Inhibition of Hepatitis Delta Virus RNA Editing by Short Inhibitory RNA-Mediated Knockdown of ADAR1 but Not ADAR2 Expression

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Hepatitis delta virus (HDV) requires host RNA editing at the viral RNA amber/W site. Of the two host genes responsible for RNA editing via deamination of adenosines in double-stranded RNAs, short inhibitory RNA-mediated knockdown of host ADAR1 expression but not that of ADAR2 led to decreased HDV amber/W editing and virus production. Despite substantial sequence and structural variation among the amber/W sites of the three HDV genotypes, ADAR1a was primarily responsible for editing all three. We conclude that ADAR1 is primarily responsible for editing HDV RNA at the amber/W site during HDV infection.

Hepatitis delta virus (HDV) infection causes severe acute and chronic hepatitis in those infected with its helper, hepatitis B virus (32). A central event in the HDV replication cycle is an RNA editing event that allows the virus to produce two forms of the sole viral protein, hepatitis delta antigen (HDAg), that have different and opposed functions in the HDV replication cycle (reviewed in reference 14). Editing involves the specific deamination of the amber/W site adenine to inosine and changes the stop codon of HDAg-S to a tryptophan codon for HDAg-L (4, 7, 26, 30).

In mammals, the ADAR1 and ADAR2 genes encode proteins that edit specific adenosines in double-stranded RNA segments (reviewed in references 15, 20, and 33), and ADAR1 and ADAR2 proteins can specifically edit the amber/W site in HDV RNA (18, 33, 36) as well as adenosines in several cellular pre-mRNA substrates (15, 20, 34). The product of a third related gene, ADAR3, has no apparent deaminase activity on other ADAR1 or ADAR2 substrates (9, 27) and is unlikely to edit HDV RNA. ADAR1 is expressed in many tissues, while the highest level of ADAR2 expression is found in the brain (21, 28). The relative levels of ADAR1 and ADAR2 RNA expression have been analyzed by Northern blotting for some tissues (9, 22) but not for the liver. Using Northern blot hybridization and reverse transcription-PCR (RT-PCR), we analyzed ADAR1 and ADAR2 expression both in cultured Huh-7 human hepatoma cells and in HDV-infected liver tissue and found that the expression level of ADAR1 is 10- to 20-fold higher than that of ADAR2. These data are consistent with the general pattern of ADAR1 and ADAR2 expression (9, 21, 27) and could suggest that ADAR1 is principally responsible for HDV amber/W editing in infected hepatocytes. However, these enzymes can exhibit differential activities on some substrates (28, 33, 36). Although previous studies (18, 33, 36) showed that both ADAR1 and ADAR2 can edit HDV RNA when overexpressed in Huh-7 cells, their relative activities on the HDV amber/W site were not investigated: amber/W editing activities were analyzed only at very high, possibly saturating, levels of ADAR expression.

We sought to determine the extent to which ADAR1 and ADAR2 and their splice variants are responsible for HDV RNA editing in vivo by using short inhibitory RNAs (siRNAs) (2, 10) to specifically knock down expression of ADAR1 or ADAR2 in cultured Huh-7 cells. siRNAs (Table 1) were designed as double-stranded RNAs with 19 or 20 bp and 2-nucleotide 3’ overhangs, as described previously (2, 11). GenBank searches (1) indicated that only the targeted genes matched the siRNA sequences perfectly; the closest nontargeted genes were mismatched with the siRNAs in at least two positions and would not likely be targeted for siRNA-mediated knockdown of expression (12). siRNAs were obtained as annealed duplexes from Dharmacon Research Inc. (Lafayette, Colo.) (11) and transfected into cultured Huh-7 cells as reported previously (2).

Transfection with siAD1 dramatically inhibited expression of endogenous targeted ADAR1 in Huh-7 cells (Fig. 1A). This suppression was specific: siRNAs shown to be functional against luciferase (10) and ADAR2 (Fig. 1B) did not suppress ADAR1 protein expression, and siAD1 had a minimal effect against luciferase (10) and ADAR2 (Fig. 1B). We also observed that siAD1 could suppress expression of ADAR1 from a cotransfected ADAR1 expression construct (data not shown), further confirming the specificity of ADAR1 suppression by siAD1. Because the level of endogenous ADAR2 expression in Huh-7 cells is too low to accurately quantify the reduction of expression, we demonstrated the efficacy of siAD2, which targets ADAR2, by cotransfection with an ADAR2 expression construct, pMS040 (36). Similar to the effect of siAD1 on ADAR1 expression, siAD2 dramatically and specifically inhibited ADAR2 protein expression (Fig. 1B). Suppression of ADAR1 and ADAR2 by these siRNAs was greatest between days 2 and 4 posttransfection but was still evident 6 days posttransfection.

