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Structure and Sequence Determinants Required for the RNA Editing of ADAR2 Substrates*

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T. Renee Dawson‡, Christopher L. Sansam§, and Ronald B. Emeson‡§¶

From the Departments of ‡Molecular Physiology and Biophysics and §Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

ADAR2 is a double-stranded RNA-specific adenosine deaminase involved in the editing of mammalian RNAs by the site-specific conversion of adenosine to inosine. We have demonstrated previously that ADAR2 can modify its own pre-mRNA, leading to the creation of a proximal 3′-splice junction containing a non-canonical adenosine-inosine (A-I) dinucleotide. Alternative splicing to this proximal acceptor shifts the reading frame of the mature mRNA transcript, resulting in the loss of functional ADAR2 expression. Both evolutionary sequence conservation and mutational analysis support the existence of an extended RNA duplex within the ADAR2 pre-mRNA formed by base-pairing interactions between regions ~1.3-kilobases apart in intron 4 and exon 5. Characterization of ADAR2 pre-mRNA transcripts isolated from adult rat brain identified 16 editing sites within this duplex region, and sites preferentially modified by ADAR1 and ADAR2 have been defined using both tissue culture and *in vitro* editing systems. Statistical analysis of nucleotide sequences surrounding edited and non-edited adenosine residues have identified a nucleotide sequence bias correlating with ADAR2 site preference and editing efficiency. Among a mixed population of ADAR substrates, ADAR2 preferentially favors its own transcript, yet mutation of a poor substrate to conform to the defined nucleotide bias increases the ability of that substrate to be modified by ADAR2. These data suggest that both sequence and structural elements are required to define adenosine moieties targeted for specific ADAR2-mediated deamination.

The conversion of adenosine to inosine (A-to-I) by RNA editing is catalyzed by hydrolytic deamination (1) via the enzymatic activity of a family of adenosine deaminases that act on RNA (ADARs)[1] (2, 3). These proteins are double-stranded RNA (dsRNA)-specific enzymes that contain variable N termini, multiple copies of a dsRNA-binding motif (DRBM) and conserved C-terminal sequences encoding a catalytic adenosine deaminase domain (2, 4, 5). Two enzymes in this family, ADAR1 and ADAR2, have been shown to be involved in the site-selective deamination of adenosines within multiple RNA transcripts (2, 4), yet the basis for their distinct, but overlapping, specificities at particular adenosine moieties is not well understood (6, 7). The DRBMs of ADAR1 and ADAR2 are similar to the domains that mediate dsRNA interactions in a large variety of proteins, including dsRNA-dependent protein kinase, *Drosophila* staufen and *Escherichia coli* RNase III (8), yet binding of these DRBMs appears to be independent of RNA sequence (8–11). Previous studies of A-to-I editing have revealed that an RNA duplex interrupted by single-strand bulges and loops is critical for site-specific A-to-I conversion (2, 5, 12, 13). This imperfect duplex structure is generally formed by intramolecular base-pairing interactions between exon and intron sequences in pre-mRNA transcripts that can be in close proximity to one another or as many as 1700 nucleotides (nt) apart (14–18). It has been proposed that selective binding of the RNA by the DRBM, as well as complementarity between the RNA structure surrounding the targeted adenosine and the catalytic domain, may determine the adenosine(s) that are selectively modified *in vivo* (19). While there is no discrete sequence motif common among substrates to direct the deamination of specific adenosine residues, distinct 5′-nearest neighbor sequence preferences have been identified for both ADAR1 (U = A > C > G) and ADAR2 (U = A > C = G) using *in vitro* editing assays with artificial RNA duplexes and a 3′-nearest neighbor preference for ADAR2 has also been suggested (U = G > C = A) (7). While up to 50% of the adenosine residues within a perfect RNA duplex may be modified by ADAR1 and ADAR2 *in vitro*, RNA substrates whose duplex structures are interrupted by mismatches, bulges and loops are edited more selectively (20, 21). Recent *in vitro* studies with synthetic RNA substrates have indicated that internal loops within ADAR substrates may serve to uncouple adjacent helices to convert long, promiscuously deaminated substrates into a series of short, selectively modified RNA targets (22).

Previous studies have demonstrated that ADAR2 edits a specific adenosine moiety within intron 4 (also referred to as intron 1, Ref. 23) of its own pre-mRNA to generate a non-canonical 3′-splice acceptor containing an adenosine-inosine dinucleotide that effectively mimics the highly conserved AG sequence normally found at 3′-splice junctions (16). Splicing to this proximal acceptor site (site −1) alters the reading frame of the ADAR2 mRNA and results in loss of expression for the catalytically active 78 kDa protein, suggesting that autoediting may represent a negative feedback loop by which ADAR2 can modulate its own level of protein expression (16). Further support for this model was recently reported by Maas *et al.* (24) in which a direct and specific correlation between ADAR2 activity and the extent of autoediting at the −1 position was observed. A-to-I editing has also been identified in mRNAs encoding *Drosophila* ADAR (dADAR) at a conserved residue of the catalytic domain, suggesting a common paradigm for ADAR regulation by autoediting (25). Like all characterized ADAR sub-

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¶ To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University, 465 21st Avenue South, 8160 Medical Research Bldg. 3, Nashville, TN 37232-8548. Tel.: 615-936-1688; Fax: 615-936-1689; E-mail: ron.emeson@vanderbilt.edu.

[1] The abbreviations used are: ADAR, adenosine deaminases that act on RNA; ds, double-stranded; nt, nucleotide; BSA, bovine serum albumin; DRBM, dsRNA-binding motif.

strates, ADAR2 pre-mRNA is predicted to form an RNA duplex as a result of intramolecular base pairing between two halves of an imperfect inverted repeat (−1512 to −1416 and −61 to +34, relative to the proximal 3′-splice site) (16, 23).

In this study, we report that nucleotide sequence conservation between five vertebrate ADAR2 pre-mRNAs supports the base-pairing features underlying the predicted RNA secondary structure and we identify a total of 16 editing sites within ADAR2 pre-mRNA transcripts isolated from adult rat brain. The effects of mutations designed to disrupt base-pairing interactions were consistent with local structures surrounding editing sites and further suggested a model of two independent ADAR2 editing domains separated by a large internal loop. Competition analyses with mutant RNAs revealed that ADAR2 preferentially modifies its own pre-mRNA (site −1) based upon sequence/structure information contained immediately surrounding this region of the duplex. These findings correlate with increased evolutionary sequence conservation surrounding site −1 and suggest that ADAR2 is specifically targeted to the region of the alternate 3′-splice acceptor. Analysis of sequences surrounding fourteen ADAR2 sites within the ADAR2 pre-mRNA revealed a significant nucleotide bias at eight positions and three of these positions coincided with increased levels of editing *in vitro*. Furthermore, the ability of natural ADAR2 substrates to compete for ADAR2 editing (site −1) correlated with their conformity to this nucleotide bias, suggesting that both a consensus sequence and structural elements are required to define the preference and efficiency of ADAR2-mediated deamination.

EXPERIMENTAL PROCEDURES

Analysis of ADAR2 Secondary Structure and Nucleotide Distribution—The secondary structure of rat ADAR2 pre-mRNA, in a region encompassing previously identified A-to-I editing events (−1668 to +200 relative to the proximal 3′-splice site) (16), was predicted using two RNA-folding algorithms, RNAFOLD (26) and mfold (27, 28). Sequences from *ADAR2* genes extending from nucleotides −1512 to −1416 and −61 to +34 (relative to the proximal 3′-splice site for rat ADAR2) from human (accession no. AL133499), rat (16), mouse (accession no. AF411054), and pufferfish (*Takifugu rubripes*) (accession nos. AF124050 and AF124049) were aligned using the Megalign program of the DNASTAR software suite (DNASTAR, Inc.). To identify potential nucleotide preferences in sequences surrounding ADAR2 editing sites, the distribution of sequence information was analyzed surrounding 14 adenosine moieties that were edited by ADAR2 *in vitro* (Table I). Forty nucleotides flanking each edited adenosine were manually aligned and the nucleotide distribution, purine/pyrimidine ratio and GC content at each position were determined. This information was then compared by a χ^2-test of independence to the nucleotide distribution observed at equivalent positions for all 50 non-edited adenosines in the predicted region of the ADAR2 duplex. Positions at which the χ^2-analysis indicated a nucleotide preference ($p < 0.05$) were considered statistically significant.

Tissue Culture and Transfection—Human embryonic kidney (HEK293) cells were transiently co-transfected by calcium phosphate precipitation (29) with rat ADAR1a (rADAR1a) or rat ADAR2b (rADAR2b) cDNAs containing an N-terminal epitope (FLAG) tag in the presence of a 3606-nt *rADAR2* minigene, a 3360-nt *rADAR2* minigene, or a control eukaryotic expression vector (pRC-CMV; Invitrogen) (Fig. 3). Crude nuclear extracts and total RNA were prepared ~60 h post-transfection, as previously described (16).

