Steric antisense inhibition of AMPA receptor Q/R editing reveals tight coupling to intronic editing sites and splicing

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ABSTRACT

Adenosine-to-Inosine (A-to-I) RNA editing is a post-transcriptional mechanism, evolved to diversify the transcriptome in metazoa. In addition to wide-spread editing in non-coding regions protein recoding by RNA editing allows for fine tuning of protein function. Functional consequences are only known for some editing sites and the combinatorial effect between multiple sites (functional epistasis) is currently unclear. Similarly, the interplay between RNA editing and splicing, which impacts on post-transcriptional gene regulation, has not been resolved. Here, we describe a versatile antisense approach, which will aid resolving these open questions. We have developed and characterized morpholino oligos targeting the most efficiently edited site—the AMPA receptor GluA2 Q/R site. We show that inhibition of editing closely correlates with intronic editing efficiency, which is linked to splicing efficiency. In addition to providing a versatile tool our data underscore the unique efficiency of a physiologically pivotal editing site.

INTRODUCTION

Adenosine-to-Inosine (A-to-I) RNA editing, the post-transcriptional conversion of single nucleotides in pre-mRNA, is a unique mechanism for protein diversification particularly in the nervous system (1–3). This process requires RNA secondary structures within primary mRNA transcripts that are recognized by adenosine deaminases [adenosine deaminases acting on RNA (ADARs)], which catalyze the conversion of adenosine to inosine. During translation of edited coding sequences, inosine is recognized as guanosine thus resulting in a change to the RNA codon and often the protein sequence (4,5). ADARs are essential, their deletion results in premature death in vertebrates and to severe nervous system dysfunction in invertebrates (2). In addition to fine-tuning function of central signaling molecules RNA editing abundantly targets non-transcribed regions, particularly in vertebrates and thereby regulates RNA metabolism (1).

The first A-to-I site to be described was the Q/R site in AMPA-type glutamate receptors (AMPArs) (6): cation channels mediating the bulk of fast excitatory neurotransmission in vertebrate brains (7). This editing site locates to the channel pore where it determines ion flux and channel assembly (6,8). Edited varieties render the channel Ca\(^{2+}\) impermeable, and by disfavoring assembly of edited homotrameric GluA2 the formation of AMPAR heteromers is enabled. GluA2 Q/R editing is essential for survival, the site is edited to $\geq99\%$ (6). Reduced GluA2 Q/R editing in gene-targeted mice results in severe seizures and premature death, which is linked to altered Ca\(^{2+}\) permeability through AMPARs (9,10). The Q/R site is exclusively edited by the editase Adar2; deletion of the Adar2 locus resembles the severe phenotype of editing-deficient Gria2 alleles, and is rescued by expression of a Q/R-edited Gria2 allele (in the Adar2\(^{-/-}\) background) (11). Moreover, underediting of the Q/R site is associated with a variety of diseases in humans, such as epilepsy, ischemia and amyotoleral sclerosis (ALS) (2,12,13). ALS has been studied in some detail, where it appears that reduced expression of ADAR2 are linked to reduced Q/R editing resulting in motor neuron degeneration (13). Together, these findings highlight the unique nature of the GluA2 Q/R site, with efficient editing being pivotal to survival of the organism. The question of why this critical position ‘relies’ on editing, rather than being hardwired into the genome, remains a mystery (14).
High-throughput approaches increasingly demonstrate a prominent role for A-to-I editing in development, metabolism and in disease (15). Contrasting with the rapid pace of these technical developments is a good understanding of the biology of individual editing sites. Antisense probes provide unique versatility to interfere with RNA-based processes including splicing and translation, where this approach has provided key insights. In the context of editing, a steric block antisense oligonucleotide (oligo) could be designed to hybridize to the editing site complementary sequence (ECS) and/or double stranded RNA binding sites of ADARs. As the secondary structure is essential for Adar binding, strand invasion and hybridization of the antisense oligo would inhibit A-to-I editing (Figure 2). This substrate-targeted approach could permit site-specific manipulation of endogenous editing sites. A widely used antisense oligo for steric antisense applications is the morpholino oligo. Morpholinos have been used extensively to modify pre-mRNA splicing, block mRNA translation and inhibit miRNA maturation or activity (16,17). Importantly given the duplex structure of the Q/R editing substrate, potent antisense activity of morpholinos has been demonstrated on highly structured RNA targets (18–20).

Here, we first provide an in-depth characterization and evolutionary relationships of the GluA2 AMPAR Q/R editing substrate. We go on to describe experiments that provide a proof-of-principle for substrate-targeted competitive inhibition via antisense probes. We then use the antisense strategy to characterize the link between Q/R editing and intron 11 splicing and demonstrate the remarkable resilience of the GluA2 Q/R site. This approach will facilitate an in-depth characterization of individual metazoan A-to-I RNA editing sites.

