

# Regulation of ADAR2 Activity by RNA Editing

*Jay Shrestha*

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## Introduction

**R**NA editing is a posttranscriptional modification of gene-encoded sequence, which allows organisms to produce functionally distinct proteins from a single gene. It can involve single- or multiple-base deletion, insertion, and/or substitution in the pre-mRNA (mRNA prior to splicing). This is a widespread phenomenon in eukaryotes.

Modification by deletion and/or insertion is found mainly in mitochondria of primitive eukaryotes.<sup>1</sup> Editing in mammals is comprised mostly of cytidine-to-uracil (catalyzed by RNA-dependent cytidine deaminase) and adenosine-to-inosine (catalyzed by RNA-dependant adenosine deaminase) conversions. These conversions are a result of hydrolytic deamination reactions catalyzed by the respective enzymes. These base conversions may give rise to changes in codons and thus in the amino acid sequence of the gene, which in turn can affect the function of the encoded protein. As editing occurs prior to splicing of the pre-mRNA, these conversions can also result in alternative splicing. The increase in variation of protein sequence encoded by a single gene can be better understood by looking more closely at such edited proteins. Glutamate receptor protein subunit GluR-6 in the central nervous system has three potential exonic editing sites in its pre-mRNA: isoleucine/valine, tyrosine/cysteine, and glutamine/arginine. Editing can give rise to a mixture of eight alternative sequence configurations.<sup>2,3</sup> In transcripts of *cacophony*, which encodes the voltage-gated calcium channel in *Drosophila*, the presence of 10 different editing sites gives the potential to generate more than 1,000 different protein isoforms by RNA editing. This number can increase if we take alternative splicing into consideration.<sup>4,5</sup>

Three different RNA-dependent adenosine deaminases (ADAR) have been determined: namely ADAR1, ADAR2, and ADAR3 (RED2) (Figure 1). These enzymes contain a deaminase domain near the C terminus and two or three double-stranded RNA binding domains 5 feet to the deaminase domain. They also contain nuclear localization sequences (NLS) that bind to different nuclear transport proteins. ADARs have homology to DNA methyltransferases, which methylate DNA by a base flipping mechanism. It has been suggested that ADARs also follow this “flip out” mechanism for editing dsRNA.<sup>6</sup>