Western Blotting—Expression levels of ADAR proteins in crude nuclear extracts from transfected cells were monitored by Western blotting analysis (29) using an anti-FLAG M2 monoclonal antibody (Sigma) followed by secondary antisera conjugated to horseradish peroxidase (Jackson Immunoresearch). The secondary antibody was detected using the SuperSignal West Dura Extended chemiluminescence reaction kit (Pierce, Inc.) in accordance with the manufacturer's instructions. Chemiluminescence was monitored using the Bio-Rad image detection system and quantitation was performed using Quantity One® software (Bio-Rad) on serially diluted samples that fell within the linear range of detection.

Quantification of RNA Editing—To quantify the editing of ADAR2 transcripts in RNAs isolated from rat brain or transiently transfected HEK293 cells, first-strand cDNA was synthesized from 3–5 μg of total RNA, amplified using the polymerase chain reaction (PCR), and assessed by either direct DNA sequencing of individual cDNA isolates subcloned into pBKSII$^-$ (Stratagene) or by a modified primer-extension analysis (16). Primer extension analysis of site −1 was performed as previously described (16). For site −1428, primer-extension was performed using a sense primer (5′-CTTTGTCAGCTGGGAG-3′) in the presence of 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dTTP, and 5 mM ddGTP. Site −1476 editing was measured by extension of an antisense primer (5′-TCTTTTGCTGGAGGATG-3′) in the presence of 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dGTP and 5 mM ddTTP. Analysis of editing site concurrence was determined by direct sequence analysis of 100 independent rat brain ADAR2 cDNA clones, extending the full-length of the predicted duplex, and assessed by a χ^2-test of independence.

Site-directed Mutagenesis—ADAR2 intron 4 mutations were introduced by oligonucleotide-directed site mutagenesis in pBSKII$^-$ (Stratagene) as described (30) using synthetic oligonucleotides to introduce restriction site-based mutations.

In Vitro Transcription of RNA Substrates—All RNA substrates were synthesized *in vitro* using the Megascript T7 RNA transcription kit (Ambion). To determine RNA concentrations, RNA synthesis was performed in the presence of [α-^{32}P] adenosine 5′-triphosphate as a trace label (final specific activity = 6×10^5 cpm/mmole UTP); RNA was precipitated with 2-propyl alcohol and quantified by scintillation spectrometry. The ADAR2 substrate was synthesized from an 873 bp template linearized at an FspI restriction site, while ADAR2 competitor RNA was transcribed from the 257-bp template linearized at ApaI (Fig. 2*A*). A PCR fragment containing the R/G duplex sequence was amplified from mouse genomic DNA using the synthetic oligonucleotide primers (5′-ACACCTAAAGGATCCTCATTAAGG-3′) and (5′-TAAGAGTCTTAAAGACACATCAGGG-3′), and cloned into pBSKII$^-$ (Stratagene). This construct was linearized at an EcoRI restriction site and used as template for R/G competitor RNA synthesis. 5-HT$_{2C}$R competitor RNA was transcribed from a 288-bp genomic DNA fragment as previously described (14). The sense and antisense strands of the nonspecific dsRNA substrate were synthesized separately from a fragment of the rat α_2-adrenergic receptor and the single-stranded RNAs were annealed as described (31) to generate the nonspecific duplex substrate.

Rat ADAR2 Expression and Purification—The isolation and expression of recombinant FLAG-rADAR2b was achieved using the Multi-Copy *Pichia* expression kit (Invitrogen). The FLAG-rADAR2b cDNA (16) was subcloned into the *Pichia pastoris* pPIC3.5K vector, introduced into the GS115 yeast strain by electroporation and selected in 3 mg/ml neomycin; yeast cell lysates were prepared at 4 °C per manufacturer's guidelines. Briefly, cell pellets from 1-liter cultures were resuspended in 100 ml breaking buffer (50 mM $NaPO_4$, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol), homogenized with glass beads by 20 cycles of vortexing for 30 s and clarified by high speed centrifugation. The supernatant was then exchanged into standard purification buffer (20 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 mM DTT + 10% glycerol). Recombinant FLAG-rADAR2b protein was purified from 100 ml of supernatant using a protocol modified from O'Connell *et al.* (32) for the purification of human ADAR2 from HeLa cell nuclear extracts. Recombinant proteins were first resolved by SP-Sepharose cation exchange followed by Source Q anion exchange fast-performance liquid chromatography (Amersham Biosciences). For the 25-ml SP-Sepharose column, proteins were eluted in 240 ml of purification buffer over a gradient of 100–500 mM NaCl. Peak fractions were then resolved on a 1-ml Source Q column over a gradient of 50–500 mM NaCl in a volume of 35 ml of buffer. FLAG-rADAR2b peak fractions, as assessed by Western blotting analysis, eluted between 280 and 350 mM NaCl. Finally, the recombinant protein was purified by Poly(I)*-Poly(C)* dsRNA affinity chromatography as described (32) and dialyzed into standard purification buffer containing 0.05% Igepal and 50% glycerol.

The peak fraction of FLAG-rADAR2b was purified to near homogeneity according to silver staining and Western blotting analysis using the M2 monoclonal anti-FLAG antibody (Sigma). The peak fraction of FLAG-rADAR2b was serially diluted in the presence of 100 μg/ml bovine serum albumin (BSA) and resolved by SDS-PAGE alongside BSA standards of known concentration. The protein concentration was then quantified on a Molecular Dynamics PhosphorImager by comparing the density of the ADAR2 band to the BSA standard curve.

In Vitro Editing/Deamination assays—Editing assays were performed under single turnover conditions at 37 °C with 7.5 nM FLAG-rADAR2b protein and 0.3 nM *in vitro* transcribed RNA as substrate in a buffer containing 25 mM Tris, pH 7.8, 100 mM NaCl, 1 mM dithiothreitol, 6% glycerol, 100 μg/ml RNase-free BSA, 0.003% Igepal, and 0.8

units of RNasin/μl. Reactions were stopped by addition of Proteinase K, SDS and EDTA as described previously (33). To analyze the editing of ADAR2 at site −1, first-strand cDNA was synthesized with an oligonucleotide primer complementary to the unique 3′-extension of the ADAR2 RNA substrate that was not present in competitor RNAs. First-strand cDNA was amplified by PCR and the extent of editing was quantified by primer-extension analysis as described (16). Apparent K_m values were measured for ADAR2, 5-HT_{2C}R and R/G duplex substrates by assessing the A-to-I conversion at 5 min over a concentration range of 0–316 nM RNA by primer-extension analysis. Primer extension of 5-HT_{2C}R transcripts was performed as previously reported (14). For the R/G site, extension proceeded using an antisense primer (5′-GTTATACTATTCCACCC-3′) in the presence of 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dGTP, and 5 mM ddTTP. For competition studies, the editing of 0.3 nM ADAR2 (site −1) was assessed in the presence of competitor at concentrations ranging from 10^{-13} M to 10^{-5} M RNA.

RESULTS

Predicted Secondary Structure for ADAR2 pre-mRNA—We demonstrated previously that intronic sequences critical for ADAR2 pre-mRNA editing (site −1) were contained between −1668 and −1154, relative to the proximal 3′-splice site (16). Further analysis of ADAR2 pre-mRNA sequences using RNA-folding algorithms (26–28) identified an imperfect inverted repeat that formed a putative RNA duplex as a result of base-pairing interactions between a portion of this critical upstream region and the 3′-end of the intron (Fig. 1). Comparisons of ADAR2 genomic sequences between rat, mouse, human, and pufferfish revealed >90% intronic sequence conservation in the predicted region of the inverted repeats (Fig. 1), with a majority of the nucleotide differences clustered in predicted bulge regions within the duplex. Furthermore, 100% sequence identity was maintained in both halves of the inverted repeat for a region extending 18-nt upstream and 16-nt downstream from the edited splice junction (site −1) (Fig. 1), suggesting that the structure of this region is conserved to maintain ADAR2 autoediting among multiple vertebrate species (23).