MATERIALS AND METHODS

Bioinformatics

The imperfect inverted repeat containing the Q/R site was identified from the rat Gria2 gene sequence (exons 11–12) using the EINVERTED application of a locally installed European Molecular Biology Open Software Suite (EMBOSS) [Version 6.0.1; (21)] interfaced with Jemboss Graphical User Interface (GUI) [Version 1.5] (22). The imperfect inverted repeat of rat along with some flanking sequence was used as a Basic Local Alignment Search Tool (BLAST) or BLAST-Like Alignment Tool (BLAT) search query to indentify homologs from the online Ensembl and Pre-Ensembl vertebrate assemblies (23–25). Likewise, shark sequence was retrieved from the whole genome shotgun sequence database on the Institute of Molecular and Cell Biology server (26). The sequence coordinates and associated information are documented in Supplementary Table S1. Sequences were aligned using Multiple Alignment based on Fast Fourier Transform (MAFFT) by iterative refinement with pairwise alignment information [L-INS-i; Version 6.903; (27)]. Sequence similarity was calculated and data output from the PLOTCON application in EMBOSS using the EDNA scoring matrix and a window size of 1. The γ-centroid consensus structure was predicted from the alignment using CentroidAlifold with a default inference engine [Version 0.0.9; (28)]. The common structure was superimposed onto the rat GluA2 pre-mRNA sequence and visualized with Visualization Applet for RNA (VARNA) [Version 3.8; (29)]. The posteriors were used to plot the probabilities of base pairs shown in the consensus structure.

Cell culture

HEK-293T, HeLa and SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (containing glutamax 1; GIBCO) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) and penicillin/streptomycin (Pen/Strep). MIN-6 cells were cultured in DMEM (GIBCO) containing 10% heat-inactivated FBS and (in mM): dextrose (25), l-Glutamine (1), β-mercaptoethanol (0.0715) and Pen/Strep. Plasmid DNA constructs were transfected into Hek 293T and HeLa cells using Effectene (Qiagen) and Lipofectamine 2000 (Invitrogen), respectively. Morpholinos were delivered into all cell lines (except MIN-6) using Endo-Porter (Gene Tools) (30). Transfections into MIN-6 cells were by electroporation using solution kit R and program T-027 (Amaxa).

Molecular biology

Morpholino 25-mer oligos with a 3’-carboxyfluorescein end modification were ordered from GeneTools, LLC (www.gene-tools.com) Morpholino sequences are detailed in the Supplementary Table S2. The standard negative control morpholino (control) is expected not to cause effect as it only shows complementarity to an aberrant 5’ splice site created by a point mutation in the second intron of the β-globin gene (IVS2705) in a subset of human β-thalassemia patients (31). DNA and RNA were extracted from cells and tissues by the acid guanidinium thiocyanate-phenol-chloroform procedure, using Trizol according to the manufacturers’ instructions (15596, Invitrogen). For RNA, the pellet was resuspended in buffer (in mM): Tris-Cl (40), NaCl (10), CaCl2 (1), MgCl2 (6) supplemented with 0.5 U/μl human placental ribonuclease inhibitor (N2111, Promega) and 0.1 U/μl RNase-free DNase I (Roche). DNA digestion proceeded at 37°C for ∼0.5 h. Samples were diluted to 0.5 ml with RNase-free water (Ambion) and RNA was repurified by mixing with one volume of 5:1 acid phenol:chloroform (pH 4.3) and spinning down at 10000g for 5 min at room temperature in a bench top centrifuge. The aqueous supernatant was transferred to a new microcentrifuge tube and residual phenol was removed by mixing with one volume of chloroform and spinning down as before. RNA was precipitated with 20 μg glycogen, 0.3 M sodium acetate (pH 5.2) and one volume of isopropanol at room temperature and pelleted at 10000g for 10 min at 4°C or room temperature. The RNA pellet was washed with 75% ethanol, air-dried and resuspended in RNase-free water (Ambion). Ribosomal RNA integrity was assessed by native agarose gel electrophoresis and cDNA synthesis was carried out
using random hexamers (Invitrogen) and Avian Myeloblastosis Virus reverse transcriptase according to the Gübner and Hoffman procedure in the manufacturers instructions (SuperRT, HT Biotechnology). PCR amplons from cDNA templates were subjected to restriction digestes with either BbvI (GCAGC) or TauI (GCGGC ), or products were sequenced with the Sanger method and the relative peaks of nucleotides at the position of the editing sites were quantified using PeakPicker (32) or BioEdit software (http://www.mbio.ncsu.edu/bioedit/ bioedit.html).