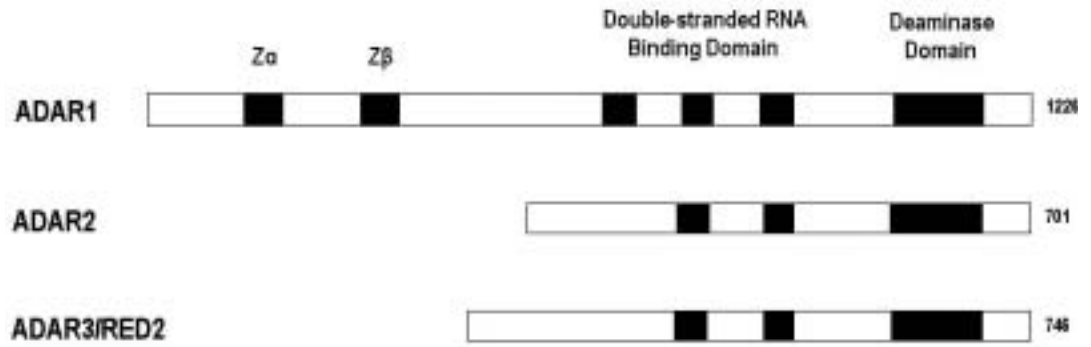


Figure 1

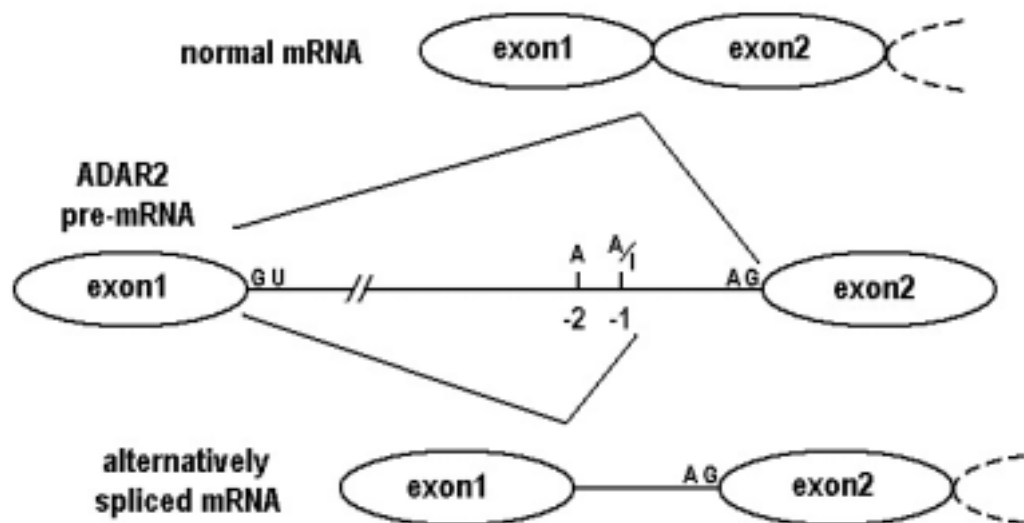


Figure 2

ADAR1 also contains two Z-DNA binding domains called Z $\alpha$  and Z $\beta$  near its N terminus. The presence of the Z-DNA binding domains in ADAR1 has led to an interesting deduction of mechanism by which ADAR1 may carry out its function. Z-DNA forms transiently due to the torsional strain generated on the DNA as RNA Polymerase travels down during transcription. It has been proposed that ADAR1 binds to this trail of transiently formed Z-DNA. This brings ADAR1 in close proximity to its substrate, which is the pre-mRNA being transcribed by the RNA Polymerase.<sup>7</sup>

ADAR1 and ADAR2 are almost ubiquitously expressed (Wagner et al). ADAR3 (RED2) is expressed exclusively in the brain, but it cannot

catalyze deamination of adenosine in dsRNA nor can it edit any known pre-mRNAs.<sup>8,9</sup>

There is degeneracy seen in the substrates that are edited by ADAR1 and ADAR2. A number of editing sites can be edited by both of these enzymes with varying efficiencies. There are also some editing sites that are specific to any one of the enzymes. One of the most important substrates for adenosine deamination by ADAR2 is transcript of glutamate receptor (GluR) channel subunit B10 found in the brain. In the pre-mRNA of this subunit, GluR-B, RNA editing changes the glutamine (Q) codon CAG to a CIG (I is recognized as U during translation), which specifies arginine (R). This single editing position (the Q/R-site) controls the Ca<sup>2+</sup> permeability of

the glutamate receptor.<sup>11</sup> Almost 99.9 percent of the transcripts of GluR-B are edited at this site.<sup>12</sup> The glutamate receptor is characterized by a low Ca<sup>2+</sup> permeability.<sup>13</sup> Presence of glutamine at this site causes influx of Ca<sup>2+</sup> ions through the channel. In addition to its function in cell-to-cell signaling, it is known that influx of Ca<sup>2+</sup> ions upon activation of Glu-R modulates gene statement and cell proliferation in the neurons and glial cells.<sup>14</sup> It has been shown that in tissues from malignant human brain tumors GluR-B is substantially underedited, with 12 to 31 percent present in the GluR-B(Q) form.<sup>12</sup>

The importance of the Q/R editing site in GluR-B became evident when mutant mouse line, heterozygous for GluR-B(Q), was generated. These mice displayed a severe epileptic phenotype and died within three weeks of birth as a result of the increased Ca<sup>2+</sup> permeability of the glutamine receptors.<sup>15</sup> Epileptic seizures are prevalent in patients with gliomas, which could at least partly be a result of change in Q/R-site editing.<sup>12</sup>

Mice heterozygous at the ADAR2 locus were normal and showed editing in 99 percent of the GluR-B pre-mRNA.<sup>12</sup> ADAR2 knockout mice showed a normal embryonic growth but died during infancy (Higuchi et al). These mice were prone to epileptic seizures, and only 40 percent of GluR-B pre-mRNA molecules were edited at the Q/R site. When alleles encoding the edited version of GluR-B, GluR-B(R) in which arginine was genetically encoded were expressed in the ADAR2 knockout mice, the knockout phenotype was rescued.<sup>16</sup> This experiment showed that the GluR-B Q/R site is physiologically the most important editing site for ADAR2.<sup>16</sup>

Interestingly, an editing site is present in the first intron of ADAR2 RNA transcript (position -1 in Figure 2), which is specifically edited by the ADAR2 protein in vivo. The -1 position is the splice acceptor site in the ADAR2 pre-mRNA transcript. The A-to-I conversion in the -1 position and the presence of an A at the -2 position give rise to a new splice acceptor junction at this site (I is recognized as G, and AG is a conserved consensus sequence for a splice acceptor junction). Edited pre-mRNAs are spliced at this site to produce mRNAs that have a 47 nucleotide

intronic sequence present in between exons 1 and 2 (Figure 2). This splice variant of ADAR2, when translated, produces a nonfunctional protein due to a frame shift caused by the alternative splicing event.

## Materials and Methods

Stably transfected human cell lines<sup>12</sup> with ectopic statement of different levels of ADAR1 and ADAR2 were used for this study. Due to its low endogenous ADAR activity, Human embryonic kidney (HEK293) cells were used as the parental cell line.

Cell lines N5, N8, and N6 showed increasing levels of ADAR1 statement (with the same level of ADAR2 statement). Cell lines R10, R14, and R13 showed increasing levels of ADAR2 statement (with the same level of ADAR1 statement).