Editing Sites within ADAR2 pre-mRNA Transcripts—In addition to the ADAR2 editing event responsible for generating a proximal 3′-splice site within intron 4 (site −1), four additional A-to-I modifications were observed previously at positions −2, +10, +23 and +24 (relative to the proximal 3′-splice site) (16). To further examine editing events within the predicted rADAR2 duplex, sequence comparisons of rat ADAR2 genomic DNA and adult rat brain cDNA clones, generated from RT-PCR amplification of ADAR2 pre-mRNA, were performed to identify additional A-to-G discrepancies. In total, 16 RNA editing sites were identified (Fig. 1) and the frequency of editing at each position was quantified (Table I); nucleotide discrepancies that were observed in less than 5% of the isolated cDNA clones were not considered for subsequent analysis. In addition to the five editing sites previously identified within the ADAR2 duplex (16), three additional sites were observed in the 3′-portion of the inverted repeat (positions −4, −27, and −28) and eight sites in the 5′-half of the duplex. The frequency of editing at these 16 sites varied dramatically from a low of ~6% at positions −1428, −28, and −27 to greater than 75% at sites −1500, −1476, and +24 (Table I). Analysis of 100 independent cDNA clones isolated from rat brain also revealed a complex and varied combination of editing events within single pre-mRNA transcripts raising the possibility of an ordered pattern of adenosine deamination within ADAR2 pre-mRNAs. To address whether editing at specific sites was statistically linked to A-to-I conversion at other positions, the coincidence of editing between any two sites was assessed using the χ^2-test of independence (Table I). Results from this analysis suggested that editing at the proximal 3′-splice acceptor (site −1) was independent of editing at all other sites within the duplex, with the exception of its immediate neighbor (site −2). A statistically significant coincidence of editing was also observed for six other site pairs (+10/+23, +24−1476, +24/−1500, −1476/−1500, −1476/−1505, −1500/−1505), suggesting a hierarchical pattern of editing within the duplex that could result from intrinsic ADAR site-selectivity or as a consequence of the "unwinding" activity of ADARs (34, 35) on local duplex structure.

Numerous *in vitro* studies using biologically relevant or model ADAR substrates have demonstrated that ADAR1 and ADAR2 have overlapping specificity (6, 7, 14, 36). While the use of such *in vitro* or transfected tissue culture model systems cannot completely define which ADAR protein(s) are responsible for editing a specific adenosine moiety *in vivo*, analysis of altered editing patterns in ADAR2-null mice (37) were generally consistent with previous *in vitro* analyses of ADAR2 specificity. To determine the ability of ADAR1 and ADAR2 to modify each of the 16 editing sites identified within ADAR2 pre-mRNA transcripts isolated from rat brain, a 3360-bp ADAR2 minigene (Fig. 2*A*) was transiently co-transfected with either rADAR1a or rADAR2b cDNAs in HEK293 cells and quantitative analysis of editing frequency at each position was assessed by a combination of primer extension and/or sequence analysis of 100 independent cDNA clones from each transfection (Table I). In the absence of exogenous ADAR1 or ADAR2, a minimal level of editing was seen at all positions (data not shown), consistent with previous observations that HEK293 cells contain a low level of endogenous editing activity (6, 14, 16, 38). While ADAR1 and ADAR2 exhibited overlapping patterns of specificity at multiple sites (−1511, −1505, −1504, −1500, and +24) editing frequencies at positions −1484, −1476, −1468, −1428, −1, +10 and +23 indicated a more exclusive pattern of ADAR specificity in which these adenosine moieties were preferentially edited by ADAR2 (Table I). In contrast, ADAR1-mediated editing was preferred at sites −2 and −4 even though quantitative Western blotting of transfected HEK293 cells revealed that the level of ADAR1 protein expression was only 75% of that demonstrated by ADAR2 (data not shown).

Nucleotide Bias and Preferences in Sequences Surrounding ADAR2 Editing Sites—To further examine ADAR2 specificity, we took advantage of an *in vitro* editing system using purified, recombinant epitope (FLAG)-tagged rADAR2b and a 704-nt transcript derived from the ADAR2 pre-mRNA substrate (Fig. 2*A*). The *in vitro* editing activity for site −1, using recombinant FLAG-rADAR2b, was assessed under single turnover conditions by primer-extension analysis (16), revealing that the initial kinetic rate was linear from 0–10 min and reached a maximum by 20 min (Fig. 2*B*). Using similar conditions, a total of 14 adenosines within the ADAR2 pre-mRNA substrate were shown to be modified at levels greater than 5% when reactions were allowed to proceed to completion (Table II). The rank-order of preferred ADAR2 sites using the *in vitro* system was similar to that observed in transfected cells (Table I), suggesting that the identified sites are modified by ADAR2 *in vivo*.

The large number of potential ADAR2 sites in this substrate provided a unique opportunity to assess the distribution of specific nucleotides surrounding ADAR2-modified adenosine residues as compared with non-edited adenosine moieties within the same duplex. Forty nucleotides flanking each edited adenosine were manually aligned and the nucleotide distribution, purine/pyrimidine ratio and GC content at each position were determined. This data was then compared with the nucleotide distribution observed at equivalent positions for all 50 non-edited adenosines in the predicted region of the ADAR2 duplex (Fig. 1 and Table II). Results from this analysis revealed a statistically significant ($p < 0.05$) nucleotide bias at eight positions in the surrounding sequence in which nucleotide

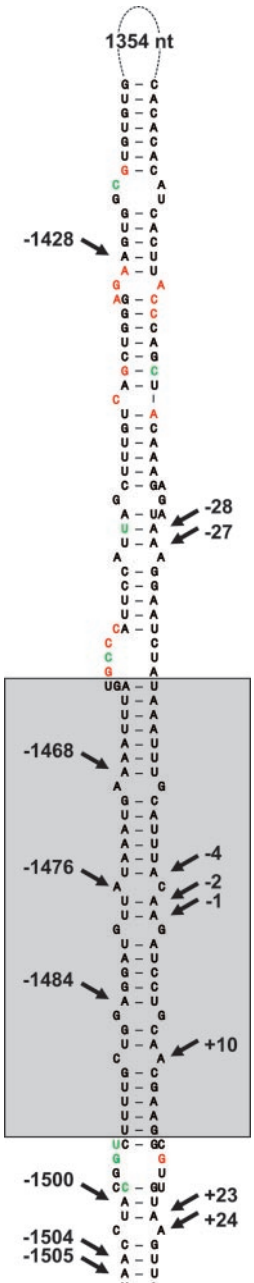


FIG. 1. **Predicted secondary structure and evolutionary conservation of ADAR2 pre-mRNA sequence in the region of major A-to-I editing modifications.** The nucleotide sequence and predicted RNA secondary structure for the rat ADAR2 pre-mRNA is presented showing the positions of 16 editing sites identified in ADAR2 pre-mRNA transcripts isolated from adult rat brain; coordinates of the editing sites are relative to the proximal 3′-splice junction in intron 4 (16) and the number of nucleotides omitted from the figure is indicated in the loop. Comparisons of evolutionary sequence conservation between human, rat, mouse and two pufferfish *ADAR2* genes are presented in which the percentage of species demonstrating 75–100% (*black*), 25–50% (*green*), and 0% (*red*) sequence identity to the rat *ADAR2* gene are indicated by *colored lettering*. The *shaded box* denotes a region of the predicted RNA duplex where 100% sequence identity has been maintained in all five vertebrate *ADAR2* genes.

TABLE I
Editing frequency in rADAR2 pre-mRNA

Site	Editing Frequency (%)		
	Rat Brain	HEK293	
		ADAR1	ADAR2
-1511	29.6	7.7	6.0
* -1505	51.0	17.3	14.0
* -1504	38.1	9.6	10.0
* -1500	76.8	21.2	33.3
* -1484	40.4	7.7	41.8
* -1476	85.0	26.9	70.6
-1468	16.2	3.9	32.7
-1428	6.2	0.0	11.5
-28	6.0	*nd*	*nd*
-27	6.3	*nd*	*nd*
-4	10.3	2.2	0.2
-2	22.9	8.0	1.8
-1	47.0	7.7	37.5
+10	42.3	11.0	38.0
+23	35.1	7.0	22.0
+24	83.8	37.0	47.0

* Brackets indicate sites in which editing events were linked according to a χ^2-test of independence. nd, not determined.

identity at positions −18 (C = U>G), +10 (A = U>G = C) and +15 (G>U>A = C) correlated with the frequency of editing observed *in vitro* (Table II). The remaining five positions did not correlate with *in vitro* editing frequency, but included the 5′-nearest neighbor preference (U = A>G) previously described for ADAR2 using artificial substrates (7) as well as biases at positions −7 (G>U>A>C), +7 (U = C>G = A) and a bias against GC content at positions +9 and +13. These results suggest that in addition to secondary structural requirements, a "consensus" sequence surrounding ADAR2-modified residues may also be an important contributor to ADAR2 preference and editing efficiency.

