) the identity of the 5′ nucleotide neighbor—G is strongly disfavored; and (2) the distance from the 3′end of the RNA—adenosines less than 20 nt from the 3′end are not deaminated (Polson and Bass 1994). Despite the role of its activity on dsRNAs in the initial characterization of ADAR, it is not yet clear to what extent editing on dsRNAs is an important cellular function. However, it is clear that ADARs edit several RNAs with high specificity and that some of these editing events are highly important (Bass 2002; Gott and Emeson 2000; Seeburg 1998, 2002).

3.1
RNA Sequence and Structural Requirements for Editing

Inspection of the predicted structures of known sites for specific editing reveals several common features. All include at least six contiguous base pairs around the editing site, and many substrates contain more. In most cases the target adenosine occurs as either an A–U pair or an A–C mismatch pair. Mutational analyses of some substrates, including the HDV genotype I amber/W site, have indicated that editing levels are higher when the adenosine occurs as an A–C mismatch rather than an A–U pair (Casey et al. 1992; Herb et al. 1996; Lomeli et al. 1994; Polson et al. 1996; Wong et al. 2001). Moreover, at least for HDV genotype I, any change in the position opposite the amber/W site (deletion, or substitution by A or G), led to markedly reduced editing levels. (Casey et al. 1992) None of the known substrates for specific editing contain the disfavored G as the 5′ neighbor of the editing site.

Outside the immediate vicinity of editing sites the requirements for base pairing are distributed asymmetrically along the RNA (Lehmann and Bass 1999; Polson and Bass 1994). Base-pairing on the 5′side of sites varies between two and five base-pairs. On the 3′ side base-pairing is greater, in most cases extending for at least about 20 base-pairs. Analysis of editing on dsRNA templates in vitro has led to a model in which ADAR1 interacts with a base-paired region extending about 20 nt to the 3′ side of edited adenosines (Lehmann and Bass 1999; Polson and Bass 1994); this interaction most likely occurs via the three DRBMs of ADAR1 (Liu et al. 1998; Liu and Samuel 1996). In dsRNA substrates, editing sites could tolerate small disruptions of base pairing in the region downstream, but the presence of a 6-nt internal loop strongly diminished editing (Lehmann and Bass 1999; Polson and Bass 1994). In most cases base pairing 3′of sites that are substrates for highly specific editing are

disrupted by mismatches, bulges and internal loops, but these disruptions are generally not as large as a 6-nt internal loop. The role of these disruptions may be to orient the ADAR protein via the double-stranded RNA binding domains such that the deaminase domain is positioned correctly at the editing site (Bass 2002; Lehmann and Bass 1999; Ohman et al. 2000; Polson and Bass 1994).
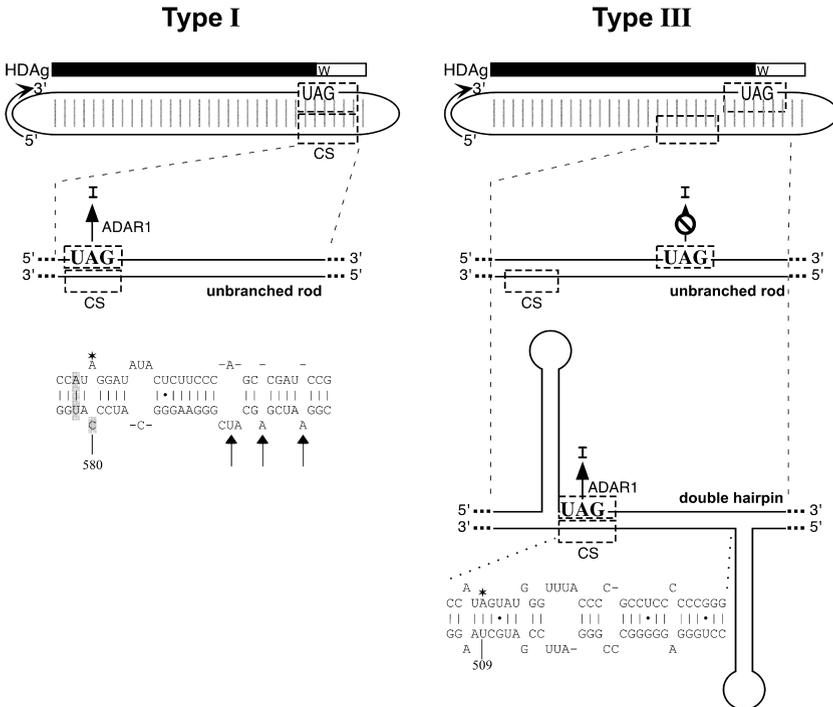