To examine the contributions of ADAR1 and ADAR2 to HDV amber/W site editing, siAD1 and siAD2 were cotrans-
fected with the HDV genotype I replication construct pHDV·I(+). Editing at the amber/W site was assessed on days 4 and 6 posttransfection using a well-characterized RT-PCR restriction digestion assay in which the amount of RT-PCR products indicated the percent editing because editing creates a SstI digestion site that is not present prior to editing (7, 18, 31). Transfection with siAD1, but not siAD2, strongly inhibited amber/W editing (Fig. 1C and D) on days 4 and 6 posttransfection. The extent of this inhibition by siAD1 was similar to the extent to which ADAR1 expression was reduced (Fig. 1A). No inhibition of editing was seen with siLuc, which has been shown to be an effective inhibitor of firefly luciferase expression (10) but has no sequence homology to ADAR1 or ADAR2. This result indicates that the HDV amber/W site is edited primarily by ADAR1 in Huh-7 cells.

Several splice variants of ADAR1 have been identified that may have different activities on some substrates (24, 25). To determine the roles of ADAR1 splice variants in HDV amber/W editing, we analyzed the relative abundance of the ADAR1a, ADAR1b, and ADAR1c forms by RT-PCR using the primers 5’-CGACCAACTCCATGCTTCTGA-3’ and 5’-GGTGCCTGCCCAGTGAGGGAG-3’ (nt positions 2238 to 2259 and 2684 to 2704, respectively; GenBank accession no. XM_036845), which amplify a fragment spanning segments that are deleted from ADAR1b and ADAR1c (Fig. 2A and B). The relative abundance of ADAR1a- and ADAR1b-specific PCR products indicated that these variants were present in a ratio of approximately 4:1 (ADAR1a/ADAR1b) in Huh-7 cells and in HDV-infected liver; ADAR1c was not detected. We tested the abilities of siRNAs to selectively target the ADAR1a and ADAR1b splice variants and analyzed their effects on HDV RNA editing (Fig. 2). Transfection with siAD1a and siAD1b, which targeted ADAR1a and ADAR1b, respectively, effectively reduced expression of their respective targets (Fig. 2B). Because ADAR1a is the predominant splice variant, siAD1a had a more pronounced effect on total ADAR protein levels. In accord with this difference, we observed that knockdown of ADAR1a expression substantially reduced HDV amber/W editing, while knockdown of ADAR1b had little effect (Fig. 2C). Thus, ADAR1a is primarily responsible for HDV amber/W editing in Huh-7 cells. It is not possible to tell from these results whether ADAR1b could edit the amber/W site, because the lack of a substantial effect of siAD1b on amber/W editing could simply be due to the lower level of ADAR1b expression.

We also attempted to use siRNAs to suppress expression of another variant of ADAR1, p150 (13, 19, 29), but were unable to do so. This variant is expressed at approximately 1/10 the level of the predominant form, p110, both in Huh-7 cells and in infected liver tissue, and is not preferentially associated with either the ADAR1a or ADAR1b splice variant. Although overexpression studies suggest that the p150 form is not more active than the p110 form, a more definitive study will be required to determine the role of ADAR1 p150 in HDV amber/W editing.

Three genotypes of HDV have been identified, each with different geographic distributions and associated disease severities (5, 17, 35, 37). These genotypes exhibit functional differences in RNA replication and in RNA editing (3, 6, 17, 37). In particular, the RNA structures required for amber/W editing are dramatically different for genotypes I and III (3). For example, the A-C mismatch that is required for editing in the genotype I site occurs as an A-U pair in genotype III, and a highly conserved A-U or G-C base pair in genotype I (4, 31) occurs as an A-A pair in genotype III (3). The structure around the editing site in HDV genotype II has not yet been defined, but there are fewer base pairs in the predicted structure in a region that is important for editing in genotype I (17). These structural variations raise the question of whether the same deaminase is active for all three genotypes (3).

To determine the roles of ADAR1, ADAR2, and ADAR1 splice variants in amber/W editing for HDV genotypes II and III, we cotransfected Huh-7 cells with either replication-competent genotype II constructs or genotype III constructs and our panel of ADAR-targeted siRNAs. In results similar to those for genotype I (Fig. 1), transfection with siAD1 and siAD1a inhibited amber/W editing in HDV genotype II and III, whereas siAD2 had no detectable effect (Fig. 3). Thus, despite structural variations around the amber/W site, ADAR1a is primarily responsible for HDV amber/W editing for all three HDV genotypes. It is worth noting that the levels of amber/W editing differ among the three genotype clones tested. It is not yet clear to what extent these differences are due to the intrinsic activity of the different editing substrates or other factors, such as different regulatory mechanisms. Overall, these data indicate that the sequence and structural determinants for RNA editing are complex. Examination of the predicted structure around the HDV amber/W site (3, 4, 17) and several cellular (34) and synthetic (23) substrates for RNA editing suggests that primary sequence, base pairing, and internal bulges and loops can all contribute to highly specific