Mutational Analysis of ADAR2 Secondary Structure—To assess the validity of the predicted RNA secondary structure determined using RNA folding algorithms (27, 28), a series of mutants were generated using an *ADAR2* minigene extending from exon 4 through exon 5 (two-exon *ADAR2* minigene, 3606 bp; Fig. 2*A*). Mutant minigene constructs were transiently cotransfected with the rADAR2b cDNA in HEK293 cells and the extent of editing at sites −1, −1476, and −1428 was quantified by primer extension analysis. Substitutions of 3–5 nucleotides in length were generated to introduce unique restriction sites that would disrupt predicted regions of base-pairing within the extended RNA duplex (Fig. 3*A*, *mutation 1*). A second series of mutations, designed to restore predicted base-pairing interactions, were also generated by introduction of compensatory mutations in the downstream portion of the inverted repeat (Fig. 3*A*, *mutation 2*). Introduction of a HpaI restriction site (HpaI (a)) reduced editing by 85% at site −1, yet editing was not significantly restored by introduction of the compensatory mutation (Fig. 3*B*). These results suggested that either the predicted secondary structure for this region of the ADAR2 pre-mRNA was incorrect, the identity of specific nucleotides in the region of the mutation were critical for efficient adenosine deamination, or the reduced GC content in the double mutant affected the structural stability of the modified region. To address these hypotheses, a second HpaI-based mutation (HpaI (b)) was generated to maintain the GC content of the duplex while equally disrupting the predicted RNA secondary structure. Introduction of the HpaI (b) mutation decreased editing at site −1 by >75%, yet the compensatory mutation restored editing to wild-type levels (Fig. 3*B*), thereby validating the predicted structure of ADAR2 pre-mRNA in the region of the proximal 3′-splice site.

To further examine the structure of the ADAR2 duplex, a

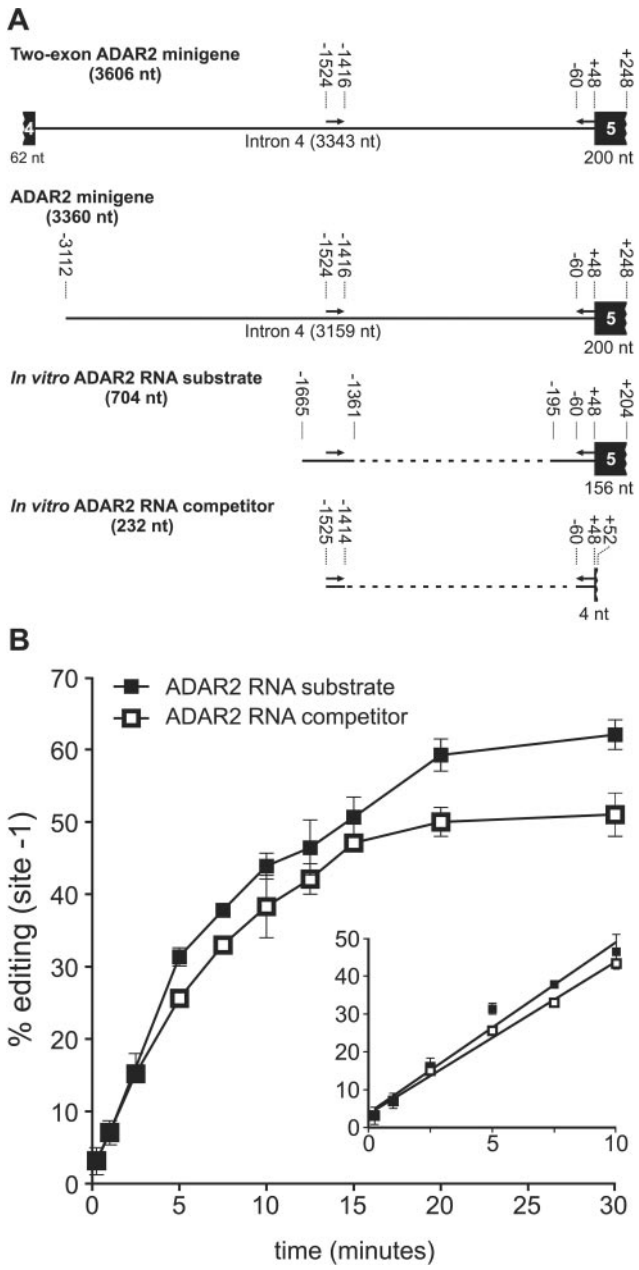


FIG. 2. ***In vitro* time course analysis of ADAR2 editing.** *A*, schematic diagram is presented indicating the structure of ADAR2 genomic fragments (3606- and 3360-nt minigenes) used for transfection studies and corresponding 704 and 232 nt transcripts used for *in vitro* editing analysis; the position of exon and intron sequences are indicated by *closed boxes* and *solid lines*, respectively, while *dashed lines* indicate sequence information deleted from the *in vitro* transcripts. The position of the imperfect inverted repeat, predicted to form an extended RNA duplex (see Fig. 1), is designated with *arrows*; the specific coordinates of the inverted repeat and sequences included in the *in vitro* substrates are indicated relative to the proximal 3′-splice site. *B*, time course of *in vitro* editing (site −1) for ADAR2 substrates using recombinant FLAG-rADAR2b under single turnover conditions (ADAR2 RNA = 0.3 nM, FLAG-rADAR2b protein = 7.5 nM). The *inset* represents linear regression analysis of initial rates determined between 0 and 10 min.

second restriction site-based mutation (EcoRV) and its corresponding compensatory mutation were introduced upstream from site −1 (Fig. 3*A*). Primer-extension analysis revealed that editing at site −1 was decreased to an intermediate level (43 ± 1%) with the single EcoRV mutation (mutation 1), whereas editing was restored to control levels by inclusion of the compensatory mutation (mutation 1 + mutation 2; Fig. 3*B*). Previous analyses of GluR-6 editing (Q/R site) have indicated that the extent to which editing is decreased in response to duplex disruption correlates with the distance of the mutation from the editing site (18). Mutational analysis of the predicted ADAR2 duplex is consistent with these observations, as introduction of a proximal HpaI site reduced editing to a greater extent than a more distant EcoRV mutation.

To examine the predicted structure of the ADAR2 duplex in a region further from the proximal 3′-splice acceptor, an AatII restriction site was introduced near editing site −1428 (Fig. 3*A*). An internal loop is predicted to separate the introduced mutation from site −1 (Figs. 1 and 3*A*) and such large bulges have been proposed to uncouple editing between adjacent regions in duplex RNA structures (22). In the mutant ADAR2 substrate, editing at site −1 was unaffected by introduction of the single AatII site (Fig. 3*B*), yet editing at a nearby editing site (−1428) was reduced by 75%. Introduction of the compensatory AatII mutation had no effect on editing at site −1, while the editing efficiency at −1428 was fully restored to wild-type levels in the double mutant. Conversely, editing at site −1428 was unaffected by introduction of the HpaI or EcoRV mutations on the distal side of the predicted duplex bulge (Fig. 3*B*). The apparent independence of these mutational perturbations is consistent with a model in which the ADAR2 duplex contains at least two separable domains of ADAR2 binding/catalysis and supports previous studies using artificial duplex substrates where large bulges have been shown to uncouple editing between adjacent regions in an extended duplex (22).

