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Substrate recognition by ADAR1 and ADAR2

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ABSTRACT

RNA editing catalyzed by ADAR1 and ADAR2 involves the site-specific conversion of adenosine to inosine within imperfectly duplexed RNA. ADAR1- and ADAR2-mediated editing occurs within transcripts of glutamate receptors (GluR) in the brain and in hepatitis delta virus (HDV) RNA in the liver. Although the Q/R site within the GluR-B premessage is edited more efficiently by ADAR2 than it is by ADAR1, the converse is true for the +60 site within this same transcript. ADAR1 and ADAR2 are homologs having two common functional regions, an N-terminal double-stranded RNA-binding domain and a C-terminal deaminase domain. It is neither understood why only certain adenosines within a substrate molecule serve as targets for ADARs, nor is it known which domain of an ADAR confers its specificity for particular editing sites. To assess the importance of several aspects of RNA sequence and structure on editing, we evaluated 20 different mutated substrates, derived from four editing sites, for their ability to be edited by either ADAR1 or ADAR2. We found that when these derivatives contained an A:C mismatch at the editing site, editing by both ADARs was enhanced compared to when A:A or A:G mismatches or A:U base pairs occurred at the same site. Hence substrate recognition and/or catalysis by ADARs could involve the base that opposes the edited adenosine. In addition, by using protein chimeras in which the deaminase domains were exchanged between ADAR1 and ADAR2, we found that this domain played a dominant role in defining the substrate specificity of the resulting enzyme.

Keywords: **adenosine deaminase; GluR-B; hepatitis delta virus; inosine; RNA editing**

INTRODUCTION

ADAR1 (adenosine deaminase that acts on RNA) and ADAR2 belong to a family of RNA editing enzymes that catalyze the hydrolytic deamination of adenosine to inosine in completely or partially double-stranded RNA. The ADAR family also includes a third member, ADAR3; however, this protein is either catalytically inactive or has a substrate(s) that has yet to be identified (Melcher et al., 1996a; Chen et al., 2000). A related subfamily, the ADATs (adenosine deaminases that act on tRNA), also deaminate specific adenosines within RNA but act on tRNAs (Gerber et al., 1998; Gerber & Keller, 1999; Maas et al., 1999).

ADAR1 was first discovered in *Xenopus* as an RNA unwinding activity (Bass & Weintraub, 1987). It was later shown that unwinding resulted from the covalent modification of adenosines to inosines (Bass & Weintraub, 1988) within double-stranded RNA. ADAR1 was subsequently cloned from mammalian cells and characterized (Kim et al., 1994; Lai et al., 1995; O'Connell et al., 1995). At roughly the same time, RNA editing was detected within the rat GluR-B pre-mRNA at several locations, the Q/R, +60, and R/G sites (Sommer et al., 1991; Higuchi et al., 1993; Lomeli et al., 1994). The GluR-B message encodes a subunit of a glutamate-gated ion channel. In mammals, the genome-encoded CAG (Q) is found converted to CGG (R) in cDNA at the Q/R site and similarly AGA (R) is converted to GGA (G) at the R/G site. These changes result in proteins with altered functional properties. Because A was converted to G (inosine is reverse-transcribed and translated as G) and because the process required double-stranded structure that resulted from base-pairing with downstream intronic sequences, editing was proposed to be catalyzed by an ADAR. This hypothesis was later supported by experiments, both in vitro and in vivo, where ADAR1 was demonstrated to be able to efficiently edit the +60 and R/G sites but not the Q/R site (Dabiri et al., 1996; Maas et al., 1996).

The inability of ADAR1 to edit the Q/R site led to the search for another activity/coactivity that could edit this site. A homologous enzyme, ADAR2, was identified and shown to have distinct and overlapping editing proper-

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ties compared with ADAR1 (Melcher et al., 1996b); that is, ADAR1 edits the +60 site, ADAR2 edits the Q/R site, and both enzymes edit the R/G site efficiently. Recently, using ADAR2-null mice, the GluR-B Q/R site was shown to be the only essential target of ADAR2 (Higuchi et al., 2000). ADAR1, on the other hand, is required for development, as heterozygosity for this gene in the mouse results in an embryonically lethal phenotype (Wang et al., 2000).

Hepatitis delta virus (HDV) is a subviral human pathogen that uses RNA editing as an essential step in its life cycle. HDV expresses two viral proteins from a single open reading frame. The small delta antigen is expressed throughout infection and is required for replication (Kuo et al., 1989) whereas the large delta antigen is expressed only late during infection and is required for virion assembly (Chang et al., 1991). Large delta antigen expression is mediated by editing of antigenomic RNA where the small delta antigen amber stop codon (UAG) is converted to a tryptophan codon (UIG; Casey & Gerin, 1995). *Xenopus* ADAR1 specifically and efficiently edits the HDV Amber/W site in vitro (Polson et al., 1996), and both human ADAR1 (hADAR1) and hADAR2 were found able to edit this site in vivo with similar efficiencies (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

ADAR1 and ADAR2 have two common functional domains, an N-terminal double-stranded RNA-binding domain and a C-terminal deaminase domain. The N-terminal domain of ADAR1 has three double-stranded RNA-binding motifs (dsRBM) whereas ADAR2 has two. The C-termini of both ADARs contain conserved histidine-cysteine-cysteine (HCC) residues that are thought to coordinate zinc ions. It has not been determined which of the two functional domains contributes to the preference of ADAR1 for the +60 site and ADAR2 for the Q/R site. By exchanging their double-stranded RNA-binding domains, here we found that the fusion with the deaminase domain from ADAR1 was more proficient than the fusion with the deaminase domain from ADAR2 at editing ADAR1-specific targets whereas the converse was true with ADAR2-specific targets.

All known editing substrates of ADARs are predicted to have extensive duplex structure at and adjacent to the site of editing, yet these duplexes are invariably interrupted with loops, bulges, and mismatches. It is thought that these structural features enable ADARs to recognize specific adenosines (Lehmann & Bass, 1999). However, the exact sequences and structures required to constitute an editing site have not been defined. Hence it is not possible to predict which adenosine in a given RNA might serve as a substrate.

Here we monitored editing of 20 different substrates derived from four known editing sites, in an effort to better understand the attributes of RNA sequence and structure involved in defining the substrate. We found that when an A:C mismatch occurred at the editing site, editing by both ADAR1 and ADAR2 was more efficient as compared to when an A:U base pair occurred at that site. Hence the base-pairing status of the targeted adenosine can affect the efficiency of editing by both ADAR1 and ADAR2. In addition, for both ADARs, we observed that the identity of the mismatched base opposing the edited adenosine significantly influenced the efficiency of editing.

RESULTS

Experimental design

We were interested in defining protein and RNA structural features involved in Amber/W site recognition and editing by ADAR1 and ADAR2. In such a study, it would be useful to compare several different Amber/W sites so that any shared structural features important for editing might be identified. To date, the only known Amber/W site occurs within HDV antigenomic RNA. However, the GluR-B +60 hot-spot site, which resides within an intron, is an editing site in which UAG is edited to UIG. In addition, Higuchi et al. (1993) showed that the GluR-B Q/R site could be converted to an Amber/W site without interfering with editing by ADAR1. Here, we reasoned that it should also be possible to convert the GluR-B R/G site to an Amber/W site, and this enabled us to study the properties of four different Amber/W sites.

Recently, we developed a convenient reporter system to monitor editing of the HDV Amber/W site inside transfected cells. In this system, the editing reporter is a mRNA that encodes the small delta antigen (HDAg-S) and includes approximately two-thirds of the HDV antigenomic structure (S. Sato, S.K. Wong, and D.W. Lazinski, submitted). The message includes an intact Amber/W site and, when it is edited, expresses the large delta antigen. Thus, the extent of editing is reflected by the ratio of large delta antigen to small delta antigen as detected by western analysis. Using this reporter, we showed that, when overexpressed, both hADAR1 and hADAR2 can edit the HDV Amber/W site in vivo. We also found that the specific activities of HA-tagged ADAR1 and ADAR2 were very similar to those of the untagged proteins and these tags enabled us to directly compare the levels of expression of ADAR1 and ADAR2.

