RNA editing in *Drosophila melanogaster*: New targets and functional consequences

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ABSTRACT

Adenosine deaminases that act on RNA [adenosine deaminase, RNA specific (ADAR)] catalyze the site-specific conversion of adenosine to inosine in primary mRNA transcripts. These re-coding events affect coding potential, splice sites, and stability of mature mRNAs. *ADAR* is an essential gene, and studies in mouse, *Caenorhabditis elegans*, and *Drosophila* suggest that its primary function is to modify adult behavior by altering signaling components in the nervous system. By comparing the sequence of isogenic cDNAs to genomic DNA, we have identified and experimentally verified 27 new targets of *Drosophila* ADAR. Our analyses led us to identify new classes of genes whose transcripts are targets of ADAR, including components of the actin cytoskeleton and genes involved in ion homeostasis and signal transduction. Our results indicate that editing in *Drosophila* increases the diversity of the proteome, and does so in a manner that has direct functional consequences on protein function.

Keywords: adenosine deaminase; *Drosophila*; RNA editing; ADAR; nervous system; transcriptome

INTRODUCTION

RNA editing is a well-established mechanism in which precursor messenger RNA transcripts are subject to re-coding by the enzyme adenosine deaminase, RNA specific (ADAR) (Bass 2002). ADAR catalyzes the deamination of adenosine to inosine. Inosine mimics guanosine in its base-pairing properties, and the translational machinery interprets I as G. In this way, an A-to-I conversion in the mRNA can alter the genetic information that can lead to changes in mRNA splicing and stability as well as changes in protein function. ADAR is essential in all animals examined to date, and studies in mouse, *Caenorhabditis elegans*, and *Drosophila* suggest that the function of pre-mRNA editing is to modify adult behavior by altering signaling components in the nervous system (Higuchi et al. 2000; Palladino et al. 2000; Wang et al. 2000; Tonkin et al. 2002). Considerable progress has also been made in understanding the mechanism of action of the ADAR enzyme (Cho et al. 2003; Haudenschild et al. 2004; Athanasiadis et al. 2005; Macbeth et al. 2005). The molecular details of target site specificity of RNA editing are drawn from studies of the mammalian glutamate receptor gene, *GluR-B*. Receptor pre-mRNA forms a double-stranded stem structure by imperfect base-pairing between an exonic sequence, where the edit(s) occur, and a noncoding intronic element called the editing site complementary sequence (ECS) (Higuchi et al. 1993). Most targets, however, are found serendipitously when isolating genes from cDNA libraries. Computational identification of ADAR substrates is difficult, and little progress has been made in designing algorithms for predicting putative target transcripts. ADAR targets in the human genome have been identified and are located predominantly in embedded *Alu* sequences, suggesting a role of ADAR in mRNA stability (Kim et al. 2004; Levanon et al. 2004). Editing can also introduce new splice sites (Rueter et al. 1999), and editing that occurs in 5′- and 3′-untranslated regions (UTRs) presumably alters the stability, localization, or translatability of the target mRNA (Morse et al. 2002; Prasanth et al. 2005). Recently, Yang and coworkers described an additional role of RNA editing in the biogenesis of select miRNAs involved in mammalian hematopoiesis (Yang et al. 2006).

The majority of verified targets of ADAR in *Drosophila melanogaster* come from a comparative genomic approach examining a distinct subset of genes consisting of ion channels, G-protein-coupled receptors (GPCRs), and proteins involved in synaptic transmission (Hoopengardner

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et al. 2003). Using this approach, conservation of the putative ECS site within neighboring introns was not an informative predictor; however, exons with the highest evolutionary conservation within a gene seemed to be the best predictive tool to identify ADAR targets for this subset of genes. The prevalence of editing sites found within the coding portions of *Drosophila* genes, as well as the stage-specific "self-tuning" of ADAR, could be a principal mechanism of increasing neuronal protein diversity (Keegan et al. 2005).