For experiments with reporter constructs, PCR amplification of the minigene transcript was achieved from the cDNA of cell RNA extract. In addition to the use of DNase I digestion of RNA samples, contamination of plasmid DNA was minimized by amplification using primers spanning efficiently spliced introns native to the pCI-neo and pET01 vectors, which expressed the GluA2 Q/R and R/G site minigenes, respectively. The Q/R site minigene was a cloned BglII–XbaI fragment of the rat Gria2 gene encompassing the 3′ half of exon 11 and the 5′-end of intron 11–12. The R/G site minigene was a cloned fragment of the mouse Gria2 gene encompassing exons 13–16.

RESULTS

Properties of the Q/R substrate

The Q/R site is located near the end of exon 11 of the Gria2 locus (Figure 1A). Here, it exists within an imperfect inverted repeat, which extends into intron 11–12 and forms a complex pre-mRNA secondary structure subject to deamination by Adar2 (Figure 1A: inset) (33). To investigate attributes of the Q/R site substrate underlying its efficient editing we used a bioinformatics approach to examine genetic variations that are permissive for editing in situ. For this, we focused on genome sequencing projects from 50 vertebrate species with diploid genomes, including 1 cartilaginous fish (Chondrichthyes), 1 amphibian (Amphibia), 2 turtles (Anapsida), 1 scaled reptile and 5 birds (Anapsida), 1 egg laying mammal (Prototheria), 2 marsupials (Metatheria) and 37 placental mammals (Eutheria) (Supplementary Table S1). Polyplody in bony, ray-finned fish (Actinopterygii) had introduced a second GluA2 subunit genomically encoding an arginine at the Q/R site position (gria2β), which could reduce evolutionary pressure to maintain high A-to-I editing efficiency in the primary subunit (gria2α) and so were not included. Aligned DNA sequences of the imperfect inverted repeat indicated that sequence similarity was high in the 3′-end of the exon and the splice donor site, and in specific regions of the imperfect inverted repeat. In contrast, the region separating the repeats was poorly conserved and of variable length, consistent with earlier deletion experiments demonstrating it to be dispensable for in vitro editing (Figure 1A and B) (33–35).

Our phylogenetic sequence analysis was used to predict the consensus secondary structure and identify the fundamental structural features required for the essential, high-efficiency editing reaction at the Q/R site (Figure 1A). Calculated base pairing probabilities were highest around the Q/R and intronic editing sites (>0.8). Closer inspection of the Q/R substrate revealed that most sequence variation that does occur comprises consistent mutations that do not alter basepairing (e.g. AU to GU; including a genomically encoded guanine at the +4 editing site; Figure 1C: green), or a mutation that maintains a mismatch position (GA to AA; Figure 1C: blue) to conserve overall RNA secondary structure. In contrast, a small cluster of mutations in the triplet guanosine (+302 to +304) appears mostly to disrupt base pairing (Figure 1C). Consistent with this finding, introducing an N2-benzyl modification into the minor groove at the guanosine +303 position does not impact on in vitro editing at the Q/R site (36). Together, these data reveal novel aspects of this unique editing substrate and highlight specific regions of double strandedness critical for Q/R editing, thus providing a guide for antisense target design.

Antisense inhibition of an exogenous Q/R editing reporter system

The double stranded nature of A-to-I editing sites implies a substrate requirement for editing that could be targeted by competitive inhibition: hybridization of a steric antisense probe to the ECS to disrupt Adar2 binding and deamination. Potent steric antisense activity of morpholino oligos has been demonstrated on highly structured RNA targets (18–20). Given the extensive duplex structure of the Q/R editing substrate this site was chosen as a proof-of-principle target. A 25-mer morpholino was designed to hybridize to the entire Q/R site ECS (+315 to +324), the binding site of dsRBD1 (+307 to +317) and five unstructured base positions outside the structured RNA to assist in strand invasion of the antisense oligo (Figure 1A: outlined in green; Figure 2; Supplementary Table S2).

To test the antisense approach, we initially used an exogenous reporter system (33–35,37) (Figure 3A: top). This constitutes a fragment of the rat GluA2 gene, encompassing the 3′ half of exon 11 and ~0.5 kb of the proximal portion of the proceeding intron (33). The reporter was transfected into HeLa cells, which have been shown previously to have some A-to-I RNA editing activity (34) and respond well to the Endo-porter morpholino delivery reagent (30). Amplons of the minigene reporter transcript showed both adenine, and to a lesser extent, guanine at the editing site in the chromatograms obtained by Sanger sequencing (Figure 3B). In addition, digestion with BbvI (GCAGC) restriction enzyme gave two detectable bands corresponding to the predicted fragment sizes (Figure 3C). In contrast, a construct with the edited residue genomically encoded showed only guanine and a ΔECS construct exhibited only an adenine at the editing site (Figure 3A–C). These results validate the test system and confirm the essential role of the ECS in Q/R editing.