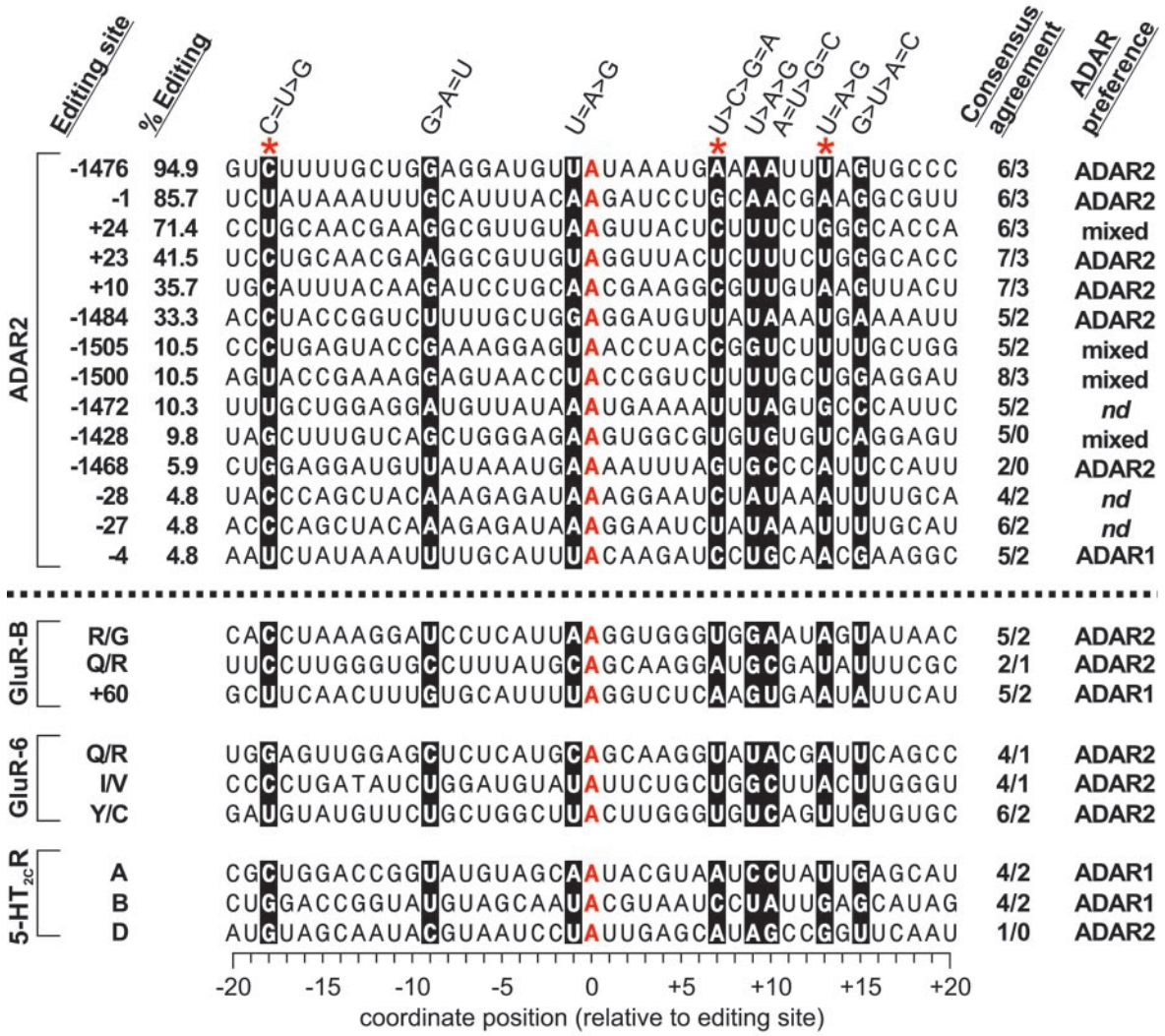
Contribution of Sequence/Structure to ADAR2 Site Recognition and Editing Efficiency—Since the binding of DRBM-containing proteins, such as ADAR1 and ADAR2, is thought to be independent of primary RNA sequence (8–11,39), the molecular mechanism(s) by which targeted *versus* non-targeted adenosine residues are selected for deamination are largely unknown (21, 22). Recent studies using scanning force microscopy have revealed that while ADAR2 can bind and edit any double-stranded RNA, the binding to selectively edited sites occurs more frequently than to regions demonstrating nonspecific or "promiscuous" editing (40), indicating that subtle differences in the structure of duplex RNAs may affect ADAR binding and subsequent catalytic deamination. To test whether the unique structural character of ADAR substrates can influence their ability to compete for editing, the editing of ADAR2 RNA (site −1) was measured *in vitro* in the presence of several competitor RNAs possessing vastly different sequence and structural characteristics; competing RNA transcripts included a perfect, artificial RNA duplex derived from a portion of the α_2-adrenergic receptor (D79N), wild-type and mutant ADAR2 duplexes and dsRNA structures derived from pre-mRNAs encoding the R/G site of GluR-B (17) and the 2C-subtype of serotonin receptor (14).

To distinguish between the *in vitro* transcribed ADAR2 substrate and ADAR2-derived RNA competitors, a 232 nt competitor substrate was developed in which the 1.3-kilobase region between the 5′- and 3′-halves of the inverted repeat was replaced by an 8-nt loop formed by the introduction of a NotI restriction site (Fig. 2*A*). The *in vitro* editing activity (site −1) for wild-type competitor RNA was assessed under single turnover conditions and shown to have an initial rate identical to the 704 nt ADAR2 RNA species (Fig. 2*B*). *In vitro* editing of ADAR2 RNA was measured in the presence of wild-type or mutant competitor RNA, and competition curves were generated across a range of concentrations from 10^{-13} M to 10^{-5} M RNA competitor (Fig. 4*A*). A concentration-dependent decrease in editing at site −1 was observed for the 704 nt ADAR2 substrate when using the 232-nt competitor (K_i = 0.65 nM), whereas introduction of an EcoRV restriction site (Fig. 3, *mu-*

TABLE II

Analysis of nucleotide bias surrounding ADAR2 editing sites

Forty nucleotides flanking each adenosine residue edited by ADAR2 (indicated in red) were manually aligned, and the nucleotide distribution, purine/pyrimidine ratio and GC content at each position were compared by a χ^2 test of independence to the nucleotide distribution surrounding all 50 non-edited adenosines in the predicted region of the ADAR2 duplex (Fig. 1). The coordinate of each editing site within ADAR2 pre-mRNA was defined based upon its position relative to the proximal 3′-splice junction; the efficiency of editing upon completion of an *in vitro* reaction (3 h) is indicated. Nucleotide positions at which the χ^2 test indicated a statistically significant difference ($p < 0.05$) are presented in inverse lettering along with the corresponding nucleotide preference. Positions at which the nucleotide bias correlated with increased editing efficiency are indicated with a red asterisk. The nucleotide sequences surrounding editing sites within other substrates (GluR-B, GluR-6, and 5-HT$_{2c}$R) are also presented. Consensus agreements represent the number of positions matching the 8 nucleotide consensus and the number of nucleotides matching the three positions (−18, +10, and +15) correlating with increased editing efficiency. ADAR preferences were defined based upon Refs. 6, 14, 37, 49, and 62; *nd*, not determined.



tation 1) into the 232 nt competitor transcript reduced the potency of the mutant RNA to compete for editing by 27-fold. Introduction of a compensatory EcoRV mutation (Fig. 3, *mutation 2*), which is predicted to restore the secondary structure of the competing RNA transcript, completely restored the potency of the mutant RNA to inhibit editing at the −1 position. These observations indicate that competition for RNA editing reflects the recognition of specific RNA duplex features, since even subtle changes in the predicted structure can dramatically affect the potency of the competitor RNA.

Other RNA substrates of varying length and structure were also analyzed to determine if they would demonstrate discrete competitor profiles. Prior to these analyses however, the *in vitro* editing of each RNA substrate was first characterized (Fig. 4*B*). Thin layer chromatographic analyses revealed that the D79N substrate was non-selectively deaminated at ~50% of adenosine residues by purified FLAG-rADAR2b *in vitro* (data not shown). Saturation analysis of ADAR2-mediated editing for the 704 nt ADAR2-derived substrate (−1 site; K_m = 4.4 nM), a 60-nt duplex derived from the R/G site of GluR-B pre-mRNA (R/G site; K_m = 41 nM) and a 288-nt duplex derived from the 5-HT$_{2C}$R precursor RNA transcript (D-site; 5-HT$_{2C}$R K_m = 25 nM) revealed that all of these substrates were effectively edited by ADAR2 *in vitro* (Fig. 4*B*). *In vitro* analyses designed to assess the ability of these ADAR2 substrates to compete for ADAR2 (site −1) editing revealed that each duplex RNA demonstrated a concentration-dependent inhibition of ADAR2 editing (Fig. 4*A*). As expected, the nonspecific, perfect RNA duplex (D79N) acted as a relatively strong competitor for editing at site −1 (K_i = 2.6 nM), albeit to a lesser degree than ADAR2 RNA itself. Competition by the GluR-B derived RNA was shifted rightward 15-fold (K_i = 9.5 nM), while the 5-HT$_{2C}$R