Although directed approaches to identify ADAR targets have been employed successfully, we have taken a different approach based on a systematic analysis of the *Drosophila* Gene Collection (DGC; http://www.fruitfly.org/DGC). The DGC contains cDNAs representing 10,398 of the predicted 13,449 genes in *D. melanogaster* (Release 4.1). This collection originates from cDNA libraries derived from a variety of tissues and developmental stages (Rubin et al. 2000; Stapleton et al. 2002a,b). Utilizing high-quality sequence data from full-insert sequences of adult head cDNA clones, we have identified 27 new targets of ADAR, doubling the total to 55. The edited sites verified in our analysis are within coding regions and in the 3′ UTRs of these targets. Previous studies (Dutzler et al. 2002, 2003) on one of our targets (*CG31116*), a member of the ClC family of chloride channels, suggest a clear functional consequence of editing upon the gating properties of the ion channel. Some target genes, like the G-protein-coupled receptor *bride-of-sevenless* (*boss*) and the calcium-activated potassium channel *small conductance calcium-activated potassium channel* (*SK*), have clear roles in neuronal development and signaling, while other target genes, like *spir*, which is an actin nucleating factor, represent components of the cytoskeleton. The largest class of ADAR targets consists of novel genes that have not yet been assigned a functional attribute in the Gene Ontology (GO) database (Ashburner et al. 2000).

Transcriptional analyses of the human genome (Kapranov et al. 2002), the *Arabidopsis* genome (Yamada et al. 2003), and the *Drosophila* genome (Stolc et al. 2004) suggest that upward of 50% of the predicted noncoding genome is expressed. In *Drosophila*, noncoding transcription has been well characterized and includes microRNAs (Lai et al. 2003; Lai 2005; Leaman et al. 2005), noncoding RNAs (Tupy et al. 2005), and anti-sense RNAs (Misra et al. 2002). The complexity of eukaryotic transcriptomes continues to expand, and editing of pre-mRNA transcripts adds yet another layer of intricacy.

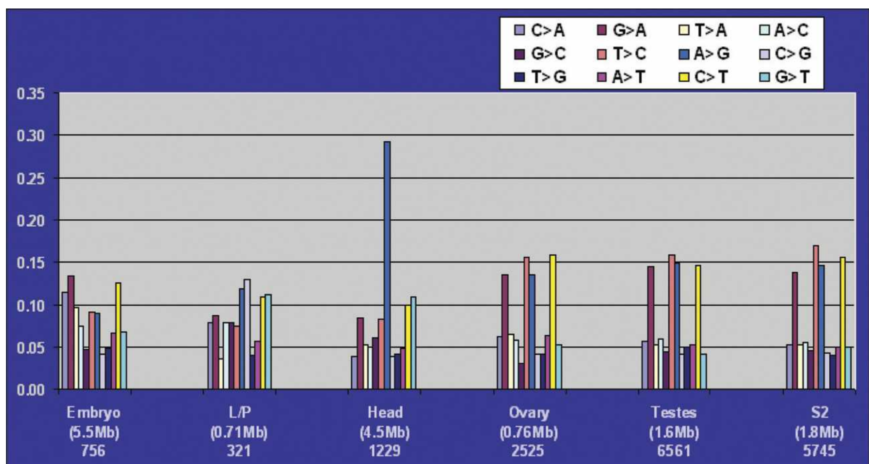

FIGURE 1. A-to-G transitions in head library cDNAs are 2.5–3-fold higher than all other types of base-pair substitutions within libraries derived from the same strain. Base-pair substitutions were identified and grouped by library and expressed as a percent of the total number of substitutions within each library. The embryo, larval/pupal (L/P), and head cDNA libraries are derived from the same isogenic strain used for delineating the genome sequence, while the ovary, testes, and the cell-line S2 libraries are not. Numbers below each library type correspond to the total number of base pairs (in parentheses, megabase pairs, Mb) and the total number of substitutions in each library.