Antisense oligo (10 μM) delivered into reporter-expressing HeLa cells resulted in significantly inhibition of Q/R editing compared with vehicle controls (Figure 3D). A dose–response experiment provided a
50% inhibitory concentration (IC\textsubscript{50}) of \(~1.9\)\,\textmu M (Figure 3E and F). In contrast, editing was not affected by any tested concentrations of a standard negative control oligo (Supplementary Table S2; Figure 3E and F) (http://www.gene-tools.com/node/23#standardcontrols). Together, these experiments indicate that Q/R editing of a minigene reporter can indeed be effectively perturbed using a substrate-targeted antisense oligo. Furthermore, we could demonstrate that this effect was sequence and editing site specific (Supplementary Figure S1A and S2; see also next section). The ability and specificity of the antisense to inhibit Q/R editing of endogenous GluA2 was examined next.

**Specific steric antisense inhibition of the endogenous Q/R editing substrate**

The neuroblastoma cell line SH-SY5Y expresses GluA2 endogenously, which is edited by Adar2 (38). Morpholinos were delivered over a 16–24\,h period before assaying the editing state of the Q/R site. In
pressed 5-HT2CR mRNA indicating that a general effect (Figure 4A and B). Furthermore, the antisense was not indicating that sequence specificity was maintained abolished the ability of the oligo to inhibit Q/R editing, to the GC content; Supplementary Table S2) completely length of the antisense sequence (with minimum change B). In addition, five mismatches distributed along the invert morpholino showed no inhibition (Figure 4A and S2). As with the standard negative control oligo, this sense, but with inverted sequence (Supplementary Table used an oligo with equivalent base content to the anti- extents of delivery between antisense and control oligos we viability that differences in solubility could result in varying vehicle delivery control or to the standard negative control oligo (Figure 4A and B). To control for the possibility that differences in solubility could result in varying extents of delivery between antisense and control oligos we used an oligo with equivalent base content to the anti- sense, but with inverted sequence (Supplementary Table S2). As with the standard negative control oligo, this invert morpholino showed no inhibition (Figure 4A and B). In addition, five mismatches distributed along the length of the antisense sequence (with minimum change to the GC content; Supplementary Table S2) completely abolished the ability of the oligo to inhibit Q/R editing, indicating that sequence specificity was maintained (Figure 4A and B). Furthermore, the antisense was not associated with inhibition of editing in endogenously expressed 5-HT2CR mRNA indicating that a general effect on A-to-I editing is unlikely with up to 10 μM of antisense morpholino (Supplementary Figure S1B). We also obtained the same result for a similar series of control experiments using the exogenous reporter system in HeLa cells (Supplementary Figure S1A and S2).

If the morpholinos are operating via a steric mechanism, the above results should be reproducible using a different backbone chemistry, which similarly does not induce RNase-H cleavage or trigger RNA interference. Accordingly, we could reproduce inhibition of the Q/R site using an alternative antisense approach: oligos comprised of 2'-O-Methyl RNA/locked nucleic acid (2'-O-Me/LNA) mixmers (39, 40) also interfered with Q/R editing, albeit with reduced efficacy (Supplementary Figure S3).

The dramatic effect of competitive inhibition by morpholinos on the high editing levels of endogenous GluA2 mRNA (~85%) was surprising given that efficacy of a competitive inhibitor would be expected to be lower when basal levels of editing (and thus competing levels of Adar2) are higher. To understand this better we examined editing in the endogenous GluA2 pre-mRNA of SH-SY5Y. Consistent with a recent report (38), the editing state of the Q/R site in SH-SY5Y under control conditions was ~3-fold lower for pre-mRNA than for spliced mRNA (Figure 4C). Analyzing the antisense dose–response data for pre-mRNA indicated that the IC50 was very similar to that of the corresponding mRNA (1.8 versus 2.8 μM: pre-mRNA versus mRNA; Figure 5A) suggesting that antisense efficacy was comparable for the two RNA populations. The relationship between editing and splicing was examined next.