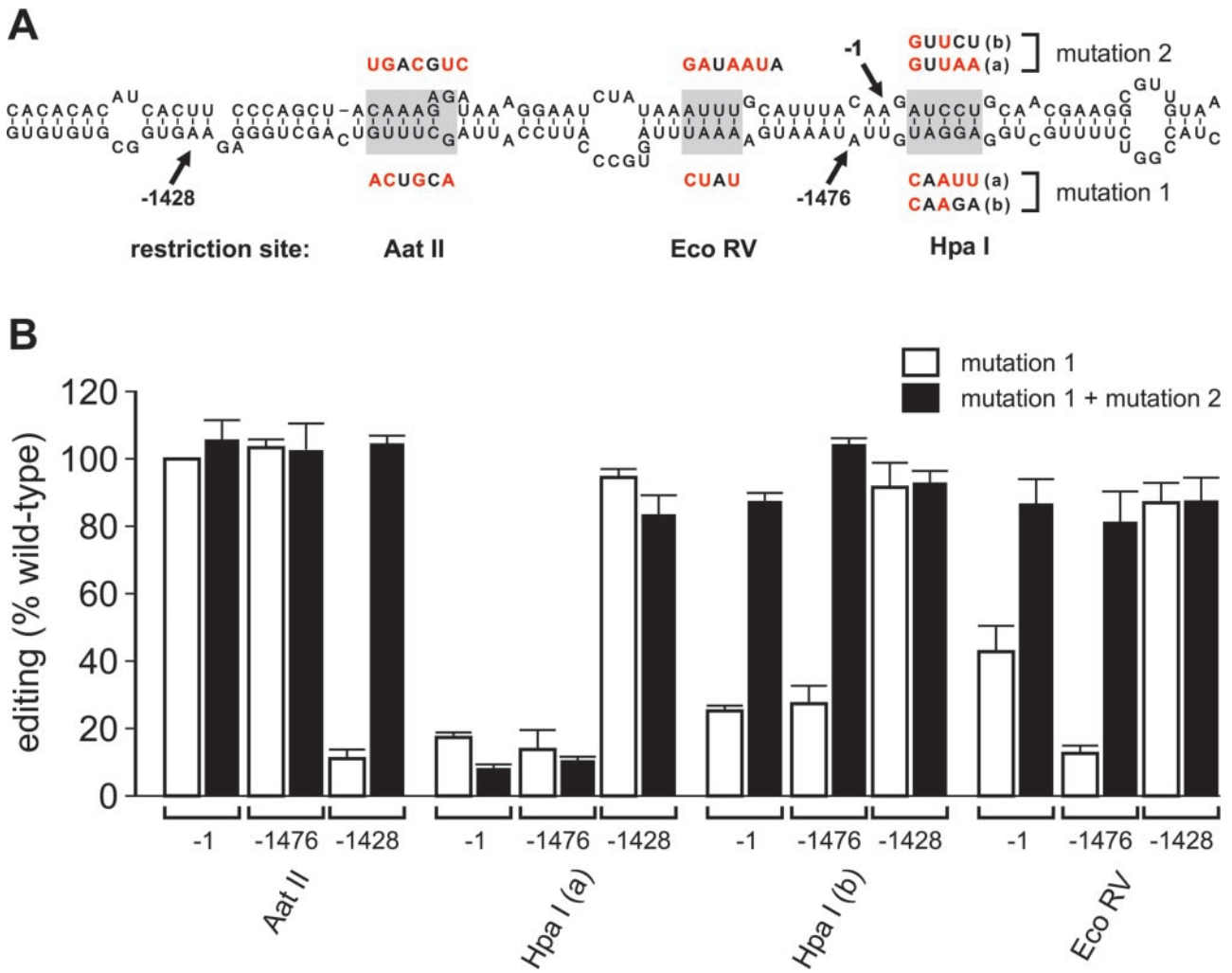


FIG. 3. **Analysis of ADAR2 editing (site −1) using two-exon minigene mutants.** *A*, schematic diagram is presented indicating the predicted secondary structure for a region of the rat ADAR2 pre-mRNA surrounding A-to-I editing events at sites −1, −1428, and −1476. Predicted duplex regions (*gray boxes*) in which restriction sites were introduced to disrupt potential secondary structure (*mutation 1*) and compensatory mutations predicted to restore base-pairing interactions (*mutation 2*) are indicated with the altered nucleotides shown in *red*. *B*, quantitative analysis of ADAR2 editing (site −1) is presented from HEK293 cells transiently co-transfected with FLAG-rADAR2b and either wild-type or mutant *ADAR2* minigenes (mean ± S.D., $n = 3$); all values are normalized to the percentage of editing observed for the wild-type minigene at positions −1 (67.0 ± 9.9%), −1428 (28.7 ± 5.1%), and −1476 (62.0 ± 4.2%).

RNA was the least effective competitor (K_i = 30.2 nM). These differences did not appear to reflect the overall length or predicted free-energy of the RNA duplex, the number of editing sites in the RNA or the individual K_m^{app} values determined for the Q/R, R/G, and D sites within these RNA substrates as determined by kinetic analysis (Fig. 4*A*); instead, these differences in competitor potency appeared to correlate with how well the competitor RNAs matched the consensus sequence observed for ADAR2 sites as indicated in Table II.

While the 5′ and 3′ nearest neighbor positions of the GluR-B R/G and the 5-HT_{2C}R D-sites agreed with previously determined nucleotide biases for ADAR2 (7), the two RNA transcripts differed significantly in their match to the other nucleotide preferences observed among ADAR2 sites (Table II). Among the seven additional nucleotide positions surrounding ADAR2 sites, the R/G duplex conformed to the putative nucleotide preferences at 4 of these positions, including two of three sites where the nucleotide bias correlated with *in vitro* editing efficiency (*red asterisks* in Table II). By contrast, the sequence surrounding the D site of 5-HT_{2C}R was a poor match for the consensus at all remaining positions and contained disfavored guanosine residues at positions −18 and +10 and a less preferred uridine at position +15. To examine whether mutation of these positions to the preferred nucleotide bias would increase the efficiency of editing in a modified 5-HT_{2C}R transcript (m5-HT_{2C}R) and increase subsequent potency in competition analyses, the disfavored nucleotides were changed to C, A, and G at positions −18, +10 and +15, respectively, and compensatory changes in the complementary strand were introduced to maintain the predicted secondary structure of the 5-HT_{2C}R duplex (Fig. 5*A*). The extent and rate of editing at the D-site were significantly increased for the m5-HT_{2C}R substrate (Fig. 5*B*) and competition analysis (Fig. 5*C*) revealed a 6-fold increase in the potency to compete for ADAR2 (site −1) editing (K_i = 4.7 nM), suggesting that the primary sequence surrounding ADAR2 sites contributes directly to editing preference and efficiency. Analysis of other ADAR2 substrates revealed a varying degree of conformity to the eight putative nucleotide preferences (Table II). The Y/C site of GluR-6, implicated as an ADAR2-specific site by editing analysis in ADAR2-null mice, matches the ADAR2 consensus at six of eight positions, while the ADAR2-specific Q/R site of GluR-B agrees solely at positions −18 and +13. The identified consensus sequence also matched well with the predominantly ADAR1-specific +60 site of GluR-B and A site of 5-HT_{2C}R, suggesting that the observed nucleotide biases may apply to both ADAR1 and ADAR2 site specificity (Table II).