RESULTS AND DISCUSSION

Identifying putative targets of ADAR

In order to identify putative new targets of ADAR, we took advantage of our full-insert sequenced gene collection. As part of our cDNA analysis pipeline, we aligned each high-quality cDNA sequence with the genome sequence using Sim4 (Florea et al. 1998) and recorded the base-pair discrepancies (see Materials and Methods). Figure 1 represents the results of tabulating all possible substitutions from ~15 Mb of data from full-insert sequenced cDNAs. When substitutions are grouped by library and expressed as a percentage of the total, clones from the isogenic head library possess a threefold increase in the level of A-to-G transitions when compared to clones from the other two isogenic libraries. Moreover, the A > G transition frequency in the head libraries is the only type of substitution within the isogenic libraries that has a discernible frequency above background substitutions. This analysis led us to generate a list of clones with the highest likelihood of having an A-to-G substitution due to editing and reducing the possibility of strain-dependent polymorphisms. This approach identifies both authentic editing sites as well as errors introduced by the reverse transcriptase used to generate the cDNA libraries. To distinguish these events, further experimental validation of the sites is necessary and is described below.

The analysis resulted in a list of 198 putative editing targets. Most of the known edited genes in *D. melanogaster* are involved in rapid electrical and chemical neurotransmission (Stapleton et al. 2002a; Hoopengardner et al. 2003;

Xia et al. 2005). This observation is, in part, due to the neurological phenotype associated with *ADAR*-null mutants coupled with the fact that genes of this type have been specifically evaluated for the presence of editing. Although our approach is not biased in this manner, we identified 11 of the known 28 targets of ADAR as putative targets and did not pursue them further. Additionally, 10 of the remaining 17 known target genes are represented in our collections, but do not possess the A-to-G transition, suggesting that since editing is <100% efficient, we captured the unedited mRNA in our cDNA collection. The remaining seven known targets do not have a representative EST or cDNA in our gene collection. The Sim4 alignment program encounters difficulties near the edges of sequence alignments, and putative targets with these alignment artifacts were removed from the list, further collapsing the set to a total of 108 clones containing 149 potential editing sites.

We interrogated these 149 putative editing sites experimentally by performing reverse-transcription–polymerase chain reaction (RT-PCR) followed by sequencing of the amplified products. RT-PCR was performed with total RNA isolated from adult heads of the isogenic strain. Amplicons ranged in size from ~300 to 500 bp spanning the region(s) surrounding the potential editing site(s). Sequence traces from 27 of the 108 genes yielded 58 positions with either a mixed A and G signal (n = 51) or a pure G signal (n = 7), indicating the presence of edited transcripts (Fig. 2B; Supplemental Material, contact staple@fruitfly.org). We successfully amplified regions from all 108 putative targets and obtained high-quality sequencing data for all but one (*CG7569*). For targets whose chromatograms showed "weak" evidence of editing (predominant A at the putative editing site), we cloned the products from two independent RT-PCR reactions and sequenced 48 isolates per reaction. *CG31116* is an example of this weak editing, where three of the four putative sites displayed almost no mixed signal. Cloning revealed these sites are edited with frequencies ranging from 4.5% to 10.4% (Supplemental Fig. S12 and table therein, contact staple@fruitfly.org). To rule out the unlikely possibility that the mix of A/G signals or the pure G signal seen in the RT-PCR was due to genome polymorphisms that have accumulated in the isogenic strain, we PCR-amplified and sequenced the same regions from genomic DNA isolated concomitantly with the RNA. None of the 58 sites is due to polymorphisms in the genomic DNA (Fig. 2B; Supplemental Material, contact

FIGURE 2. (Legend on next page)

staple@fruitfly.org). Similar analysis was performed on a subset of these 27 new targets using RNA isolated from *ADAR*-deficient animals, and all sites tested ($n = 23$) showed a pure A signal, indicating the unedited transcript (data not shown). Although this method of measuring the ratio of edited and unedited transcripts has been reported to be accurate to as low as 5% editing (Palladino et al. 2000), it cannot be used to determine the relative distribution of sites in cases where a transcript contains more than one edited site.