Here we have adapted the HDV Amber/W reporter to allow the study of Glur-B based Amber/W sites. cDNA from the editing site of interest was inserted into the reporter plasmid replacing the HDV Amber/W site. The GluR-B R/G and Q/R editing sites were mutated from AAG and CAG respectively to UAG and this amber codon was fused in-frame to HDAg to allow immunodetection (Fig. 1B). The resulting transcript, when edited, should be translated into a larger protein product, which can then be detected by western analysis. The pre-

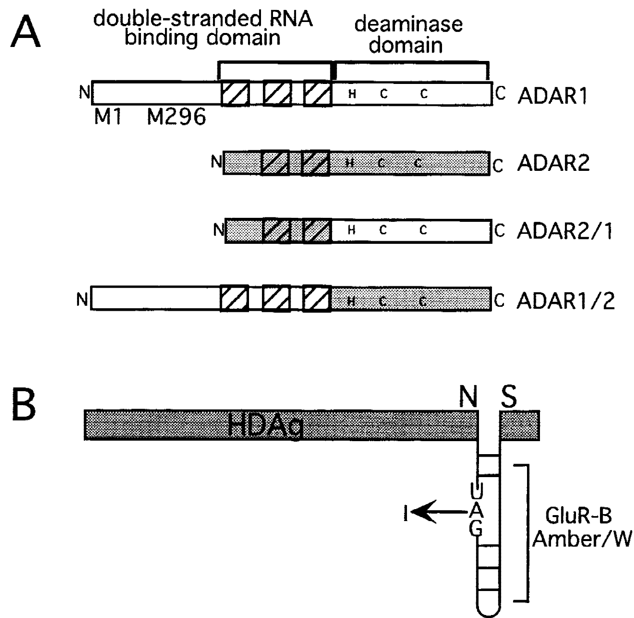


FIGURE 1. **A:** ADAR1 and ADAR2 and their chimerical fusions. The diagram shows the two major functional domains of ADAR1 and ADAR2, the N-terminal double-stranded RNA-binding domain and the C-terminal deaminase domain with the conserved HCC zinc-binding motif. ADAR1 has three dsRB motifs (dsRBMs) shown as hatched boxes and ADAR2 has two dsRBMs. The chimera ADAR2/1 had amino acids 1–323 of ADAR2 fused to amino acids 841–1226 of ADAR1 and the chimera ADAR1/2 had amino acids 1–840 of ADAR1 fused to amino acids 324–701 of ADAR2. M1 and M296 denote methionines at positions 1 and 296 from which the two forms of ADAR1 initiate (Fig. 3, top panel). **B:** Design of editing reporters derived from the GluR-B premessage. The reporter was an mRNA with the GluR-B editing sites converted to Amber/W sites (in the case of R/G and Q/R but not +60; see text) and fused in-frame to the ORF of Hepatitis Delta Antigen (HDAg) for immunodetection. N and S are the *Ngo*AIV and *Sal*I sites of HDAg where the 199-bp fragment of HDAg was replaced by GluR-B sequences. Editing of this reporter at the Amber/W site would result in the translation of a larger protein product.

dicted structures of reporter derivatives of the GluR-B R/G, GluR-B +60 hotspot, GluR-B Q/R, and HDV Amber/W sites are shown in Figure 2.

Preedited versions (UGG derivatives) of these reporters were also constructed to determine if the larger protein expressed from an edited message was as stable as the smaller one expressed from the unedited message. The level of protein expressed from the preedited UGG derivative was similar to that of the unedited version for three of the four reporters. In the case of the GluR-B +60 UGG derivative, the protein was observed at an approximately twofold lower level than was the wild-type (UAG) derivative (data not shown). Hence this reporter underestimates the absolute level of editing. However, because the same underestimation should occur regardless of which ADAR is overexpressed, this systematic error should not affect the relative comparison of editing efficiency by different ADARs.

A second form of error could arise from deamination of editing reporter transcripts at adenosines other than that in the Amber/W site. Such editing might alter either the stability of the mRNA or the amino acid composition and stability of the resulting protein. Furthermore, it is possible that secondary sites could be edited differently by different ADARs and this, if accompanied by differences in mRNA or protein stability, would complicate the comparison of editing at the Amber/W site by these enzymes. If a significant difference in message or protein stability were to result from ADAR-mediated editing at a secondary site(s), then a significant difference in reporter protein expression should be observed. For this reason, the total protein expression (edited + unedited) was quantified for all the editing reporters. The total protein expression of each sample was then normalized to the level of expression observed with the control transfection in which no ADAR was expressed.

The GluR-B R/G, GluR-B +60, GluR-B Q/R, and HDV Amber/W reporters were assayed for their ability to be edited by ADAR1 and ADAR2. In addition, the activities of two chimeric ADAR proteins were similarly assayed. These chimeras were constructed by fusing the N-terminal double-stranded RNA-binding domain of ADAR1 or ADAR2 to the C-terminal deaminase domain of ADAR2 or ADAR1 respectively (Fig. 1A).

HA-tagged ADARs were transiently cotransfected with each reporter in HEK293 cells. Following western analysis using anti-delta antigen polyclonal sera, the resulting percentage of editing was quantified by the ratio of the large protein to the total protein signal. ADAR expression was monitored by western analysis using anti-HA monoclonal sera and was normalized to a HA-tagged ADAR1 standard. Equal aliquots of this standard were loaded on all anti-HA westerns so that the relative level of protein expression could be compared within and between experiments.

Two forms of ADAR1 have been detected in human cell lines, the larger full-length form (150 kDa) and the smaller N-terminally truncated form (110 kDa; Patterson & Samuel, 1995). The HA-tagged ADAR1 expression vector used in this study also expresses both forms in a ratio similar to that expressed in human cell lines (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

Editing of wild-type derivatives

Figure 3 shows editing by ADAR1 and ADAR2 and their chimeras ADAR2/1 and ADAR1/2 of the wild-type derivatives of the four editing sites. In this context, we are defining the wild-type derivative as the derivative whose sequence is closest to that of the natural site. ADAR1 and ADAR2 edited the HDV Amber/W wild-type derivative (~40%) and the GluR-B R/G wild-type derivative (~70–80%) with similar efficiencies. Editing of the GluR-B +60 wild-type derivative was 10-fold higher with ADAR1 than with ADAR2 and the chimera ADAR2/1 edited this reporter more efficiently than did