**Coupling of intronic hotspot 2 and splicing to Q/R site substrate integrity**

The data above uncovered a disparity between Q/R editing before and after splicing, which is consistent with earlier reports describing greater splicing efficiency for the Q/R-edited GluA2 pre-mRNA (11, 41, 42). The antisense approach provided an opportunity to test this relationship further. We plotted dose–response data for ratios of editing in mRNA against those of pre-mRNA; the slope of the resulting plot providing the relative splicing efficiency (Supplementary Figure S6). The data were significantly correlated (r = 0.86, P < 0.0001, Spearman’s rank) and were fit well by a linear function (R² = 0.8) with a slope corresponding to ~3.8 times more efficient splicing of edited pre-mRNA (Figure 5B). It has been demonstrated recently that the combination of editing at the Q/R site and at intronic hotspot 2 (position +262) is responsible for more efficient splicing of edited GluA2 pre-mRNA (42). Therefore, we analyzed the extent of editing at the intronic hotspots (Figure 5C). Given that these lie in an independent helical element (Figure 1A: helix II) to the Q/R site (Figure 1A: helix I) and antisense target we were surprised to find that hotspot 2 editing (in particular +262) was inhibited by the antisense as efficiently as the Q/R site (Figure 5D). Moreover, the editing at both sites was well-correlated (r = 0.81, P < 0.01; Spearman’s rank; Figure 5E). Therefore, there appears to be a direct coupling of the Q/R site and hotspot 2 that maintains preferential splicing of the Q/R-edited GluA2 pre-mRNA (42).

**Editing-coupled splicing efficiency and high basal editing activity safeguard the Q/R site**

The low level of editing at the Q/R site of GluA2 pre-mRNA in SH-SY5Y is unlike that in brain, where it often exceeds 95% (Figure 6A; Supplementary Table S3). Similarly, MIN-6, a β-pancreatic cell line, expresses GluA2 with near 100% editing of the Q/R site in GluA2 pre-mRNA (Figure 6A; Supplementary Table S3). In
addition, intronic pre-mRNA editing hotspots and the R/G editing site of GluA2 mRNA are processed to levels mirroring neural tissue (Supplementary Table S3). Therefore, we used MIN-6 as a tractable cell system for testing the ability of the antisense to perturb Q/R editing in the background of more neuron-like levels of editing activity. The extent of inhibition by morpholinos was compared with inhibition via dominant negative Adar2 mutants. In particular, we over-expressed catalytically inactive (E396A) and dominant negative (EAA) forms of Adar2 fusion protein with enhanced green fluorescent protein (EGFP) (Figure 6B). As MIN-6 cells were insensitive to the delivery vehicle used for the other cell lines, we delivered morpholinos using electroporation and assayed the editing state of GluA2 pre-mRNA.

MIN-6 cells were electroporated with the Adar2 mutants, allowed to express for 3 days, then EGFP-positive cells were collected by fluorescence-activated cell sorting (Figure 6C). The E396A mutant of Drosophila Adar has been shown previously to inhibit editing up to 60% for some targets in vivo (43). For the Q/R site, we observe only a modest 5% drop in Q/R pre-mRNA editing compared to EGFP alone (Figure 6C). In the dominant negative construct, mutations of key lysine residues (KKXXK) in both dsRBDs to EAAXA has been proposed to act in a dominant negative fashion by sequestering endogenous Adar2 into non-functional heterodimers (44); we detected a /C24 3.5-fold greater inhibition by this mutant compared with E396A. However, overall levels of pre-mRNA editing were still as high as 83%, reflecting the unique editing efficiency of the AMPAR Q/R site. Similarly, the antisense oligo reduced pre-mRNA editing to 86%. To confirm that the results were not due to one of the Gria2 alleles having a
genomically encoded guanine at the Q/R editing position (as occurs in the Gria2β paralogue in Teleost fish) we examined the editing state of the genomic DNA. Restriction digest with TauI (GCGGC) revealed complete editing of pre-mRNA but only one band (corresponding to the unedited sequence: GCAGC) in genomic DNA (Figure 6E), which ruled out this possibility. To account for GluA2 mRNA turnover in MIN-6 cells we tested a range of harvesting time points post-electroporation for the antisense oligo and found that the largest drop (~45%) in Q/R editing of GluA2 pre-mRNA at the earliest time point (at 12 h), and a gradual increase in editing over time, which likely reflects dilution of the antisense oligo during cell divisions (Figure 6D). The changes in Q/R editing of pre-mRNA was accompanied by only small changes in editing of the spliced mRNA (Figure 6D).

**DISCUSSION**

Here, we describe a strategy to selectively target specific A-to-I editing sites for inhibition. Steric antisense morpholinos designed to hybridize to the ECS of the GluA2 Q/R site substrate specifically and effectively perturbed editing at this site in the background of lower A-to-I editing activity, which is commonly seen with most other A-to-I editing targets. In addition, the antisense approach revealed a tight coupling of intronic hotspot 2 to the Q/R site, which likely ensures more efficient splicing of Q/R edited pre-mRNA. This ‘safe-guard’, potentially unique to the Q/R site (Supplementary Figure S4), contributes to the difficulty in perturbing Q/R editing in the background of high-editing activity. This novel antisense approach could be used to identify currently unexplored functions of select A-to-I editing sites.