Fig. 2 Comparison of amber/W sites in genotypes I and III. *Upper*: schematic diagram of HDV genome, indicating the unbranched rod and the location of the HDAg coding region. The *filled bar* above the genome indicates the S-HDAg coding region; the *open bar*, including the W indicates the additional amino acids added to make L-HDAg. *Dashed boxes* indicate sequences from the coding and noncoding regions that make up the amber/W editing site. *Middle*: the amber stop codon (UAG) is edited by ADAR1 in the unbranched rod conformation of genotype I RNA; for type III, however, editing occurs in the double hairpin structure, not the unbranched rod. *Lower*: the predicted secondary structures around the genotype I and III amber/W sites are shown. *Vertical lines* indicate A–U and G–C base-pairs; *dots* indicate G–U pairs. Positions that have been shown to be critical for editing are *shaded* in the genotype I structure. *Arrows* indicate internal loops and bulges; improved base-pairing at these locations increased editing (Jayan and Casey 2005)

The RNA secondary structure downstream of the HDV amber/W site in HDV genotype I contains base-paired segments, but is more frequently disrupted by bulges and mismatches than other editing substrates. Mutations that improved base-pairing, particularly in the region 15–25 nt 3′ of the editing site (see arrows in Fig. 2, left panel), increased editing significantly (Jayan and Casey 2005; Sato et al. 2004). However, the increased editing resulted in dramatically lower levels of HDV RNA replication, principally due to excessive L-HDAg production (Jayan and Casey 2005; Sato et al. 2004). These results led to the suggestion that the HDV editing site (at least for genotype I) may have been selected to be suboptimal in order to prevent the rapid accumulation of too much L-HDAg, which could inhibit viral RNA replication.

In light of the model for ADAR1 substrate activity, the bulges and mismatches 3′ of the HDV genotype I amber/W site raise questions about the role of this region in editing at the amber/W site. Some studies have suggested that extensive base-pairing 3′ of editing sites may not be essential for efficient editing. Sato and Lazinski (Sato et al. 2001) found that a minimal substrate that was derived from the HDV amber/W site and that contained only eight base-pairs could be efficiently edited when ADAR1 was overexpressed. Herbert and Rich (2001) showed that ADAR1 could efficiently edit even when the three DRBMs were removed. Perhaps consistent with these findings, more extensive disruption of the base-pairing 3′ of the HDV genotype I amber/W site had no apparent effect on editing due to endogenous ADAR1 in Huh-7 cells (Jayan and Casey 2005). These results could indicate that the deaminase domain itself possesses some RNA binding activity that can be effective under certain conditions. Alternatively, the role of the ADAR1 DRBMs in editing at the HDV amber/W site may be more complex than previously thought; perhaps these domains can also recognize and bind RNA segments that do not exhibit significant dsRNA character. Further analyses of editing under more controlled conditions, such as in vitro, and using variant forms of ADAR1 and amber/W substrates may be necessary to resolve the role of dsRNA segments and the ADAR1 DRBMs in editing at the HDV amber/W site.

3.2
Variations in Amber/W Site Structures Among HDV Genotypes

It is interesting to note that both the RNA secondary structure around the amber/W site and the C-terminal sequences specific to L-HDAg are distinguishing features of some HDV genotypes (Casey 2002; Casey et al. 1993; Hsu et al. 2002; Ivaniushina et al. 2001; Niro et al. 1997; Shakil et al. 1997) (see the chapter by P. Dény, this volume). The genotypic differences in the RNA secondary structure required for editing fall into two categories: (1) the dif-

ferent portions of the HDV antigenome that form the structure; and (2) the specific RNA secondary structure formed around the amber/W site. Despite these variations, all three of the genotypes analyzed (I, II and III) are edited by the same enzyme—ADAR1 (Jayan and Casey 2002b).