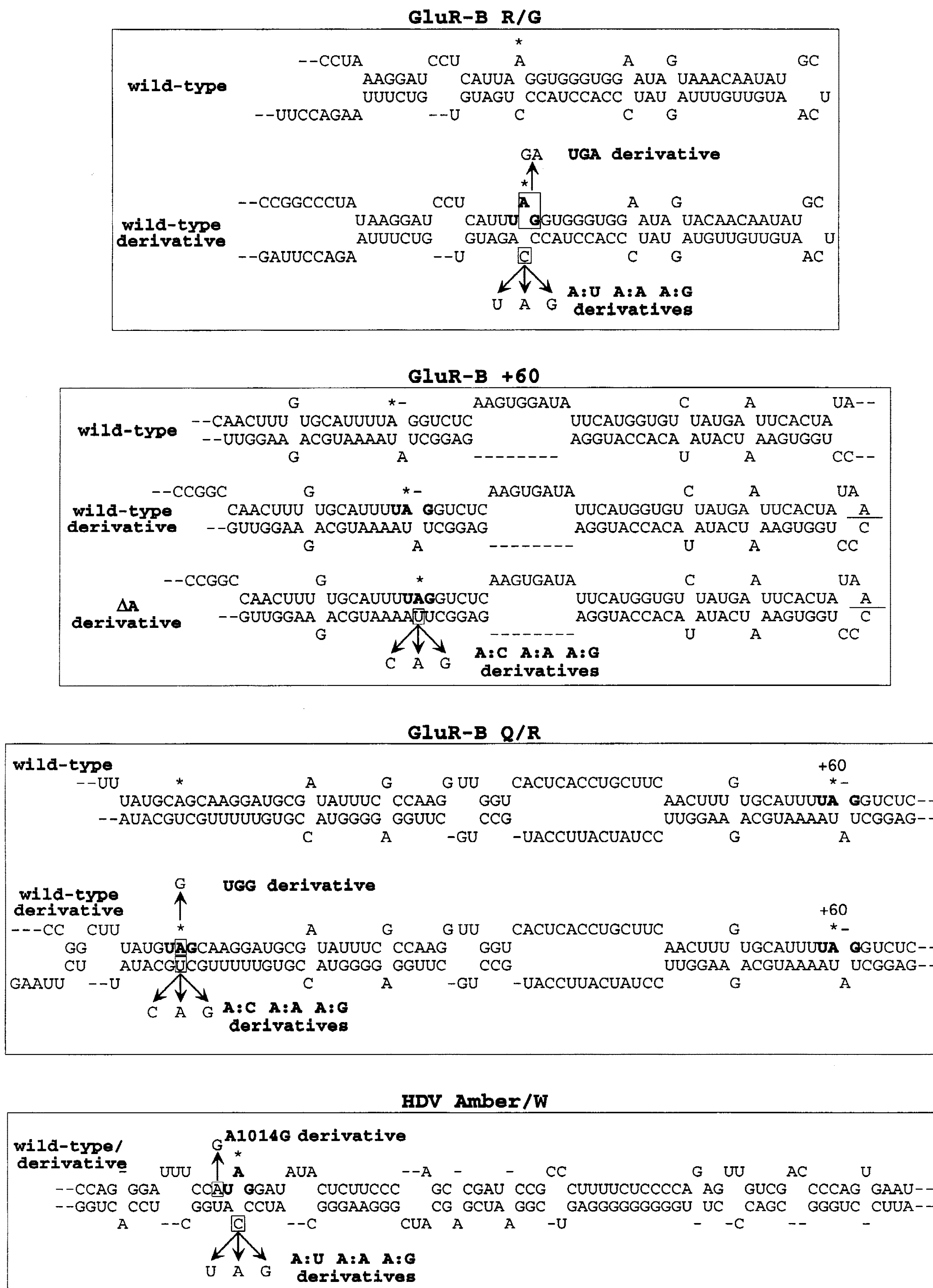


FIGURE 2. Reporters derived from the R/G, +60, and Q/R sites of the GluR-B premessage and the Amber/W site of HDV antigenomic RNA. Structures were predicted using mfold (Le & Zuker, 1991). For the GluR-B derivatives, the wild-type sequences are shown on the first line of each box. The regions inserted into the *Ngo*AIV and *Sal*I sites of HDAg to create their derivative are shown below (for GluR-B R/G and +60). For the GluR-B Q/R, only part of this region is shown (Yang et al., 1995). The HDV reporter has approximately two-thirds of the HDV antigenomic rodlike structure (S. Sato, S.K. Wong, and D.W. Lazinski, submitted). The editing reporters that most resemble the wild-type structures and sequences were named wild-type derivatives. Arrows denote mutations made from these wild-type derivatives. The Amber/W site of each derivative is shown in bold and the asterisk denotes the targeted adenosine.

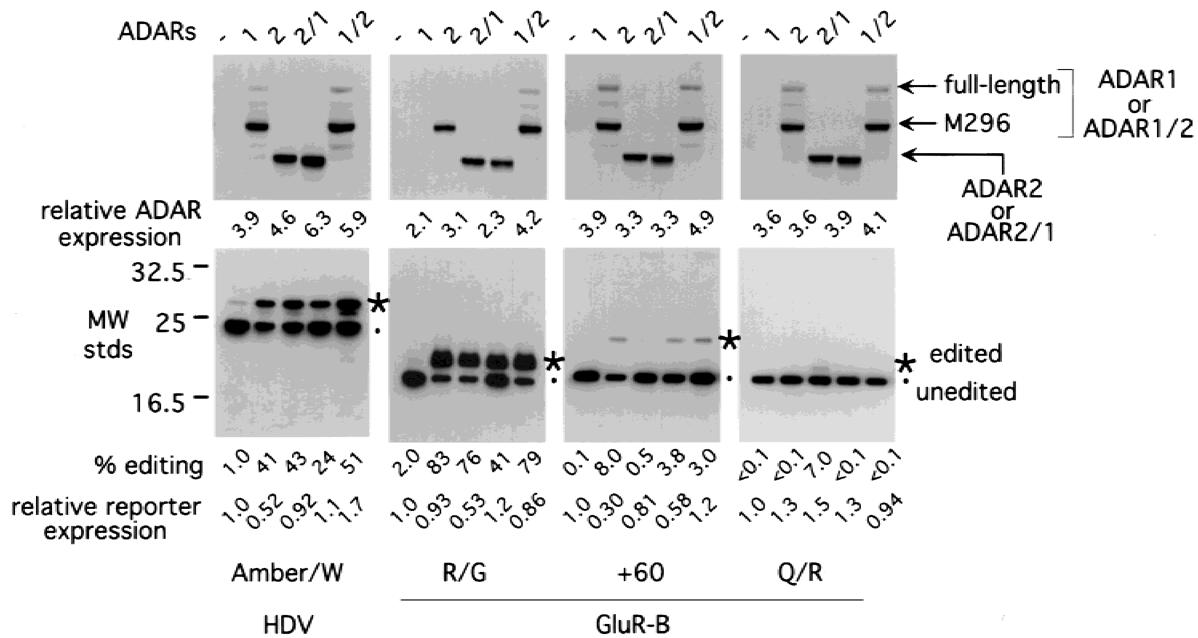


FIGURE 3. Western analyses showing editing of wild-type derivatives of HDV Amber/W and GluR-B R/G, +60, and Q/R sites by HA-tagged hADAR1 and hADAR2 and their chimeras. Total protein lysates from transient cotransfection of HEK296 cells with ADARs and editing reporters were immunoblotted with anti-HA antibody (top panel) to show relative expressions of ADARs and with anti-delta antigen (HDAg) antibody (bottom panel) to monitor editing. The relative ADAR expression was obtained by comparison with a HA-tagged hADAR1 standard, which was given an arbitrary value of 10. The percentage of editing was calculated from the ratio of the edited (denoted with an asterisk) product to the sum of the edited and unedited (denoted with a dot) products. The total protein expression of the each editing reporter was normalized to the no-ADAR control, whose total protein expression was defined as 1. The sizes of the unedited and edited products for each reporter were 195 and 214 (HDV Amber/W), 162 and 185 (GluR-B R/G), 161 and 197 (GluR-B +60), and 158 and 178 (GluR-B Q/R) amino acids.

ADAR1/2 despite being expressed at a lower level. For the GluR-B Q/R wild-type derivative, editing by ADAR1 was undetectable and editing by ADAR2 was 7%. This low level of editing could be due to at least two reasons. First, the U of the amber UAG site was shown in 1993 by Higuchi et al. to inhibit editing at the Q/R site when it was paired with a G as is the case in this study. Second, additional upstream sequences were shown by the same authors to be required for efficient editing of the Q/R site.

The trend of editing of the Glur-B-derived reporters by ADAR1 and ADAR2 was consistent with previous studies (Dabiri et al., 1996; Maas et al., 1996; Melcher et al., 1996b; Burns et al., 1997). Both ADAR1 and ADAR2 efficiently edited the R/G wild-type derivative, ADAR1 more efficiently edited the +60 wild-type derivative, and ADAR2 more efficiently edited the Q/R wild-type derivative. Thus, conversion of the GluR-B R/G and Q/R editing sites into Amber/W sites did not alter the relative editing efficiency of ADAR1 compared with that of ADAR2.

In Figure 3 as well as in subsequent figures, the maximum variation in total reporter expression was roughly within a factor of two for all reporters excepting the GluR-B +60 derivatives. This range is consistent with the variation observed in ADAR expression and is to be expected in transfection experiments of this type. We therefore conclude that if one or more of the ADARs do edit a secondary site(s) in any of the reporters, such editing does not significantly alter the stability of the message or the resulting protein. For the GluR-B +60 derivatives, however, in this figure as well as in subsequent figures, consistently lower levels of reporter protein were observed with samples that showed high levels of editing. This was observed regardless of which enzyme was used for editing. These results are consistent with data obtained from the GluR-B +60 UGG derivative and reflect the instability of the larger protein expressed from this reporter. Because the systematic error applies to all four editing enzymes tested in this study, it should not affect their relative comparison.