We used morpholino backbone chemistry to make steric block antisense oligos. Unmodified DNA and RNA oligos are rapidly degraded in biological systems by enzymatic cleavage of the phosphodiester bond (Supplementary Figure S3B, R = O) and are prone to hydrolysis due to the presence of a hydroxyl group on the 2′-carbon of the ribose sugar (Supplementary Figure S3B, X = OH). Some leading backbone modifications for steric antisense applications include peptide nucleic acids, morpholinos and 2′-O-methyl RNA/LNA mixmers. For example, morpholinos are widely used to modify pre-mRNA splicing, block mRNA translation and inhibit miRNA maturation or activity (16,17) and have been used in several model organisms, such as sea urchins, zebrafish, frogs, chicks and mice (45). We add another application to this list: steric antisense inhibition of RNA editing. The application of new antisense delivery systems [e.g. Tat peptides (39,40)] with the current strategy will facilitate evaluation of editing sites in vivo.

In our application of the antisense tool, our data emphasizes the robust nature of this functionally critical A-to-I
editing substrate. Intact preferential splicing of edited pre-mRNA combined with high-editing activity will make transient fluctuations of Adar2 unlikely to lead to significant changes in editing status of spliced (protein coding) transcript (42). This is supported by our experiments using siRNAs to knockdown Adar2 in cultured neurons (Supplementary Figure S5). Recently, it was shown that increased splicing efficiency requires edited positions at both the Q/R site and hotspot 2 (43). Our antisense approach provides evidence for a coupling between hotspot 2 and Q/R substrate integrity. Consistent with the essential requirement for editing the Q/R site, we show that the hotspot 2 sequence and structure of the associated helical element (Figure 1A: helix II) is almost as well-conserved as the Q/R site substrate itself (Figure 1A: helix I; Figure 1B). This observation for coupling between the editing sites is also supported by earlier ECS mutagenesis experiments (33). The absence of obvious coupling to the +60 site on the complementary strand of hotspot 2 is not surprising given that it is almost exclusively an Adar1 target (11).

Positive and negative coupling between relatively distant editing sites and RNA secondary structures has been shown recently for the Adar2, 5-HT2CR, GluK2 and Gabra3 substrates (47,48). One possible explanation for the positive coupling observed in GluA2 could be as follows: Adar2 binds the Q/R substrate and dimerizes (44,45), the second subunit then binds and edits hotspot 2. Such avidity would be consistent with the high levels of pre-mRNA editing typically observed for the Q/R site compared to some other A-to-I editing sites. Consistent with this in vitro editing of a minimal Q/R substrate (like the one shown in Figure 1C) is less efficient than that of the complete pre-mRNA in vivo (36). However, in the absence of structural information, proof for such a mechanism remains unexplored.
Morpholino oligos are typically used to disrupt splicing by sterically blocking splice sites. For example, by hybridizing to the donor splice site morpholinos prevent base pairing of the U1 snRNA and thus assembly of the spliceosome. The antisense target in our study is around 290 nucleotides downstream of the donor splice site. In addition, although the target and splice donor are on the same helical element of the secondary structure, the nearest end of the target is around 12 bp from the start of the splice site. Furthermore, splicing occurs more rapidly for Q/R edited pre-mRNA because editing and splicing are coordinated by the C-terminal domain of RNA polymerase II (41,49). As a consequence, binding of a high-affinity antisense probe is less likely for the edited pre-mRNA. Therefore, a direct effect of the antisense on donor splice site recognition could be biased for the unedited pre-mRNA and predict differences in the IC_{50} for editing in pre-mRNA versus mRNA resulting in dose-dependent deviations from the model described in Supplementary Figure S6, which we do not observe (Figure 5A and B). Together, it seems unlikely that the antisense has a direct impact on splicing but rather exerts a specific effect on editing.

In summary, this antisense approach should open up in-depth characterization of other A-to-I editing sites.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figures 1–6.
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REFERENCES