In HDV genotype I the structure required for amber/W site editing is part of the unbranched rod structure characteristic of HDV RNA (Fig. 2; Casey et al. 1992). The eight Watson–Crick base-pairs flanking the amber/W site and the A–C mismatch pair involving the amber/W site are highly conserved among over 50 genotype I sequences (Niro et al. 1997; Yang et al. 1995). The role of this structure in editing has been confirmed by site-directed mutagenesis studies (Casey et al. 1992; Casey and Gerin 1995; Polson et al. 1996; Wong et al. 2001). HDV genotype III RNA also forms an unbranched rod structure, which is required for RNA replication; however, the base-pairing in the immediate vicinity of the amber/W site is disrupted such that this structure does not function as a substrate for amber/W editing (Casey 2002). Rather, editing in genotype III requires an alternative, 'double hairpin' structure that creates better base-pairing in the immediate vicinity of the amber/W site (Fig. 2; Casey 2002). This structure, which differs from the unbranched rod structure by about 80 base-pairs, contains two stem–loops that essentially shift the positions of the noncoding side of the HDV antigenome that are base-paired with the amber/W site region. In the unbranched rod structure the amber/W site is opposite position 580; in the double hairpin structure, the paired position is 509. The structure required for editing in the other HDV genotypes has not yet been determined. Comparative analysis of the predicted secondary structure in the vicinity of the amber/W site in the unbranched rod reveals structures similar to the genotype I structure but more disrupted (Hsu et al. 2002; Ivaniushina et al. 2001; Radjef et al. 2004).

Inspection of the predicted RNA secondary structures around the amber/W sites of genotypes I and III indicates that the genotype III amber/W site differs from the type I site. In some cases the differences occur at positions that have been shown to be essential for efficient editing in genotype I (Fig. 2). For example, the A–C mismatch pair that involves the amber/W adenosine and which is highly conserved among genotype I isolates, occurs as an A–U pair in genotype III; when introduced by site-specific mutagenesis into a genotype I genome, this specific change substantially reduces both editing and virus production (Casey et al. 1992; Jayan and Casey 2005). The significance and effect of these variations on editing, RNA replication and virus production in genotype III remains to be determined. Perhaps the variations at the genotype III site can be explained by compensatory effects, such as changes elsewhere in the editing site region, including sequences/structures 3′ of the editing site, or differences in the mechanisms by which HDV regulates

editing during replication. Another (but not mutually exclusive) possibility is that the differences described are responsible for variations in the levels of editing among genotypes (Hsu et al. 2002).

Defining the structural determinants for editing remains an important goal. Despite recognition of the common features among editing sites noted in Sect. 3.1, the sequence and structural determinants for highly specific editing are still not well understood. Only a handful of substrates for highly specific editing have been identified to date in mammals, and it is anticipated that many more remain to be found (Paul and Bass 1998). Knowledge of sequence and structural requirements for editing will likely facilitate the prediction of potential adenosine deamination editing sites from analysis of genomic sequences. Moreover, it is reasonable to expect that differences in editing levels among different substrate adenosines are due in part to variations in the structure of the RNA in the vicinity of the editing sites. Understanding the effects of structural variations will contribute to our understanding of how this important post-transcriptional regulatory mechanism is modulated. Variations among the amber/W sites in the HDV genotypes may provide a valuable opportunity to evaluate the effects of different sequences and RNA secondary structures on editing at the HDV amber/W site.

4
Effects of Variations in Editing on HDV RNA Replication and Virus Production

Examination of the role of editing in the HDV replication cycle (Fig. 1) suggests that varying the efficiency of editing at the amber/W site, either by altering levels of ADAR expression or by the introduction of mutations near the amber/W site, is likely to affect HDV replication, virus production, or both. Premature editing at the amber/W site could reduce levels of RNA replication because edited antigenomes encode L-HDAg, which is a trans-dominant inhibitor of HDV RNA replication (Chao et al. 1990; Glenn and White 1991). Insufficient editing could inhibit virion production because L-HDAg is required for virus production (Ryu et al. 1992; Wang et al. 1992). Several studies have indicated just how sensitive HDV replication and virus production are to variations in editing (Casey 2002; Jayan and Casey 2002a, 2005; Sato et al. 2004).