A mismatch improves editing of the GluR-B Q/R and +60 derivatives whereas a base pair inhibits editing of the GluR-B R/G and HDV Amber/W derivatives

The HDV Amber/W wild-type derivative and the GluR-B R/G wild-type derivative were efficiently edited by both ADAR1 and ADAR2 (Fig. 3) and they resemble each other in that the target adenosine is positioned across from a cytidine and is flanked by duplexes (Fig. 2). We

next tested whether a mismatch at the editing site could be responsible for the efficient editing of these sites.

To this end, we mutated the A:C mismatch at the editing sites of the GluR-B R/G and HDV Amber/W wild-type derivatives to an A:U base pair (Fig. 2, AU derivatives). For GluR-B R/G, changing the A:C mismatch to an A:U base pair decreased ADAR1-mediated editing by approximately twofold and did not significantly alter editing by ADAR2. The A:C-to-A:U mutation had a more dramatic effect on the HDV Amber/W reporters. Here, editing was decreased ~17-fold for ADAR1 and ~5-fold for ADAR2. In both cases, editing by ADAR1 decreased more than did editing by ADAR2 (Fig. 4A).

In addition, we also mutated the editing sites of the GluR-B +60 and Q/R wild-type derivatives to resemble the GluR-B R/G and HDV Amber/W sites by mutating their editing sites so that they would contain an A:C mismatch at the appropriate position (Fig. 2, A:C derivatives). Converting the editing site of the GluR-B +60 wild-type derivative to an A:C mismatch increased editing by approximately twofold with ADAR1 and approximately sixfold with ADAR2. For GluR-B Q/R, changing the wild-type A:U base pair to an A:C mismatch at the editing site enabled us to detect editing by ADAR1 from <0.1% (undetectable) to 1.6% and increased ADAR2-catalyzed editing by approximately sixfold (Fig. 4B). Thus, for all four reporters, having A:C mismatches at the editing site enhanced editing by both ADAR1 and ADAR2.

A:C mismatches are preferred to A:A or A:G mismatches

To test if any mismatch will enhance editing by ADAR1 and ADAR2 or whether an A:C mismatch is specifically

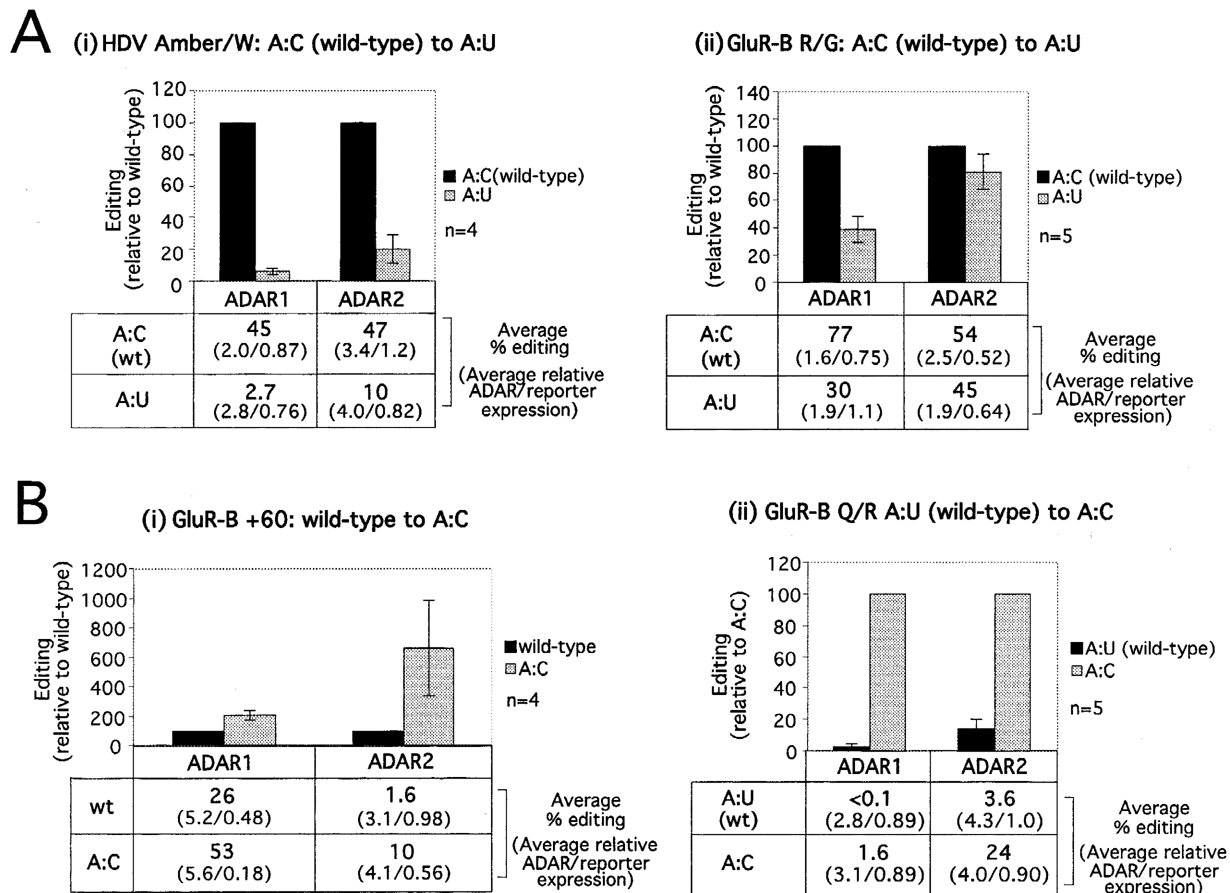


FIGURE 4. **A:** Inhibition of ADAR1- and ADAR2-catalyzed editing when an A:C mismatch was converted to an A:U base pair at the editing site. The graphs compare editing of (i) HDV Amber/W wild-type (A:C) to A:U derivatives, and (ii) GluR-B wild-type (A:C) to A:U derivatives. **B:** Enhancement of ADAR1- and ADAR2-catalyzed editing when an A:U base pair was converted to an A:C mismatch at the editing site. The graphs compare editing of (i) GluR-B +60 wild-type to A:C derivatives, and (ii) GluR-B Q/R wild-type (A:U) to A:C derivatives. Editing and relative ADAR expression were quantified as previously mentioned. For the HDV, GluR-B R/G, and +60 derivatives, editing (y axes) was normalized to the wild-type derivatives where the percentage of editing of the wild-type derivative was given a value of 100. For the GluR-B Q/R derivatives, the relative editing was normalized to the A:C derivative because editing by ADAR1 was low or undetectable in the wild-type derivative. The tables below the bar graphs show the average percentage of editing of that derivative and the average relative ADAR/editing reporter expression in parentheses. *n* denotes the number of times the experiment was performed.