1. Jepson, J.E. and Reenan, R.A. (2008) RNA editing in regulating gene expression in the brain. Biochim. Biophys. Acta. 1779, 459–470.
2. Tariq,A. and Jantsch,M.F. (2012) Transcript diversification in the nervous system: A to I RNA editing in CNS function and disease development. Front. Neurosci., 6, 99.
3. Rosenthal,J.J. and Seeburg,P.H. (2012) A-to-I RNA editing: effects on proteins key to neural excitability. Neuron, 74, 432-439.
4. Bass,B.L. (2002) RNA editing by adenosine deaminases that act on RNA. Annu. Rev. Biochem., 71, 817-846.
5. Hogg,M., Paro,S., Keegan,L.P. and O’Connell,M.A. (2011) RNA editing by mammalian ADARs. Adv. Genet., 73, 87-120.
6. Sommer,B., Kohler,M., Sprengel,R. and Seeburg,P.H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell, 67, 11–19.
7. Traynelis,S.F., Wollmuth,L.P., McBain,C.J., Menniti,F.S., Vance,K.M., Ogden,K.K., Hansen,K.B., Yuan,H., Myers,S.J., Dingledine,R. et al. (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev., 62, 405–496.
8. Greger,I.H., Khatri,L., Kong,X. and Ziff,E.B. (2003) AMPA receptor tetramerization is mediated by Q/R editing. Neuron, 40, 763–774.
9. Feldmeyer,D., Kask,K., Brusa,R., Kornau,H.C., Kolhekar,R., Rozov,A., Burnashev,N., Jensen,V., Hvalby,O., Sprengel,R. et al. (1999) Neurological dysfunctions in mice expressing different levels of the Q/R site-undedited AMPAR subunit GluR-B. Nat. Neurosci., 2, 57–64.
10. Brusa,R., Zimmermann,F., Koh,D.S., Feldmeyer,D., Gass,P., Seeburg,P.H. and Sprengel,R. (1995) Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. Science, 270, 1677–1680.
11. Higuchi,M., Maas,S., Kawahara,Y., Tamburro,K.M. and Nishikura,K. (2006) A-to-I RNA editing and human disease. RNA Biol., 3, 1–9.
12. Kwak,S. and Weiss,J.H. (2006) Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. Curr. Opin. Neurobiol., 16, 281–287.
13. Reenan,R.A. (2001) The RNA world meets behavior: A→I pre-mRNA editing in animals. Trends Genet., 17, 53–56.
14. Peng,Z., Cheng,Y., Tan,B.C., Kang,L., Tian,Z., Zhu,Y., Zhang,W., Liang,Y., Hu,X., Tan,X. et al. (2012) Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome. Nat. Biotechnol., 30, 253–260.