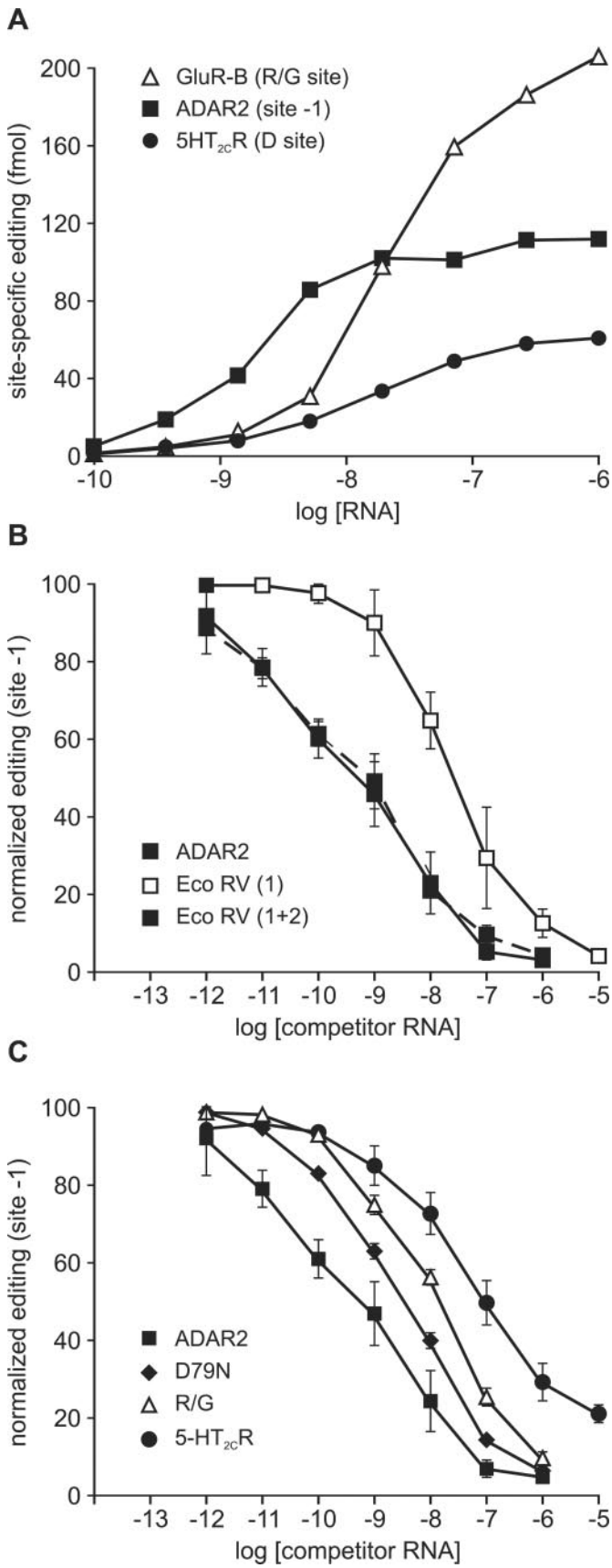


FIG. 4. **Competition analysis of *in vitro* ADAR2 (site −1) editing.** *A*, kinetic analysis of site-specific editing for *ADAR* minigene substrates derived from pre-mRNA transcripts encoding ADAR2, the GluR-B subunit of the AMPA-subtype of glutamate receptor (R/G) and the 2C-subtype of serotonin receptor (5-HT_{2C}R) is presented (FLAG-rADAR2b protein = 7.5 nM; incubation time = 5 min). *B*, ability of wild-type and mutant ADAR2 transcripts (232 nt) to compete for the *in vitro* editing of an ADAR2 transcript (704 nt; see Fig. 2) at site −1 was assessed under conditions of single enzyme turnover (ADAR2 RNA, 0.3 nM; FLAG-rADAR2b protein, 7.5 nM; incubation time, 5 min). The efficiency of editing was normalized to the extent of editing observed for the wild-type ADAR2 transcript (704 nt) in the absence of competitor RNA (mean ± S.D., $n = 3$). *C*, ability of *ADAR* minigene substrates derived from the GluR-B subunit (R/G), an artificial RNA duplex derived from the α_2-adrenergic receptor (D79N) or the 5-HT_{2C} receptor (5-HT_{2C}R) to compete for ADAR2 editing (site −1) was assessed under conditions of single enzyme turnover. The efficiency of editing was normalized to the extent of editing observed for the wild-type ADAR2 transcript (704 nt) in the absence of competitor RNA (mean ± S.D.; $n = 3$).

DISCUSSION

While the occurrence of A-to-I editing events within introns has been identified in numerous pre-mRNA transcripts (2, 12), the autoediting of ADAR2 pre-mRNA represents one of only two known examples in which editing can affect subsequent splicing patterns (16, 41). As with most identified RNA substrates that undergo A-to-I modification, the autoediting of ADAR2 requires an extended RNA duplex, which is formed via base-pairing interactions between exon and intron regions (Fig. 1) (16, 23). Unlike secondary structures formed by inverted repeat elements that are in close proximity to one another for pre-mRNAs encoding the 5-HT_{2C}R, AMPA receptor subunits, and the hepatitis delta virus (HDV) antigenome (13), the predicted RNA duplex within ADAR2 is formed by inverted repeat elements that are 1354 nucleotides apart. This observation is similar to the duplexes predicted for pre-mRNA transcripts encoding the GluR-5 and GluR-6 subunits of the kainate-subtype of glutamate receptor in which the two halves of the duplex are separated by 1713 and 1817 nucleotides, respectively (18).

Previous studies by Slavov and Gardiner (23) have demonstrated evolutionary sequence conservation for *ADAR2* genes in the region of the predicted duplex and that alternative splicing, resulting in the inclusion of a 47-nucleotide cassette via ADAR2 autoediting, occurs in multiple vertebrate species; however, no evidence was provided to confirm the validity of the proposed pre-mRNA structures or whether their perturbation could affect ADAR2 autoediting. In the present studies, the predicted RNA secondary structure of ADAR2 pre-mRNA is strongly supported by both sequence conservation (Fig. 1) and mutational analysis (Fig. 3). Mutations that disrupted base-pairing interactions within the predicted duplex (Fig. 3; *mutation 1*) significantly diminished the editing of adenosine residues near the introduced mutation, whereas the inclusion of compensatory mutations that restored the predicted dsRNA structure (Fig. 3; *mutation 2*) also restored site-specific editing to wild-type levels. It is interesting to note that sequences between −18 and +15, relative to the proximal 3′-splice site (site −1), define the outer boundaries of a duplex region in which 100% sequence identity is maintained across all vertebrate species examined. Given that ADAR2 exhibited an *in vitro* preference for editing sites surrounded by specific residues at positions −18, +10 and +15 (Table II), it is likely that the increased evolutionary conservation in this region may serve to maintain efficient editing at the proximal 3′-splice junction.

In addition to the A-to-I modification that generates the 3′-splice acceptor site, fifteen additional editing sites were identified in ADAR2 pre-mRNA transcripts isolated from adult rat brain (Table I). The identification of multiple editing events within a single duplex has been described previously for numerous pre-mRNA transcripts including GluR-B and 5-HT_{2C}R, as well as non-coding regions from mRNAs isolated from *Cae-*

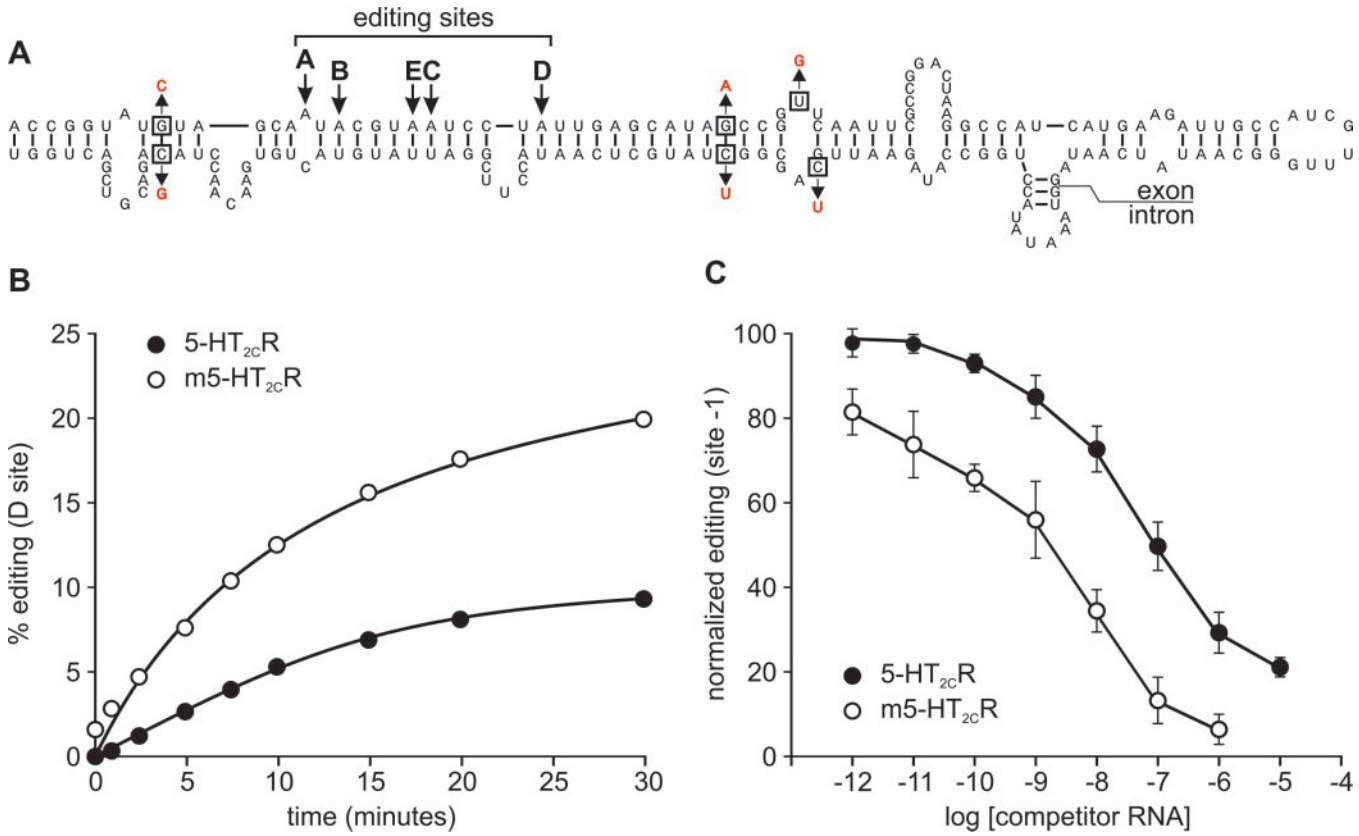


FIG. 5. **Competition analysis of wild-type and mutant 5-HT$_{2C}$R transcripts.** *A*, nucleotide sequence and predicted RNA secondary structure for a portion of the rat 5-HT$_{2C}$R pre-mRNA is presented showing the positions of five previously identified editing sites (*A–E*) and the location of the exon/intron boundary. The nucleotide residues surrounding the D-site at positions −18, +10, and +15 are highlighted with *squares* and were mutated to match the putative ADAR2 consensus sequence (Table II) indicated with *red lettering*. Nucleotides on the opposite side of the predicted duplex were also altered to maintain the predicted RNA secondary structure. *B*, time course analysis of D-site-specific editing for wild-type and mutant 5-HT$_{2C}$R transcripts; (RNA, 0.3 nM; FLAG-rADAR2b protein, 7.5 nM). *C*, ability of wild-type and mutant 5-HT$_{2C}$R transcripts to compete for the *in vitro* editing of an ADAR2 transcript at site −1 was assessed under conditions of single enzyme turnover (as in Fig. 4). The efficiency of editing was normalized to the extent of editing observed for the wild-type ADAR2 transcript (704 nt) in the absence of competitor RNA (mean ± S.D., $n = 3$).

norhabditis elegans and human brain (14, 15, 31, 42, 43). While the physiological relevance of editing at the 11 sites in ADAR2 intron 4 has not yet been determined, deamination at these sites could function to promote ADAR2 splicing by destabilizing the intron 4:exon 5 duplex that would be predicted to occlude the splicing of exon 4 to exon 5 (44, 45). Co-transfection of ADAR1 or ADAR2 cDNAs with an *ADAR2* minigene demonstrated overlapping specificity for these enzymes at multiple sites, yet sites −1 and −2 were preferentially modified by ADAR2 and ADAR1, respectively (Table I). Since conversion of an adenosine-adenosine (AA) to an inosine-inosine (II) dinucleotide, via the combined actions of ADAR1 and ADAR2, would not be predicted to generate a functional 3′-splice acceptor, ADAR1 may also play a functional role in modulating ADAR2 splicing patterns.