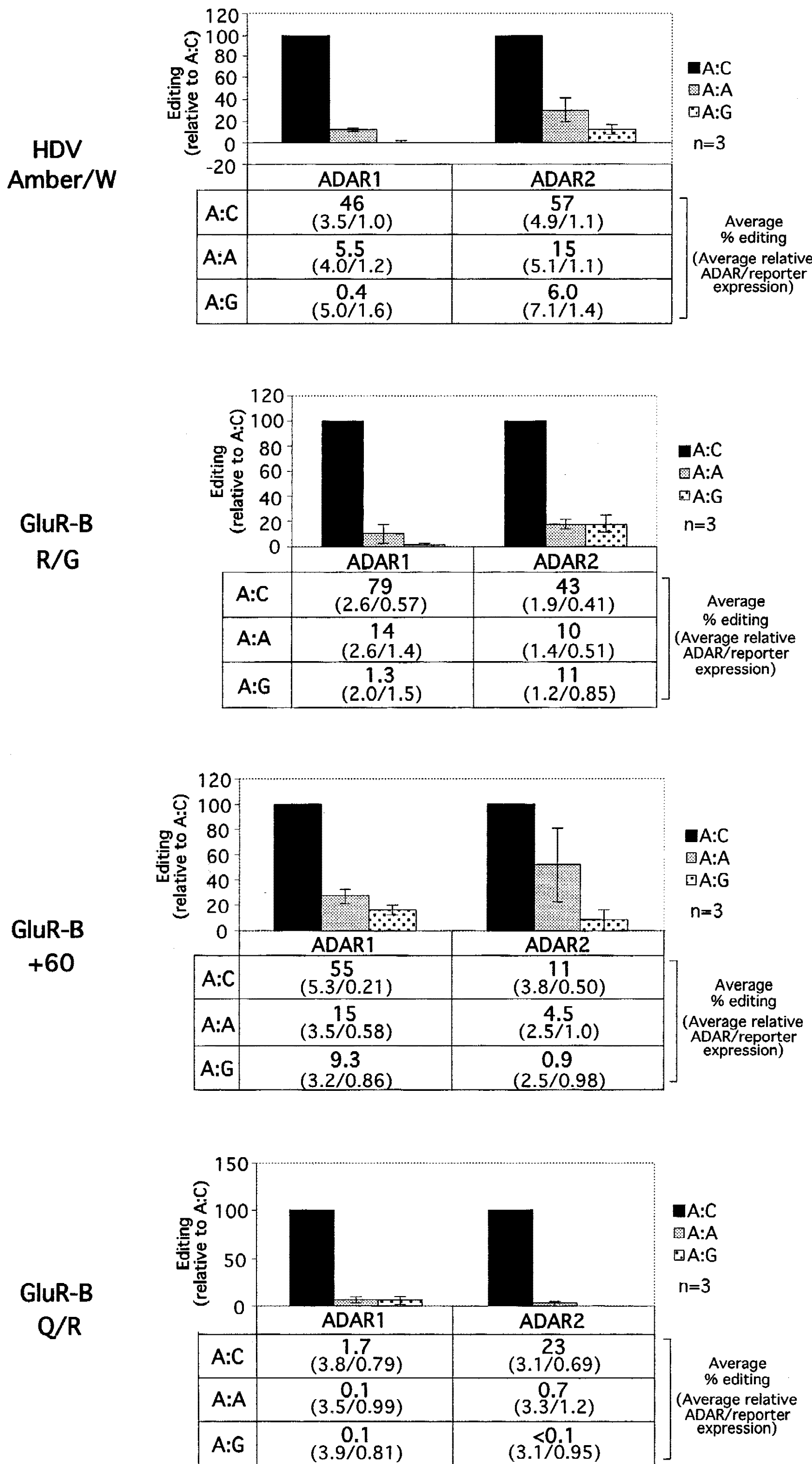


FIGURE 5. Preferential editing by ADAR1 and ADAR2 of substrates with an A:C mismatch compared to an A:A or A:G mismatch at the site of editing. The graphs compare editing of reporters derived from the HDV Amber/W, the GluR-B R/G, +60, and Q/R sites with either an A:C or A:A or A:G mismatch at their editing sites. Editing was quantified as previously mentioned. Relative editing (y axes) was editing that had been normalized to the A:C derivatives where the percentage of editing of the A:C derivative was given a value of 100.

preferred, we mutated each reporter with an A:C mismatch (A:C derivatives) to either an A:A or A:G mismatch (A:A and A:G derivatives, Fig. 2). For all four editing reporters, we observed that optimal editing by ADAR1 and ADAR2 occurred when an A:C mismatch was located at the editing site (Fig. 5). The GluR-B R/G, GluR-B Q/R, and HDV Amber/W showed drastic decreases in editing by both ADARs when the A:C mismatch was mutated. With the HDV Amber/W mutants, ADAR1-mediated editing was inhibited ~8-fold (A:A) or >100-fold (A:G). Similarly, ADAR-2 mediated editing was inhibited ~4-fold (A:A) or ~10-fold (A:G). Editing

of the GluR-B R/G derivatives decreased 5-fold (A:A) and 60-fold (A:G) with ADAR1 and 4-fold (A:A and A:G) with ADAR2. Editing of the GluR-B Q/R derivatives decreased 17-fold (A:A and A:G) with ADAR1 and 33-fold (A:A) or 200-fold (A:G) with ADAR2. The same mutations at the GluR-B +60 reporters had less dramatic consequences; a 4-fold (A:A) or 6-fold (A:G) decrease was observed with ADAR1 and a 2-fold (A:A) or 12-fold (A:G) decrease was observed with ADAR2. Nevertheless, even with this site, an A:C mismatch was still the optimal target.

Casey et al. (1992) reported that mutating the base opposite to the edited A from C to U or G or A severely inhibited editing during HDV replication, and here we attempted to reproduce those results. The mutations in question were introduced into a cDNA that expresses an ~1.1 unit-length antigenomic RNA and is competent for initiating genome replication (Lazinski & Taylor, 1994). The wild-type and mutant vectors were transfected into Huh7 cells that were then harvested 5, 10, and 15 days posttransfection. Editing was monitored by western analysis, and replication was monitored by northern analysis.

We found that none of the three mutations had a significant effect on replication (data not shown). We observed 1.3, 6.2, and 18% editing at days 5, 10, and 15, respectively, with the wild-type replicon, whereas for the three mutants, editing was less than 1% even at day 15 (data not shown). Although the vectors used to initiate HDV replication in this study differed somewhat from those used by Casey et al. (1992), the two studies obtained very similar results. We conclude that the enzyme that is endogenously expressed in HuH7 cells and that edits HDV RNA has the same substrate preference as ADAR1 and ADAR2, that is an A:C mismatch is preferred over an A:A or A:G mismatch or A:U base pair. Furthermore, this preference is observed even when the editing enzyme is not overexpressed.

The deaminase domain confers specificity to ADAR1 and ADAR2

ADARs have two common functional domains: the N-terminal double-stranded RNA-binding domain and the C-terminal deaminase domain. To determine which domain is responsible for substrate specificity, we fused the N-terminal double-stranded RNA-binding domain of ADAR1 and ADAR2 to the C-terminal deaminase domains of ADAR2 and ADAR1 respectively.

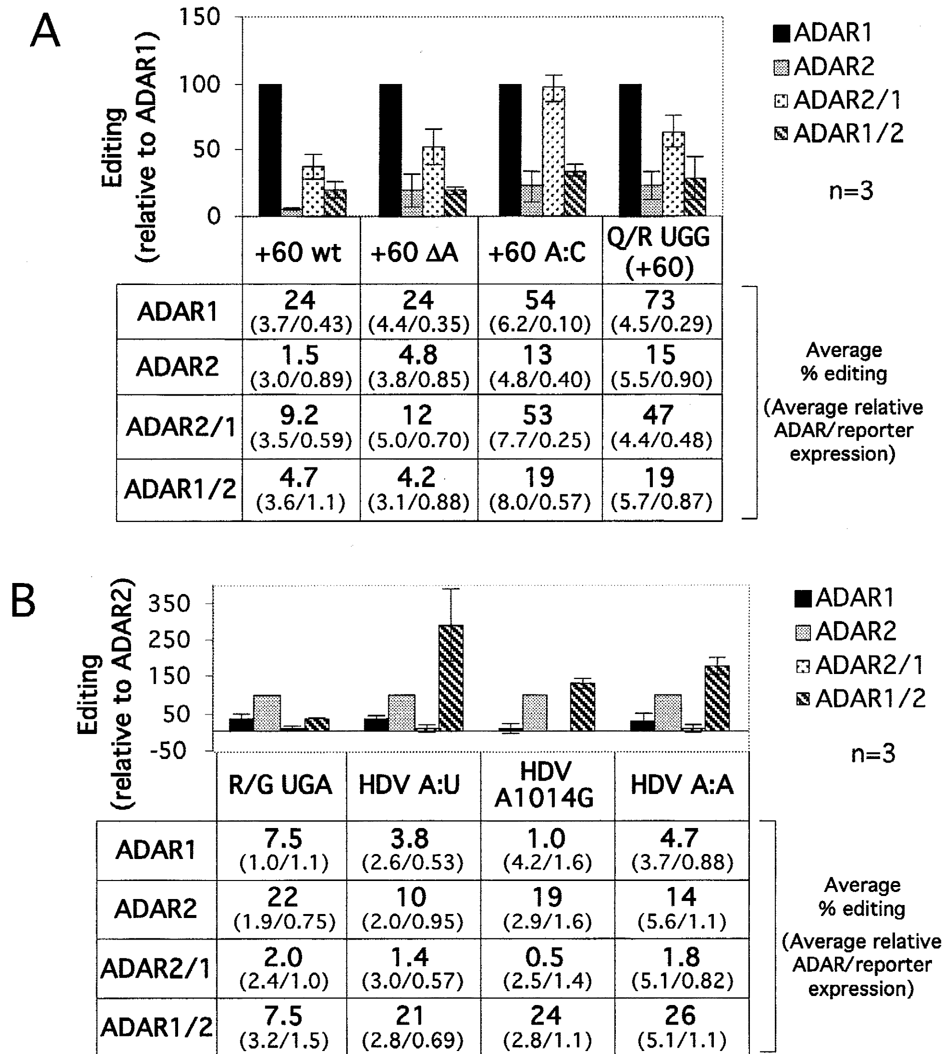
These chimeras were tested with two different categories of editing reporters. The first category is comprised of editing reporters that were edited more efficiently by ADAR1 than by ADAR2, for example, the GluR-B +60 wild-type, +60 ΔA, +60 A:C, and the GluR-B Q/R UGG derivatives. Editing of these reporters was 4- to 16-fold higher with ADAR1 compared with ADAR2 and 2- to 3-fold higher with ADAR2/1 compared with ADAR1/2 (Fig. 6A). Even though our experimental strategy assays editing only at an Amber/W site, in the GluR-B Q/R UGG derivative, the +60 site happens to be an Amber/W site in-frame with the Q/R site. Hence in this construct, we can detect editing of the +60 site within its natural context. We found that editing of the +60 site in the GluR-B Q/R UGG derivative was much higher (~70% by ADAR1 and ~15% by ADAR2) than it was with the smaller GluR-B +60 wild-type derivative (~24% by ADAR1 and ~2% by ADAR2; see Fig. 6A). This difference is at least in part due to the fact that the two reporters, when edited, express proteins with different C-termini and, as previously mentioned, the edited products of the GluR-B +60 derivatives were found to be underexpressed.