Figure 7. Summary of GluA2 Q/R substrate properties: (A) Illustrating the positive coupling between the editing at the Q/R site and hotspot 2 revealed by the antisense; (B) Illustrating that the larger fraction of editing apparent in mRNA results from more efficient splicing of edited pre-mRNA. Coupling of the intronic editing hotspot 2 to the Q/R editing site is probably responsible for the change in pre-mRNA splicing efficiency.
16. Eisen,J.S. and Smith,J.C. (2008) Controlling morpholino experiments: don’t stop making antisense. Development, 135, 1735–1743.
17. Moulton,J.D. and Yan,Y.L. (2008) Using Morpholinos to control gene expression. Curr. Protoc. Mol. Biol., Chapter 26: Unit 26.8.
18. Jubin,R., Vantuno,N.E., Kieff,J.S., Murray,M.G., Doudna,J.A., Lau,J.Y. and Baroudy,B.M. (2000) Hepatitis C virus internal ribosome entry site (IRES) stem loop IIId contains a phylogenetically conserved GGG triplet essential for translation and IRES folding. J. Virol., 74, 10430–10437.
19. Summerton,J. (1999) Morpholin antisense oligomers: the case for an RNAse H-independent structural type. Biochin. Biophys. Acta, 1489, 141–158.
20. Summerton,J., Stein,D., Huang,S.B., Matthews,P., Weller,D. and Partridge,M. (1997) Morpholin and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Antisense Nucleic Acid Drug Dev., 7, 63–70.
21. Rice,P., Longden,I. and Bleasby,A. (2000) EMBOSs: the European Molecular Biology Open Software Suite. Trends Genet., 16, 276–277.
22. Carver,T. and Bleasby,A. (2003) The design of Jemboss: a graphical user interface to EMBOSs. Bioinformatics, 19, 1837–1843.
23. Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., 25, 3389–3402.
24. Birney,E., Andrews,T.D., Bevan,P., Caccamo,M., Chen,Y., Clarke,L., Coates,G., Cuff,J., Curwen,V., Cutts,T., et al. (2004) An overview of Ensembl. Genome Res., 14, 925–928.
25. Kent,W.J. (2002) BLAT—the BLAST-like alignment tool. Genome Res., 12, 656–664.
26. Venkatesh,B., Kirkness,E.F., Loh,Y.H., Halpern,A.L., Lee,A.P., Johnson,J., Danda,N., Viswanathan,L.D., Tay,A., Venter,J.C. et al. (2007) Survey sequencing and comparative analysis of the elephant shark (Callorhinchus milii) genome. PLoS Biol., 5, e101.
27. Katoh,K., Kuma,K., Miyata,T. and Toh,H. (2005) Improvement of PSI-BLAST: a new generation of protein database search program. Bioinformatics, 21, 3047–3048.
28. Hamada,M., Sato,K. and Asai,K. (2005) VARNA: interactive drawing and editing of the RNA secondary structure. Bioinformatics, 21, 193–194.
29. Dautry,K., Denise,A. and Ponty,Y. (2009) VARNA: interactive drawing and editing of the RNA secondary structure. Bioinformatics, 25, 1974–1975.
30. Summer, J.E. (2005) Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. Ann. N. Y. Acad. Sci., 1058, 62–75.
31. Dominski,Z. and Kole,R. (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc. Natl Acad. Sci. USA, 90, 8673–8677.
32. Ge,B., Gurd,S., Gaudin,T., Dore,C., Lepage,P., Harmsen,E., Hudson,T.J. and Pastinen,T. (2005) Survey of allelic expression using EST mining. Genome Res., 15, 1584–1591.
33. Higuchi,M., Single,F.N., Kohler,M., Sommer,B., Sprengel,R. and Seeburg,P.H. (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. Cell, 75, 1361–1370.
34. Rueter,S.M., Burns,C.M., Coode,S.A., Mookherjee,P. and Emeson,R.B. (1995) Glutamate receptor RNA editing in vitro by enzymatic conversion of adenosine to inosine. Science, 267, 1491–1494.
35. Yang,J.H., Sklar,P., Axel,R. and Maniatis,T. (1995) Editing of glutamate receptor subunit B pre-mRNA in vitro by site-specific deamination of adenosine. Nature, 374, 77–81.
36. Stephens,O.M., Haudenschild,B.L. and Beal,P.A. (2004) The binding selectivity of Adar2’s dsRBMs contributes to RNA-editing selectivity. Chem. Biol., 11, 1229–1250.
37. Egebjerg,J., Kukkot,V. and Heinemann,S.F. (1994) Intron sequence directs RNA editing of the glutamate receptor subunit GluR2 coding sequence. Proc. Natl Acad. Sci. USA, 91, 10270–10274.
38. Yamashita,T., Tadami,C., Nishimoto,Y., Hideyama,T., Kimura,D., Suzuki,T. and Kato, T. (2012) RNA editing of the GluA2 gene in different cultured cell lines that constitutively express different levels of RNA editing enzyme Adar2. Neurosci. Res., 73, 42–48.
39. Arzumanov,A., Stotenko,D.A., Malakov,A.D., Reichelt,S., Sorensen,M.D., Babu,B.R., Wengel,J. and Gait,M.J. (2003) A structure-activity study of the inhibition of HIV-1 Tat-dependent trans-activation by mixmer 2′-O-methyl oligoribonucleotides containing locked nucleic acid (LNA), alpha-L-LNA, or 2′-thio-LNA residues. Oligonucleotides, 13, 435–453.
40. Arzumanov,A., Walsh,A.P., Rajwanshi,V.K., Kumar,R., Wengel,J. and Gait,M.J. (2001) Inhibition of HIV-1 Tat-dependent trans-activation by steric block chimeric 2′-O-methyl/LNA oligoribonucleotides. Biochemistry, 40, 14645–14654.
41. Ryman,K., Fong,N., Bratt,E., Bentley,D.L. and Ohman,M. (2007) The C-terminal domain of RNA Pol II helps ensure that editing precedes splicing of the GluR-B transcript. RNA, 13, 1071–1078.
42. Balik,A., Penn,A.C., Nemoda,Z. and Greger,I.H. (2012) Activity-regulated RNA editing in select neuronal subfields in hippocampus. Nucleic Acids Res., 41, 1124–1134.
43. Schoft,V.K., Schoof,S. and Jantsch,M.F. (2007) Regulation of glutamate receptor B pre-mRNA splicing by RNA editing. Nucleic Acids Res., 35, 3723–3732.
44. Gallo,A., Keegan,L.P., Ring,G.M. and O’Connell,M.A. (2003) An ADAR that edits transcripts encoding ion channel subunits functions as a dimer. EMBO J., 22, 3421–3430.
45. Valente,L. and Nishikura,K. (2007) RNA binding-independent dimerization of adenosine deaminases acting on RNA and dominant negative effects of nonfunctional subunits on dimer functions. J. Biol. Chem., 282, 16054–16061.
46. Heasman,J. (2002) Morpholino oligos: making sense of antisense? Dev. Biol., 243, 209–214.
47. Daniel,C., Veno,M.T., Ekdahl,Y., Kjems,J. and Ohman,M. (2012) A distant cis acting intrinsic element induces site-selective RNA editing. Nucleic Acids Res., 40, 9876–9886.
48. Estero,M., Daniel,C., Wahlstedt,H., Major,F. and Ohman,M. (2009) Recognition and coupling of A-to-I edited sites are determined by the tertiary structure of the RNA. Nucleic Acids Res., 37, 6916–6926.
49. Laurencikiene,J., Kallman,A.M., Fong,N., Bentley,D.L. and Ohman,M. (2006) RNA editing and alternative splicing: the importance of co-transcriptional coordination. EMBO Rep., 7, 303–307.