4.1
Effects of Excessive Editing at the Amber/W Site

Overexpression of ADAR1 by cotransfection of ADAR1 expression constructs resulted in increased editing at the amber/W site, and increased production

of L-HDAg (Jayan and Casey 2002a; Sato et al. 2004). Concomitantly, levels of HDV RNA synthesis were strongly inhibited. Another approach gave similar results: increasing the base-pairing 3′ of the amber/W site led to higher levels of editing and L-HDAg synthesis, and dramatic inhibition of viral RNA synthesis (Jayan and Casey 2005; Sato et al. 2004). In all cases, overproduction of L-HDAg accounted for a significant fraction of the inhibition. The sensitivity of replication to editing (via L-HDAg) is remarkable; in one study mutations that increased editing by approximately threefold led to a 50-fold decrease in RNA replication (Jayan and Casey 2005).

It might be expected that inhibition of HDV RNA synthesis due to increased editing and subsequent L-HDAg overproduction would automatically inhibit viral particle production (because of decreased viral RNA levels within the cell). Indeed, inhibition of virus production was observed 6–12 days post-transfection with a site-directed mutant that exhibited increased editing (Jayan and Casey 2005). However, there was no inhibition of virus production before day 6, even though intracellular RNA levels were significantly decreased. One explanation of these results is that intracellular viral RNA is not normally the limiting factor for particle production. Virus secretion was closely correlated with levels of L-HDAg, consistent with the interpretation that L-HDAg is the limiting factor for virus particle production.

ADAR1 has several isoforms, one of which is induced by interferon. Although siRNA knockdown studies have shown that the shorter form of ADAR1, which is not induced by interferon, is primarily responsible for editing at the amber/W site (Wong and Lazinski 2002), treatment of Huh-7 cells with interferon has been shown to increase ADAR1 p150 expression and increase editing (Hartwig et al. 2004). Levels of HDV RNA were not analyzed in this study; based on the inhibition of replication associated with modest increases in editing due to ADAR co-transfection or editing site mutations (Jayan and Casey 2002a, 2005, Sato et al. 2004), it might be expected that HDV RNA levels would decrease. Conversely, previous studies indicated that interferon treatment of cultured cells did not affect HDV RNA replication (Ilan et al. 1992; McNair et al. 1994); however, the effects of interferon treatment on ADAR1 and amber/W editing were not assessed. Analysis of the effects of interferon on both editing and replication in the same study is required to clarify this issue.

4.2
Effects of Diminished Editing at the Amber/W Site

The requirement of L-HDAg for production of HDV particles is clear (Chang et al. 1991; Ryu et al. 1992). Not surprisingly, inhibition of editing, either by site-directed mutagenesis (Casey 2002; Jayan and Casey 2005) or by siRNA-

mediated knockdown of ADAR1 expression (Jayan and Casey 2002b), inhibited viral particle production in transfected cells. Early studies on the effect of L-HDAg indicated that L-HDAg expression strongly inhibited HDV RNA replication (Chao et al. 1990; Glenn and White 1991). However, editing site mutations that prevent L-HDAg production do not result in increased levels of HDV RNA replication, at least for genotype I constructs (Macnaughton and Lai 2002; Sato et al. 2004; Wu et al. 1995). Partly based on this result, it has been suggested that L-HDAg might not actually inhibit HDV RNA accumulation, at least under normal circumstances in Huh-7 cells (Macnaughton and Lai 2002). However, the results discussed in Sect. 4.1 indicate that inhibition of replication does occur when L-HDAg is overproduced by excessive editing during the course of HDV RNA replication. In contrast to the observations with genotype I, genotype III RNA replication is increased at least fivefold by mutations that abolish editing (Casey 2002). One interpretation of these results is that, at least in Huh-7 cells, maximum levels of HDV RNA replication are limited by factors other than L-HDAg production; thus, decreased L-HDAg production does not increase replication. However, when L-HDAg is overproduced, replication becomes sensitive. Genotype III RNA replication may either be less sensitive to these as yet undefined limitations, or more sensitive to L-HDAg.