The second category of editing reporters is edited more efficiently by ADAR2 than by ADAR1 as shown in Figure 6B. Here editing of the GluR-B R/G UGA and the HDV amber/W A:U, A1014G, and A:G derivatives (Fig. 2) was 3- to 12-fold higher with ADAR2 compared with ADAR1 and 5- to 29-fold higher with ADAR1/2 compared with ADAR2/1. Therefore the converse was also true in that the chimera with the ADAR2 deaminase domain (i.e., ADAR1/2) edited ADAR2-preferred substrates more efficiently than did ADAR2/1. We conclude that when ADAR1 or ADAR2 preferentially edit a site, their deaminase domains can confer much of the preference for that site.

DISCUSSION

Our objective was to better understand how ADAR1 and ADAR2 recognize their substrates. We designed Amber/W editing reporters derived from known editing substrates of these enzymes to serve as tools for studying ADAR1- and ADAR2-catalyzed editing. Editing of these mutated Amber/W reporters would not necessarily be expected to correlate with editing of the native sites from which they were derived. However, we did observe such a correlation insofar as the GluR-B Q/R wild-type derivative was still edited more efficiently by ADAR2, whereas the GluR-B R/G wild-type derivative was edited efficiently by both ADAR1 and ADAR2. As expected, the GluR-B +60 wild-type derivative, which is not mutated at the editing site, was edited more efficiently by ADAR1. By making use of the characteristic features of these reporters, some of which are specific substrates of ADAR1 or ADAR2, we were able to define several aspects of substrate recognition by ADARs.

Chimeras of ADAR1 and ADAR2 were generated in which the N-terminal double-stranded RNA-binding domain of one protein was fused to the deaminase domain of the other. We found that the ADAR1 deaminase domain-containing chimera edited ADAR1-specific reporters at higher levels than did the fusion that contained the ADAR2 deaminase domain. Furthermore, with ADAR2-specific reporters, the converse was ob-



	+60 wt	+60 ΔA	+60 A:C	Q/R UGG (+60)
ADAR1	24 (3.7/0.43)	24 (4.4/0.35)	54 (6.2/0.10)	73 (4.5/0.29)
ADAR2	1.5 (3.0/0.89)	4.8 (3.8/0.85)	13 (4.8/0.40)	15 (5.5/0.90)
ADAR2/1	9.2 (3.5/0.59)	12 (5.0/0.70)	53 (7.7/0.25)	47 (4.4/0.48)
ADAR1/2	4.7 (3.6/1.1)	4.2 (3.1/0.88)	19 (8.0/0.57)	19 (5.7/0.87)

	R/G UGA	HDV A:U	HDV A1014G	HDV A:A
ADAR1	7.5 (1.0/1.1)	3.8 (2.6/0.53)	1.0 (4.2/1.6)	4.7 (3.7/0.88)
ADAR2	22 (1.9/0.75)	10 (2.0/0.95)	19 (2.9/1.6)	14 (5.6/1.1)
ADAR2/1	2.0 (2.4/1.0)	1.4 (3.0/0.57)	0.5 (2.5/1.4)	1.8 (5.1/0.82)
ADAR1/2	7.5 (3.2/1.5)	21 (2.8/0.69)	24 (2.8/1.1)	26 (5.1/1.1)

FIGURE 6. Contribution of the deaminase domains of ADAR1 and ADAR2 to their preferential editing of different substrates. **A** shows four reporters (GluR-B +60 wild-type, +60 ΔA, +60 A:C, and Q/R UGG (+60) derivatives) that were preferentially edited by ADAR1 and the chimera with ADAR1's deaminase domain, ADAR2/1. The GluR-B Q/R UGG (+60) derivative here was used to monitor editing at the +60 site of this reporter. **B** shows four reporters (GluR-B UGA, HDV A:U, HDV A1014G, and HDV A:G derivatives) that were preferentially edited by ADAR2 and the chimera with ADAR2's deaminase domain, ADAR1/2. Editing in **A** was normalized to ADAR1's editing and that of **B** to ADAR2's. Relative ADAR/reporter expression was quantified as previously mentioned.

served. Hence the deaminase domain conferred much of the specificity of a given ADAR for a particular site.

We think that this observation had been previously missed for several reasons. First, in this study we have compared the editing efficiency of ADAR1, ADAR2, and the two chimeras on four distinct editing sites as well as a total of 20 different mutant derivatives. In all cases, ADAR expression was quantified and controlled to ensure that similar levels of protein were assayed within cells. Such a careful and comprehensive investigation of ADAR specificity had not been previously attempted. Second, we constructed active chimeras of ADAR1 and ADAR2. The editing efficiency of the chimeras was comparable to that of the wild-type enzymes for most of the editing reporters in this study.

Melcher et al. (1996a) had also made chimeras of ADAR1 and ADAR2. However, in general, their fusions appeared to be much less active than the wild-type ADARs. In addition, the chimera that had the deaminase domain from ADAR2 (Dra-RED1) displayed lower activity than the other chimera with both editing substrates tested. At least two reasons might account for the differences in activity of the chimeras from that study as compared to those from this study. First, the precise positions within the proteins used for joining differed in the two cases. Second, the chimera of Melcher et al. (1996a) that included the amino-terminal domain from ADAR1 (Dra-RED1) had a deletion of ~400 amino acids such that all of the sequence unique to the 150-kDa species as well as roughly 100

amino acids common to both forms of ADAR1 were not present.

ADAR1 differs from ADAR2 in that it has three copies of double-stranded RNA-binding motifs at its N-terminus whereas ADAR2 has only two. Mutational (Liu & Samuel, 1996) and deletional (Lai et al., 1995) analyses of the ADAR1 dsRBMs show that they are not functionally equivalent and that the third motif is indispensable and the second motif is the least important. Splice variants that change the spacing among and between these motifs and the deaminase domain have also been identified. (Liu et al., 1997). Due to these findings, it was hypothesized that perhaps the specificity of ADARs might be conferred by the different versions of the double-stranded RNA binding domains that they contain. Although this remains possible, our results indicate that the deaminase domain contributes a greater role in defining the specificity of an ADAR. This is consistent with the findings that the dsRNA binding domains of ADARs bind double-stranded RNA nonspecifically and that ADATs, which lack N-terminal double-stranded RNA binding motifs, can still specifically deaminate adenosines within tRNA substrates.

We have shown that both ADARs prefer an A:C mismatch at the editing site. The generality of this observation was shown in two ways. First, when we mutated the wild-type derivatives of the GluR-B R/G and HDV Amber/W reporters from an A:C mismatch at the editing site to A:A or A:G mismatches or A:U base pairs, we observed a decrease in editing for both enzymes with both reporters. Except for ADAR2-mediated editing of the A:U derivative of GluR-B R/G, the decrease was quite pronounced. Second, when the GluR-B Q/R and GluR-B +60 wild-type derivatives were mutated to include A:C mismatches at their editing sites, we observed increased editing by both ADARs.