The DRBMs of ADAR2 are similar to the domains that mediate dsRNA interactions in a large variety of proteins, including dsRNA-dependent protein kinase (PKR), *Drosophila* staufen and *Escherichia coli* RNase III (8). Although subtle determinants of ADAR specificity have been identified in the DRBMs (46), the primary determinants of ADAR site specificity are thought to reside in the characteristics of their dsRNA substrates (38, 47). Structural selectivity of ADARs for a particular adenosine in the duplex is thought to be imparted by the pattern of bulges and base-pairing regions in the dsRNA, combined with the overall thermodynamic stability of the RNA (20, 21, 46, 48). According to observations made using artificial substrates, internal loops of greater than six nucleotides define subdomains of ADAR action (22). Mutational analyses have indicated that a large internal loop within the predicted ADAR2 pre-mRNA structure (Fig. 1) would thereby define two independent regions of editing, since mutations decreased editing efficiency for adenosines sites that were in close proximity to the sites of nucleotide alteration, yet had no effect on sites that were predicted to lie on the opposite side of the internal loop (Fig. 3).

While gross characteristics of RNA secondary structure can influence the specificity and selectivity of ADAR action, it is still unclear how this family of dsRNA-binding deaminases target specific adenosine residues for subsequent modification. Binding of the DRBM appears to be completely independent of RNA sequence (8–10) as protein:RNA co-crystal analyses have revealed that this domain interacts with the sugar-phosphate backbone of RNA duplexes without directly contacting the functional groups of the bases that are buried deep within the narrow, major groove of A-form dsRNA (9). Recent studies by Lazinski and coworkers (49) have indicated that a chimeric protein containing the DRBMs of ADAR1 and the catalytic domain of ADAR2 maintained the substrate selectivity of ADAR2, suggesting that the deaminase domain contributes significantly to the selectivity of the enzyme. Furthermore, scanning force microscopy has revealed both productive and nonproductive binding of ADAR2 in which there was no obvious correlation between ADAR2 binding and the extent of subsequent deamination (40). These observations suggest that while the DRBMs of ADAR2 may interact with duplex RNA in

a sequence-independent fashion, other regions of the protein, such as the deaminase domain, rely upon sequence/structure-based information for directing the specificity and efficiency of adenosine deamination.

Support for sequence-dependent ADAR action is based not only upon the identification of 5′- and 3′-nearest neighbor preferences surrounding sites modified by ADAR1 and ADAR2 (7), but also upon mutational analyses surrounding editing sites that dramatically affect the extent of site-specific adenosine modification without affecting ADAR binding (19, 50). Other sequence motifs include a preference for cytosine as the opposing base (22, 38) as well as A+U-rich regions like that observed for the GluR-B R/G site (63% A+U) and site −1 of ADAR2 (69% A+U) (21). In the present study, we have taken advantage of the 14 adenosine residues within the ADAR2 pre-mRNA that are edited by ADAR2 *in vitro* and simply compared the primary sequence surrounding these sites with regions surrounding the 50 non-edited adenosines in the predicted duplex (Fig. 1), completely ignoring any contribution to ADAR2 specificity that may result from gross secondary structural features (*i.e.* bulges, mismatches) within the RNA substrate. Using this statistical approach, eight nucleotide positions were identified that exhibit a nucleotide bias not observed surrounding non-edited adenosine moieties (Table II). One of the eight positions identified in this analysis is the 5′-nearest neighbor preference (U = A>G) that is shared by both ADAR1 and ADAR2 (7). Furthermore, the sequences surrounding the ADAR1-specific GluR-B +60 site and 5-HT_{2C}R A-site also matched the consensus at several additional positions, suggesting that the observed sequence biases might also direct site selectivity for ADAR1.

Three of the eight positions (-18, +10, and +15) appeared to correlate with both editing efficiency using an *in vitro* system (Table II) as well as potency to compete for ADAR2 (site −1) autoediting (Fig. 4*C*), suggesting that the identity of specific nucleotide residues surrounding ADAR2 sites may be involved in directing deaminase specificity and efficiency. While our analysis of base distribution surrounding ADAR2 sites cannot readily distinguish between sites immediately surrounding the edited adenosine residue or positions on the opposite side of the duplex, mutational analysis of a 5-HT_{2C}R RNA substrate revealed that altering the identity of the nucleotides at positions −18, +10, and +15, as well as the corresponding nucleotides on the opposite side of the predicted duplex, not only increased the rate and extent of editing for this substrate *in vitro* (Fig. 5*B*), but also increased its potency to compete for ADAR2 autoediting (Fig. 5*C*). Although the GluR-B Q/R site is specifically and efficiently edited by ADAR2 (6, 36, 37), it does not correlate well with the sequence consensus observed for other ADAR2 sites (Table II). Previous studies however, have noted that the Q/R site lacks many of the sequence characteristics that typify other efficiently edited ADAR substrates, in that it contains a disfavored cytosine at the 5′ nearest neighbor position (7), the edited region is not AU-rich (15, 21), and is not positioned in the A-C mismatch context that is preferred by both ADAR1 and ADAR2 (49). Editing at this site may rely more heavily on the structural features of the duplex since the sequence is conserved between GluR-B and the non-edited GluR-A, C, and D subunits. Mutational analyses support this idea, since mutations that improve the sequence context maintain or enhance editing at the Q/R site (15, 38, 49) while structural changes are poorly tolerated (15, 31, 51).

Sequence analysis of ADAR C termini have revealed the presence of motifs similar to those found in the family of N6 DNA methyltransferases (52) in which specific amino acid residues have been shown to complex with dsDNA to make up the binding site for a deoxyadenosine moiety that is flipped out of the helix during catalysis (53, 54). A similar base-flipping strategy has been implicated for ADAR2 function in which the DRBM not only plays a role in the recognition of potential ADAR2 sites, but also an additional role in rendering the nucleotides around the targeted adenosine more conformationally flexible, thus lowering the activation energy for base flipping (19, 48). More recent studies have demonstrated that the identity of nucleotides surrounding the targeted adenosine moiety can affect the efficiency of editing, possibly by altering whether the targeted adenosine is stacked in the RNA helix (50), suggesting that local sequences surrounding the edited adenosine moiety can affect its ability to be flipped out of the helix during catalysis.

It was first proposed in 1997 that the regulation of A-to-I editing could occur by modulating the expression of distinct ADARs that differ in their specificity of RNA recognition (55). Alterations in A-to-I editing have been implicated in physiological and neuropsychiatric disorders including schizophrenia, Alzheimer's disease, Huntington's disease and depression with suicide (56–59) as well as death (37, 44, 60). While the consequences of alterations in editing are only beginning to be appreciated as more RNA substrates undergoing A-to-I modification are identified (42, 43, 61), little is known regarding the cellular mechanisms by which ADAR2 expression is regulated. If the ability of ADAR2 to edit its own pre-mRNA does function to modulate the levels of ADAR2 activity *in vivo* (16, 24), such a mechanism must be responsive cellular changes associated with alterations in the levels of potential ADAR substrates or the expression of competing ADAR isoforms (62, 63). In this study, both the sequence and structural determinants of editing specificity were predictive of the ability for natural substrates to compete with the autoediting of ADAR2 pre-mRNA *in vitro*. The competition profiles of ADAR2, R/G and 5-HT_{2C}R RNA substrates corresponded to their degree of compatibility with ADAR2 sequence preferences at three positions (Fig. 4*C*). Based on this data, we suggest that the complement of RNA substrates in the nucleus may influence the degree with which ADAR2 targets its own RNA, providing a highly adaptive means of modulating ADAR2 expression.

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