Total RNA from N6 and R13 cell lines were extracted with TRIzol Reagent (GIBCO) and digested with RNase-free DNase I. These digests were tested for presence of any residual DNA. Successfully purified total RNAs were reverse transcribed to produce cDNA using random hexamer primers and SuperScript II reverse transcriptase. The cDNAs were amplified by PCR. For analysis of intronic editing (at the -1 position) primers A2UI1D 5'-GGAATTCTATTAGTCAC TAAGCAAAGTGTGTCAG-3' (sense, intron1) and A2E2U 5'-GCGGTACCCAGGTGTGCTGCCAT CCTTGG-3' (antisense, exon 2) were used. This set of primers amplifies the ADAR2 cDNA derived from the pre-mRNA. The PCR products were then digested with Kpn1 and EcoRI restriction endonucleases and ligated to BlueScript plasmid. The plasmids were transformed into *E.coli*. The DNA preps isolated from the successful transformants were used for gel-sequencing reaction.

For analysis of alternative splicing, primers A2D 5'-GTATTTTGCCATGGATATAGAAGATG-3' (sense, exon1) and A2U 5'-GTACTGG

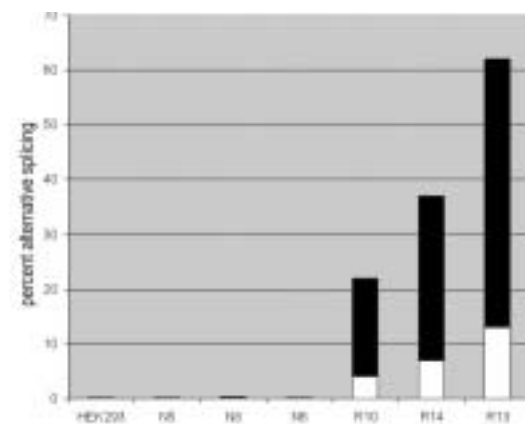


Figure 3

Cell Line	Editing Position							
	-27	-24	-2	-1	+18	+23	+24	
HEK293	12	82	0	0	83	8	8	
N5	10	89	0	102	10	10	10	
N8	12	88	0	0	8	8	8	
N6	37	77	0	0	8	8	8	
R10	1	8	0	4	8	8	8	
R14	2	8	0	7	8	103	8	
R13	2	8	0	12	8	+1	8	

Table 1

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GATCCAGGCTTGATCTCATTGAGCTG-3' (antisense, exon 2) were used. This set of primers amplifies both splice variants from the cDNA.

## Results

The gel sequence of the first intron in the ADAR2 pre-mRNA transcript revealed editing at the –1 position in R13 cell lines (Figure 2). Four out of 49 (about 8 percent) primary mRNA transcripts sequenced showed an A-to-I editing at this position. None of the 20 primary mRNA transcripts of N6 sequenced showed editing at this position. There was no editing seen at the –2 site for both the cell lines. Editing at the –2 site would make any editing at the –1 site irrelevant (as a splice acceptor site would not be formed because of an A-to-I conversion at the –1 position). None of the pre-mRNA transcripts showed any editing at the –24 position, which also lies in the intron. Additional data of editing at these two sites in different cell lines from studies conducted in our lab is listed in Table 1. The data clearly shows a direct relationship between level of ADAR2 statement and editing at the –1 site.

The semiquantitative RT-PCR of HEK293, N6, R13, and other cell lines suggested a direct relationship between ADAR2 statement and alternative splicing (Figure 3). The existence of alternative splice variant was confirmed by sequence analysis of the PCR products of ADAR2 pre-mRNA. The data also suggests that the ADAR2 pre-mRNAs that are edited in cell lines overexpressing the gene may also splice the edited pre-mRNAs preferentially. The R13 cell lines show a 13 percent increase in occurrence of pre-mRNAs edited at the –1 position (Table 1).

But the occurrence of alternatively spliced variant in increased to 53 percent in this cell line (Figure 3).

## Discussion

Evidence of self-editing of ADAR2 to produce nonfunctional splice variants was first discovered in mice.<sup>17</sup> This self-editing has also been observed in human cell lines. It has been suggested that this self-editing of ADAR2 gives rise to an autoregulatory feedback mechanism in cells. This could be an important mode of regulating ADAR2 activity.

The most important known function of ADAR2 protein is the editing of the GluR-B subunit of glutamate receptors at the Q/R site. It has been suggested that the abnormal cell proliferation seen in brain tumors and the epileptic seizures prevalent in such patients could partly be induced by the change in ADAR2 activity, which leads to an underediting of the GluR-B subunit causing Ca<sup>2+</sup> influx into the cells.<sup>12</sup> In the future, drugs could be designed to enhance RNA editing in tumor cells.<sup>12</sup> We still need to isolate additional targets of ADAR2 in order to find out if this underediting is seen in other cancer tissues. Although the autoregulatory feedback model of ADAR2 regulation is quite promising, other modes of protein regulation, particularly post-translational modification and controlled subcellular localization of ADAR2, have to be investigated to better understand the mechanism of ADAR2 regulation.

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