The biological significance of the preference for an A:C mismatch was demonstrated by evaluating the editing of wild-type and mutant HDV during replication in tissue culture. Even though Casey et al. (1992) had obtained similar results, the significance of their observations was difficult to interpret then. At that time, it was not known whether the genome or antigenome was the substrate for editing and hence, whether HDV editing involved an A-to-I or U-to-C change. Furthermore, because neither ADAR1 nor ADAR2 had yet been cloned, the authors had no way of determining how the mutations might affect the activity of these enzymes. Finally, because HDV editing and replication are coupled, it was unclear whether the mutations in HDV had directly affected the competence of the editing site or had an indirect effect on editing. For instance, the mutations could have altered the binding of the delta antigens such that the accessibility of the editing site to an ADAR was reduced.

The results reported here indicate that mutation of the base that opposes the edited adenosine in HDV does, indeed, decrease the competence of the editing site in the absence of replication and that analogous mutations behave similarly in other editing sites. In all, four mutations that were previously reported to severely inhibit HDV editing during replication (Casey et al., 1992; Casey & Gerin, 1995) were tested in this study in the absence of replication. We found that all four mutations inhibited editing by both ADAR1 and ADAR2. Thus, based on these results alone, we cannot conclude that either enzyme is more likely to be the endogenous protein that edits HDV in Huh7 cells. However, we note that for all four mutations, of the two enzymes tested, editing by ADAR1 was more severely affected.

In the case of the GluR-B R/G derivative, an A:U base pair at the editing site did not significantly alter ADAR2-catalyzed editing. This is consistent with a recent report (Stephens et al., 2000) where only a very modest decrease in the extent of deamination by ADAR2 was observed in vitro when the same mutation in the wild-type R/G site was tested. Based on this finding, the authors concluded that the identity of the base opposing the edited adenosine exerts a relatively minor effect on editing. However, if the investigators had tested ADAR1 with the same mutant or ADAR2 with a GluR-B R/G mutant that contained an A:G mismatch at the editing site, they would likely have observed a more severe effect.

We have found that, in general, the base opposing the target adenosine influences the efficiency of editing. It is noteworthy that of the known substrates of ADAR1 and ADAR2, none are predicted to have an A:A or A:G mismatch at the site of editing. However, several sites (e.g., the GluR-B R/G, HDV Amber/W, and Serotonin-2C A sites) are predicted to have A:C mismatches at the site of editing and are efficiently edited by both ADAR1 and ADAR2 (Yang et al., 1995; Dabiri et al., 1996; Maas et al., 1996; Melcher et al., 1996b; Polson et al., 1996; Burns et al., 1997).

We can propose several hypotheses to account for the role of the opposing base in editing. First, it is possible that an A:C mismatch serves as a recognition element for ADARs. This model predicts that the binding affinity of an ADAR to an A:C substrate would be greater than that for the corresponding A:G substrate. Second, it is possible that the base opposing the target adenosine might have to be accommodated within the active site of an ADAR. Because purines are larger than pyrimidines, perhaps they might sterically interfere with that process. This model predicts that if the nucleotide opposing the target adenosine contained no base (an apurinic nucleotide), in vitro editing of that substrate would be efficient. Finally, we observed a hierarchy in the contribution of the unpaired opposing base to editing efficiency, where $C > A > G$. It is known that in DNA, the hierarchy for the stability of pairing with inosine is also $C > A > G$ (Martin et al., 1985). Thus,

perhaps pairing between the opposing base and inosine might enhance the rate at which the enzyme dissociates from its product. Clearly, additional experiments will be needed to test the validity of these models.

MATERIALS AND METHODS

Expression vectors of HA-tagged hADAR1, hADAR2, hADAR2/1, and hADAR1/2

The hADAR1 (Genbank accession number NM 001111) and hADAR2 (hRED1-S) (Genbank accession number U82120) were kindly provided by Andre Gerber and Walter Keller (University of Basel). ADAR cDNA was subcloned into a mammalian expression vector, pSS43 (S. Sato, S.K. Wong, and D.W. Lazinski, submitted), which is a pUC-based plasmid with a CMV promoter and HDV polyadenylation signal flanking a cloning cassette. The *Xho*I-*Xba*I fragment of hADAR1 (4,482 bp) was inserted between the *Xho*I and *Xba*I sites of pSS43 to generate pSKW004 and the *Not*I-*Kpn*I fragment of hADAR2 (2,789 bp) was inserted between the *Not*I and *Kpn*I sites of pSS43 to generate pSKW005.

To determine the junctions of fusions of our chimeras, we used the GCG pileup program with ADAR1 and ADAR2 from several species. The ADAR1/2 expression plasmid, pSKW018, was constructed by fusing residues 1–840 of ADAR1 to residues 324–701 of hADAR2. Overlapping PCR products were generated with primers, oli182 (5′-GAAGGT GCTGCCAGTGAGAGG-3′) + oli183 (5′-CTGAATTCAAGT TGGTCGACCAG-3′) from hADAR1 (pSKW004) and with oli184 (5′-CACTGGCAGCACCTTCGCTGACGCTGTCTCAC-3′) + oli166 (5′-GTAAAGGCTGCCCAGGATGATGCT-3′) from hADAR2 (pSKW005). These PCR products were used to amplify the chimerical fragment with primers oli183 + oli166 in a second PCR. The product of the second PCR was digested with *Bsp*EI and *Blp*I to generate a 900-bp fragment, which was then ligated to the *Sfi*I-*Bsp*EI fragment of pSKW004 (3,162 bp and encodes the N-terminus of ADAR1) and the *Blp*I-*Sfi*I fragment of pSKW005 (3,253 bp and encodes the C-terminus of ADAR2). The ADAR2/1 expression plasmid, pSKW017, was constructed by fusing residues 1–323 of hADAR2 to residues 845–1226 of hADAR1. Overlapping PCR products were generated with primers oli194 (5′-GTTCAG TTTCCTAATGCCTCTG-3′) + oli177 (5′-TAAAACCTGCG GT AAATGCAGC-3′) from hADAR2 (pSKW005) and with oli180 (5′-TTTACCGCAGGTTTTACATGACCAGATAGCCA-3′) + oli181 (5′-AGTGGCGGGATTCTGTGCTTTCC-3′) from hADAR2 (pSKW004). These PCR products were used to amplify the chimerical fragment in a second PCR with primers oli194 + oli181. The second PCR product was digested with *Sfi*I and *Blp*I to generate a 537-bp fragment, which was then ligated to the *Sfi*I-*Sfi*I fragment of pSKW005 (1,598 bp and encodes the N-terminus of ADAR2) and the *Blp*I-*Sfi*I fragment of pSKW004 (4,170 bp and encodes the C-terminus of ADAR1).

DNA encoding two HA epitope tags and six histidines, $T(GYPYDVPDYAA)_2G(H)_6$, was inserted between the final amino acid and stop codon of pSKW004 (ADAR1), pSKW005 (ADAR2), pSKW018(ADAR1/2), and pSKW017(ADAR2/1) to generate pDL700, pMS040, pMS041, and pMS042, respectively.

Construction of editing reporters derived from the GluR-B R/G, +60, and Q/R sites and the HDV amber/W site

GluR-B derivatives

General strategy: The editing site of interest was generated by PCR, the product of which was then digested with *Ngo*AIV and *Sal*I and inserted between those sites in pKW42 (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

R/G

pSKW001; wild-type derivative: primer extension and PCR with primers oli136 (5′-AAAAAGCCGGCCCTATAAGGAT CCTCATTTAGGTGGGTGGAATAG-3′) and oli139 (5′-AA AAAGTCGACTAAGGTCTTAAAGACACATCTGGGTAGG TGGGATACTACAACAACATTGAGCATATTGTTGTACTA TTCCACCCAC-3′).

pSKW002; preedited derivative(UGG): as above using primers oli137 (5′-AAAAAGCCGGCCCTATAAGGATCCTCAT TTGGGTGGGTGGAATAG-3′) + oli139.

pSKW021; UGA derivative: as above using primers oli195 (5′-AAAAAGCCGGCCCTATAAGGATCCTCATTTGAGTG GGTGGAATAG-3′) + oli139.

pSKW013; A:U derivative: as above using primers oli136 + oli160 (5′-AAAAAGTCGACTAAGGTCTTAAAGACACATC TAGGTAGGTGGGATACTACAACAACATTGAGCATATTG TTGTACTATTCCACCCAC-3′).

pSKW050; A:A derivative: as above using primers oli136 + oli394 (5′-CTGGGGTCGACTAAGGTCTTAAAGACACAT CTTGGTAGGTGG-3′).

pSKW049; A:A derivative: as above using primers oli136 + oli393 (5′-CTGGGGTCGACTAAGGTCTTAAAGACACA TCTCGGTAGGTGG-3′).