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**TABLE 1. Sequence of siRNA duplexes used to knock down ADAR expression**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
<th>Region targeted</th>
<th>mRNA targeted</th>
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<tr>
<td>siAD1</td>
<td>5’ CGCAGAGGUUCCACUCCGUATT 3’</td>
<td>+271 to +293</td>
<td>All ADAR1 mRNAs</td>
</tr>
<tr>
<td></td>
<td>3’ TTGGCCUCCAGAGGGACAU 5’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siAD1a</td>
<td>5’ GGAGGAGGAGGGAGGAGUUTT 3’</td>
<td>+1563 to +1583</td>
<td>ADAR1a</td>
</tr>
<tr>
<td></td>
<td>3’ TCCUUCUCUCUCUGCAGAAAG 5’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siAD1b</td>
<td>5’ CCAGUGAGGGAGGGCGUUGTGG 3’</td>
<td>+1527 to +1533, +1572 to +1585</td>
<td>ADAR1b</td>
</tr>
<tr>
<td></td>
<td>3’ GTGGUCACUCCUCUGCAGACAC 5’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siAD2</td>
<td>5’ GCCUUGGUUAGGACACTT 3’</td>
<td>+261 to +288</td>
<td>All ADAR2 mRNAs</td>
</tr>
<tr>
<td></td>
<td>3’ TCCGGAACCAAGGGCAUGGG 5’</td>
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* Relative to the AUG start codon for the corresponding mRNA.
Editing. Clearly, a more complete understanding of these determinants will require further study.

Editing at the amber/W site is required for the production of HDAg-L, which is necessary for the formation of infectious HDV virions with a hepatitis B virus surface antigen envelope (8). To examine the effect of inhibiting editing on virion production, we cotransfected Huh-7 cells with pHDV·I(+) and siAD1, and a hepatitis B virus surface antigen expression construct; RNAs were harvested from cells and from exported viral particles 6 days posttransfection and analyzed by North-
ern blot hybridization (3). Whereas cotransfection of siAD1 had no effect on HDV RNA replication, virion production was strongly inhibited (Fig. 4), in accord with the suppression of amber/W editing (Fig. 1).

We have shown here that ADAR1a is primarily responsible for editing HDV RNA at the amber/W site in Huh-7 cells. Because the relative levels of ADAR1 and ADAR2 expression and the relative levels of ADAR1 splice variants are similar in Huh-7 cells and infected liver tissue, it is likely that ADAR1a is also primarily responsible for editing in HDV-infected liver tissue. While our results indicate a correlation between ADAR protein expression levels and participation in HDV amber/W editing during replication in Huh-7 cells and infected liver tissue, they do not directly address the relative specific activities of ADAR1, ADAR2, and their splice variants on the HDV amber/W sites. Further studies in this area could provide valuable information on the determinants of editing activity and specificity for these enzymes.

Our results further emphasize the effects of various ADAR levels on HDV and underscore the central role of RNA editing in the HDV replication cycle. Previously we showed that overexpression of ADARs inhibits HDV RNA replication by increased HDAg-L production and hyperediting of HDV RNA (18). The observed marked decrease in virus production (Fig.

FIG. 2. Effects of ADAR1 splice variants on HDV amber/W editing. (A) Illustration of the structure of ADAR1 splice variants ADAR1a and ADAR1b (24). ADAR1b is generated by alternative splicing at exon 7 and has a deletion of 78 nucleotides (shown by the dashed line) compared with ADAR1a. Locations of primers P1 and P2 used for RT-PCR analysis of ADAR1a and ADAR1b expression are indicated by leftward and rightward arrows. Locations of ADAR1a- and ADAR1b-specific siRNAs are indicated schematically. Note that the sketch is not drawn to scale. (B) RT-PCR analyses to detect the efficiency of ADAR gene targeting by siRNAs. Huh-7 cells were transfected with siAD1, siAD1a, siAD1b, or siAD2, as in Fig. 1. RNAs were harvested 4 days posttransfection and analyzed by RT-PCR. Products were run on 1% agarose gels, stained with ethidium bromide, and photographed. The image shown is an inverted image of a scanned photograph, which better illustrates the reduction of intensity of several bands. MW, molecular weight standards (1-kb ladder; Invitrogen, Carlsbad, Calif.). Sizes of selected molecular weight markers are indicated, as are the locations of the predicted RT-PCR products for ADAR1a and ADAR1b. (C) Effect of siRNA-targeted reduction of ADAR1a and ADAR1b on HDV amber/W editing. Huh-7 cells were transfected with pHDV-I(+) and the indicated siRNAs; RNAs were harvested 6 days posttransfection and analyzed for HDV amber/W editing as in Fig. 1.
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REFERENCES