5
Control of HDV RNA Editing

Control of HDV RNA editing occurs on several levels. First, the HDV antigenome contains about 337 adenosines, but editing is highly specific for the amber/W site. Second, both the rate and extent of editing appear to be carefully controlled. Some host substrates for editing exhibit modification rates approaching 100%, and this editing likely occurs rapidly; most known host substrates are pre-mRNAs that are edited prior to splicing. In contrast, for HDV, edited viral RNAs accumulate slowly and levels typically plateau at less than 30% edited after 12 days in transfected cells in culture.

5.1
Restriction of Editing to the Amber/W Site

ADAR1 and ADAR2 can extensively edit long ($\geq$ 50 base-pairs) double-stranded RNAs, in which up to 50% of adenosines may be deaminated. Clearly, promiscuous editing such as occurs on dsRNA could be deleterious to virus replication. Indeed, spurious editing on HDV RNA by overexpressed ADAR1

and ADAR2 led to the production of protein variants that inhibited repli-
cation (Jayan and Casey 2002a). However, even though HDV RNA exhibits
significant base-pairing in the unbranched rod structure, promiscuous edit-
ing does not typically occur during HDV infection; the amber/W site is edited
600-fold more efficiently than the other 337 adenosines in the RNA (Polson
et al. 1998). It is worth noting here that, although editing at nonamber/W
sites does not appear to occur at levels important for the replication cycle, the
genetic evolution of the virus may nevertheless be affected by ADAR editing
during the course of infection (see the chapter by J.L. Casey and J.L. Gerin,
this volume). It is likely that the primary and secondary structure of the HDV
RNA have evolved to avoid undesirable (for the virus) editing at sites other
than amber/W. As noted in Sect. 3, analysis of editing on dsRNAs has indi-
cated that adenosines with a 5′ guanosine neighbor are much less likely to be
deaminated than other adenosines (Polson and Bass 1994). In both the HDV
genome and antigenome, guanosine is by far the most common 5′ neighbor
for adenosine, and the ratios of observed to expected occurrences for the
dinucleotides GA and UC (which would be GA in the complementary strand)
are higher than for any other dinucleotides. This bias may be due, in part,
to selection for sequences that place nonamber/W adenosines in contexts
that are less likely to be edited. As for secondary structure, 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 (Aruscavage and Bass 2000; Lehmann and Bass
1999; Ohman et al. 2000).

5.2
Regulation of Editing Levels

HDV must regulate both the rate and the extent of editing at the amber/W
site because, as shown in Sect. 4, levels of viral RNA replication and virion
production are sensitive to the kinetics and amount of L-HDAg produced.
Moreover, as shown in Fig. 1, editing occurs not on the mRNA, but on the
antigenome, which is a replication intermediate. Hence, HDV RNA editing
levels within an infected cell at any given time are the result of the accumula-
tion of all editing events within that cell up to that time, and the percentage
of antigenomes containing the UGG codon (and genomes with ACC at the
corresponding positions) increases with time. The cost of this mechanism
to the virus is that a fraction of viral particles contain genomes that encode
L-HDAg; such genomes cannot replicate (Glenn and White 1991). Thus, HDV
must control the level of editing in order to ensure viability. HDV does not
appear to regulate editing by affecting ADAR1 expression because ADAR1

levels are unaffected by HDV replication (Wong and Lazinski 2002). Control mechanisms for editing rely on several viral components and functions, including: RNA structure, HDAg, and viral RNA replication. These mechanisms vary among genotypes, at least for genotypes I and III.

Some of the control mechanisms may be described as passive, in that they are not affected by (or responsive to) the level of editing. This category includes the secondary structure of the RNA around the amber/W site. As mentioned in Sect. 3.1, the disruptions in base-pairing 3′ of the amber/W site in HDV genotype I create a suboptimal substrate for editing. Mutations that increase base-pairing in this region increase editing, but severely reduce replication and virion production (Jayan and Casey 2005; Sato et al. 2004). It is not yet known whether the structures in the vicinity of the amber/W sites of other genotypes are also suboptimal. One potential dilemma for the virus that is posed by using a suboptimal structure to limit editing efficiency is that the specificity of editing is likely to be compromised because the specificity is determined by the ratio of the efficiency of editing at the amber/W site to the efficiency of editing at other 'non-specific' sites. The danger for the virus of nonspecific editing is the production of additional genomes defective for replication, or even the creation of dominant negative S-HDAg mutants (Jayan and Casey 2002a). Thus, there may be limits as to how much amber/W editing can be restricted by using suboptimal structures. HDV does appear to have a mechanism for minimizing the effects of editing at nonamber/W sites: in one study of HDV replicating in transfected cells, all nonamber/W changes that occurred during replication were found on genomes that were also edited at the amber/W site (Polson et al. 1998).