+60

pSKW025; wild-type derivative: as above using primers oli218 (5′-ACAACCTTCTGCATTTTATAGCCTCTCCATGGTGT ATATGATTTCACCAGGGTTATAGTGAATTCATAGACACC ATGAATATCACTTGAGA-3′) + oli213 (5′-TCCTGGGCA CCCTTGGGGG-3′) + oli219 (5′-AAAAAGCCGGCCAAC TTTGTGCATTTTAGGTCTCAAGTGATA-3′).

pSKW026; preedited(UGG): as above using primers oli218 + oli213 + oli220 (5′-AAAAAGCCGGCCAACTTTGTGCA TTTTGGGTCTCAAGTGATA-3′).

pMS043; ΔA derivative: as above using primers oli219 + oli254 (5′-ACTGGGGTCGACCAACCTTCTGCATTTTA AGCCTCTCCATGGTG-3′) + pSKW025 as template.

pMS030; A:C derivative: as above using primers oli219 + oli255 (5′-ACTGGGGTCGACCAACCTTCTGCATTTTGA GCCTCTCCATGGTG-3′) + pSKW025 as template.

pSKW063; A:A derivative; as above using primers oli219 + oli452 (5′-CTGGGGTCGACCAACCTTCTGCATTTTTAG CCT-3′) + pSKW025 as template.

pSKW064; A:G derivative: as above using primers oli219 + oli453 (5′-CTGGGGTCGACCAACCTTCTGCATTTTCAG CCT-3′) + pSKW025 as template.

Q/R

A shorter version of the Q/R reporters was generated using the above strategy (i.e., primer extension and PCR products

were digested with *Ngo*AIV and *Sal*I and replaced the *Ngo*AIV-*Sal*I fragment of HDAg). The *Bsp*MI-*Kpn*I fragment (140 bp) from the GluR-B mini gene B13 (gift from J.H. Yang & T. Maniatis) replaced the *Bsp*MI-*Kpn*I fragment of this shorter version to generate a longer version of Q/R, which were used in the editing assays here.

Short version of Q/R.

pSKW038; short wild-type derivative: primer extension and PCR with primers oli367 (5′-AAAAAGCCGGCTTTATGTA GCAAGGATGCGATA-3′) + oli366 (5′-GCAAAAACACGG TACCCCGCACAAAGTTGAAGCAGGTGAGTGACCAACC TTGGAAATATCGCATCCTTGC-3′) + oli369 (5′-AAAAAG TCGACTTAAGAATATGCAGCAAAAACACGGTA-3′).

pSKW039; preedited (UGG) derivative: as above with primers oli368 (5′-AAAAAGCCGGCTTTATGTGGCAAGGAT GCGATA-3′) + oli366 + oli370 (5′-AAAAAGTCGACTTAA GAATATGCGGCAAAAACACGGTA-3′).

pSKW040; A:C derivative: as above with primers oli367 + oli366 + oli370.

Q/R (longer version).

pSKW043; wild-type derivative: pskw038 with the *Bsp*MI-*Kpn*I fragment (140 bp) from the GluR-B mini gene B13.

pSKW044; pskw039 with the *Bsp*MI-*Kpn*I fragment (140 bp) from the GluR-B mini gene B13.

pSKW045; pskw040 with the *Bsp*MI-*Kpn*I fragment (140 bp) from the GluR-B mini gene B13.

pSKW066; A:A derivative: PCR with primers oli367 + oli455 (5′-CTGGGGTCGACTTAAGAATATGCTGCAAAAAC-3′) using pSKW045 as template. pSKW067; A:G derivative: as in pSKW066 except primer oli456 (5′-CTGGGGTCGA CTTAAGAATATGCCGCAAAAAC-3′) replaced oli455.

HDV

pSS74: wild-type derivative (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

pSS75; preedited UGG derivative (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

pSS95: A:U derivative (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

pSS92: A1014G (S. Sato, S.K. Wong, and D.W. Lazinski, submitted).

pSKW048; A:A derivative: PCR with primers oli087 (5′-GAG TTGTCGACCCCAGTGAATCCCGCGGGTTTCCACTCA CAGGT-3′) + oli392 (5′-CCCCCTTCGAAAGTGACCGG AGGGGGTGCTGGGAACACCGGGGACCAGTGGAGCC ATTGGATGCCCTTCCCG-3′) using pSS74 as template.

The PCR product was digested with *Sal*I and *Bst*BI (435 bp) and replaced the *Sal*I-*Bst*BI fragment of pSS74.

pSKW047; A:G derivative: as in pSKW048 except primer oli392 was replaced by oli391.

HDV replicons

pDL456 (Lazinski & Taylor, 1994) expresses antigenomic HDV RNA. The 120-bp *Pst*I-*Bst*BI fragment of pDL456 is replaced by the 120-bp *Pst*I-*Bst*BI fragment of: pSKW047 to generate pSKW068 (HDV A:G replicon); pSKW048 to generate pSKW069 (HDV A:A replicon); pSS95 to generate pSKW070 (HDV A:U replicon).

Editing assay

Transfection

HEK293 cells cultured in DMEM with 10% fetal calf serum were cotransfected by a modification of calcium phosphate precipitation method (Lazinski & Taylor, 1994) with 3 μg of ADAR expression vector, 0.25 μg of editing reporter and 0.3 μg of pSS15 (S. Sato, S.K. Wong, and D.W. Lazinski, submitted), which expresses secreted alkaline phosphatase (SEAP). pSS43 was used here for the no-ADAR control. The ratios of ADARs used in the transfections were 4:2:3:4 for ADAR1:ADAR2:ADAR2/1:ADAR1/2 to achieve comparable expression levels. Three days posttransfection, transfection efficiency was scored with a colorimetric assay for the SEAP activity (Berger et al., 1988) and the cells were lysed with SDS gel-loading buffer.

Immunoblot analysis and quantification of editing and ADAR expression levels

Total protein lysates were separated by electrophoresis on SDS polyacrylamide gels (7% for anti-HA and 11 or 12% for anti-HDAg westerns). The proteins were transferred electrophoretically on to nitrocellulose membranes and stained with Ponceau Red to ensure that comparable amounts of proteins were loaded and that transfer was efficient. The membranes were blocked with 5% nonfat milk in PBS for 30 min at room temperature and immunoblotted with either a 1:1,000 dilution of anti-HA antibody (BabCo; affinity-purified mouse monoclonal antibody against HA.11 epitope) or a 1:2,000 dilution of anti-HDAg antibody (S. Sato, S.K. Wong, and D.W. Lazinski, submitted). Following washes, the membranes were then blotted with either a 1:300 dilution of ^{125}I anti-mouse IgG (New England Nuclear; NEX161) for the anti-HA westerns or a 1:1,000 dilution of ^{125}I Protein A (New England Nuclear; NEX146) for the anti-HDAg westerns. The blots were exposed on phosphor screens and the radioactive signals were detected by the Molecular Dynamics Storm Phosphorimager and quantified using the ImageQuant software.

From the anti-HA westerns, relative protein expression is obtained by comparison with an HA-tagged hADAR1 standard. Equal aliquots of this standard were loaded on all the anti-HA westerns and were given an arbitrary value of 10. The ADAR1 signal was the total signal from both the full-length and M296 forms.

From the anti-HDAg westerns, the percentage of editing was calculated by dividing the signal of the edited product to the total signal (edited + unedited). The total protein expression of each editing reporter was normalized to the no-enzyme control, which was given a value of 1, for each experiment.

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