5.2.1
Effects of HDAg

HDV genotype I uses an additional mechanism to slow down editing early in the replication cycle. For this genotype, S-HDAg, which is known to bind HDV RNA (Chang et al. 1988), is a strong inhibitor of editing at the amber/W site. While editing on replicating RNA 2–3 days post-transfection is nearly undetectable, up to 40% of nonreplicating RNAs produced in transfected cells in the absence of HDAg are edited. However, co-transfection of an S-HDAg expression construct leads to markedly reduced levels of editing on nonreplicating RNAs (Polson et al. 1998). The levels of S-HDAg required for this inhibition are similar to those seen in cells replicating HDV RNA. Thus, it appears that S-HDAg prevents the rapid accumulation of editing early in the HDV genotype I replication cycle. It has been suggested that inhibition occurs by HDAg binding to HDV RNA (Polson et al. 1998); however, it is not clear whether

the inhibition is due to steric effects–such as blocking of an ADAR1 binding site by HDAg–or HDAg-mediated sequestration of HDV RNA in a cellular compartment in which ADAR1 is not active. Whether the ability of HDAg to inhibit editing varies during the course of viral RNA replication remains to be determined. In contrast to the sensitivity of genotype I amber/W site editing to S-HDAg, editing at the amber/W site in the genotype III double hairpin structure is not inhibited by S-HDAg (Cheng et al. 2003). The hairpin denoted SL2, on the 3′ side of the amber/W site, plays an essential role (Cheng et al. 2003), and might somehow interfere with S-HDAg binding near the amber/W site.

5.2.2
Effects of RNA Structural Dynamics

HDV genotype III uses the distribution of the RNA between at least two conformations to restrict editing (Casey 2002; Cheng et al. 2003). Only RNA molecules that adopt the double hairpin structure can be edited (Fig. 2). However, the majority of genotype III RNA appears to assume the unbranched rod conformation, which is not a substrate for editing (Casey 2002; Cheng et al. 2003). The introduction of mutations in the genotype III RNA that shift the distribution of the RNA to the double hairpin structure increases editing to levels comparable with those seen with nonreplicating genotype I RNA (Casey 2002). Thus, while the amber/W site itself in genotype III RNA can be edited with efficiency similar to the genotype I site, editing levels in nonreplicating genotype III RNAs are much lower because most of the RNA assumes the unbranched rod conformation, which is not a substrate for editing (Casey 2002). Preliminary data from our laboratory (Linnstaedt and Casey, unpublished results) indicates that the double hairpin structure is less stable than the unbranched rod and can only be formed co-transcriptionally. Thus, the structural dynamics of the RNA determine the amount of the double hairpin structure formed, which in turn determines how much RNA is available to be edited at the amber/W site. S-HDAg is not an effective inhibitor of editing for this genotype and likely does not play a direct role in limiting editing levels (Cheng et al. 2003). Possibly because editing in genotype III is downmodulated by the distribution of the RNA between two structures, further control by S-HDAg is not necessary.

5.2.3
Negative Feedback Regulation

Two recent studies have indicated that editing can be regulated by negative feedback (Cheng et al. 2003; Sato et al. 2004). In HDV genotype I mutants that overproduce L-HDAg, levels of L-HDAg plateau as replication is shut down—

by L-HDAg (Sato et al. 2004). This inhibition of L-HDAg production occurs because of the location of editing in the HDV replication scheme (Fig. 1). Editing occurs on the antigenome–the replication intermediate–and does not result in L-HDAg synthesis until the edited antigenome first serves as template for the synthesis of genomes, which then serve as templates for transcription of mRNAs encoding L-HDAg. Thus, L-HDAg can, under these circumstances, limit its own production.

In addition to the plateau in L-HDAg production, Sato et al. observed a plateau in editing at the amber/W site, which is not explained by the above model (Sato et al. 2004). They hypothesize that editing occurs only on newly transcribed antigenomic HDV RNA, perhaps before HDAg has a chance to bind and form a 'mature' RNP, and that L-HDAg indirectly inhibits editing by blocking new RNA synthesis. One important consideration of this model is the mechanism whereby L-HDAg prevents further antigenome synthesis. Other reports have indicated that L-HDAg does not inhibit antigenome RNA synthesis (Macnaughton and Lai 2002; Modahl and Lai 2000). Does inhibition of antigenome synthesis occur indirectly via shutdown of genomic RNA synthesis, or does L-HDAg produced as a result of editing inhibit antigenome RNA synthesis in a manner that is not obvious when L-HDAg is produced in trans? Another consideration is that the observed control of editing by L-HDAg was only observed when editing levels were artificially elevated—either by mutation or by ADAR overexpression. As mentioned above in Sect. 4, L-HDAg does not appear to limit replication of HDV genotype I RNA in transfected Huh-7 cells; rather, as yet undetermined factors limit RNA levels. Perhaps, editing in HDV genotype I is calibrated to these limitations, such that appropriate levels of editing are achieved just before replication is restricted; alternatively, L-HDAg may play a more important role limiting replication in infected hepatocytes than in Huh-7 cells.

Cheng et al. recently showed that L-HDAg is a much better inhibitor of editing in HDV genotype III than is S-HDAg, which is a very poor inhibitor (see Sect. 5.2.1). This observation led to the suggestion that genotype III L-HDAg might directly inhibit its own production by directly inhibiting amber/W site editing. However, preliminary data from our laboratory (R. Chen and J.L. Casey, unpublished results) suggest that this inhibitory activity, which is unrelated to replication, might not be the predominant factor involved in a negative feedback loop to limit editing levels (Cheng et al. 2003). Although L-HDAg is a potent inhibitor of genotype III editing, mixtures of genotype III S-HDAg and L-HDAg at ratios similar to those found in cells replicating genotype III RNA exhibit inhibitory activities similar to S-HDAg. Hence, it appears that levels of L-HDAg achieved during replication might not be sufficient to directly affect editing.

In contrast to the behavior of HDV genotype I, wild-type HDV genotype III replication is affected by levels of L-HDAg production. Genotype III mutants that do not produce L-HDAg replicate at significantly higher levels, and accumulate higher levels of editing (Casey 2002; Cheng et al. 2003). Thus, it seems likely that the predominant factor in the control of maximal editing levels in HDV genotype III is the ability of L-HDAg to inhibit replication. As discussed in Sect. 5.2.1, in the current model for genotype III, editing occurs on a metastable structure that is formed co-transcriptionally; cessation of transcription would prevent further editing because the structure required for editing would not be formed. Thus, while it remains to be determined whether L-HDAg functions to control editing of wild-type HDV genotype I RNA, it seems likely that it does in genotype III. A remaining question for HDV genotype III is why do S-HDAg and L-HDAg inhibit editing at such different levels (Cheng et al. 2003)? One possibility is that the more important aspect for the virus is that S-HDAg is not a good inhibitor. Because editing is already modulated by the conformational dynamics of the RNA, further inhibition by S-HDAg could lead to insufficient levels of editing.

6
Perspective

Editing at the amber/W site plays a critical role in the HDV replication cycle. Analysis of how the virus controls editing has led to valuable contributions to the field of RNA adenosine deamination. Thus far, amber/W site editing is the only example of specific editing that occurs in an organ other than the brain in mammals, but it is highly likely that more examples will be identified. The differences in editing sites, structures, and regulatory mechanisms between HDV genotypes I and III is remarkable, and emphasizes just how different these two genotypes are. Understanding how editing is regulated during the course of HDV replication remains an important goal. There is still much to learn in this area, which is largely undeveloped for host targets of specific editing. One of the more exciting recent developments has been the identification of the role of RNA structural dynamics in controlling amber/W site editing in HDV genotype III. Further analysis of editing at the amber/W site will advance our understanding of the determinants of viral replication and is likely to continue to contribute to the field of RNA editing.

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