New targets of dADAR

The 27 new targets of ADAR are categorized into seven classes of genes, which are listed in Table 1. The seven gene classes include vesicular trafficking, ion homeostasis, signal transduction, ion channels, cytoskeletal components, an "other" class, and a novel or unknown class. For each target, the gene symbol, the cDNA representative that initially suggested editing, the protein, the amino acid residue(s), and 3′-UTR nucleotide(s) that are affected, along with the molecular function, if known, are listed.

The vesicular traffic class of targets contains six genes, *Rab26*, *Rlip*, *rab3-GEF*, *endophilin A* (*endoA*), *α-Adaptin*, and *sunday driver* (*syd*). Small Ras-like G-proteins and their effectors are well-documented proteins that function in the intracellular transport of vesicles (Takai et al. 2001). Three of the six members fall into this category and are represented by the GTPase Rab26, the Ral GTPase activator protein Rlip, and the Rab3 guanyl-nucleotide exchange factor Rab3-GEF. The *endoA* and *α-Adaptin* genes encode proteins that are involved in the endocytic pathway: endoA promotes synaptic vesicle budding, and α-Adaptin is a subunit of the AP-2 complex (Guichet et al. 2002; Jafar-Nejad et al. 2002; Seto et al. 2002). The protein encoded by the *syd* gene was identified in a genetic screen in *Drosophila* for proteins involved in the axonal transport process (Bowman et al. 2000). Flies carrying mutations in *syd* reveal massive accumulations of various axonal membrane-bound cargoes, as well as axonal transport motors such as kinesin-I within the larval segmental nerves of multiple independent *syd* alleles. Syd is thought to function as a cargo adaptor in axonal transport by interacting directly with the kinesin light chain.

The ion homeostasis class consists of five genes involved in sodium and calcium ion exchange and calcium sequestration. Members of this group represent a new class of molecules that serve as substrates for ADAR and include *Calphotin* (*Cpn*), *Nckx30C*, and the related *CG1090*, *Na pump α-subunit* (*Atpα*), and *CG32699*. *Atpα* encodes a sodium:potassium-exchanging ATPase that undergoes a tyrosine-to-cysteine substitution due to editing. The Y390C substitution lies within the ATP hydrolase domain. The Atpα protein is predicted to contain 10 transmembrane-spanning segments and is detected in a subset of medial and lateral ventral cord neurons as well as in other larval and adult *Drosophila* tissues (Lebovitz et al. 1989). Animals carrying mutations in *Atpα* have behavioral abnormalities, reduced life span, and severe neuronal hyperexcitability along with the occurrence of age-dependent neurodegeneration (Palladino et al. 2003). The two related genes *Nckx30C* and *CG1090* encode for proteins that function as potassium-dependent sodium–calcium exchangers. Nckx30C is critical for the rapid extrusion of calcium and is expressed in adult neurons and during ventral nerve cord development in the embryo (Haug-Collet et al. 1999). *Cpn* encodes a photoreceptor cell-specific calcium ion binding protein (Ballinger et al. 1993; Martin et al. 1993), and *CG32699* is a gene that has an assigned GO functional attribute as having "calcium ion binding with acyltransferase activity."

The signal transduction and ion channel groups contain two members each. The genes *Mob1* and *boss* are grouped as signaling molecules, while the genes *SK* and *CG31116* are ion channels. The G-protein-coupled receptor *boss* and the two ion channels are discussed in greater detail in the next section. *Mob1* encodes a highly conserved protein shown to genetically and physically interact with Tricornered (*trc*), which is a member of the *Drosophila* Nuclear Dbf2 related (Ndr) subfamily of serine/threonine protein kinases (He et al. 2005). *trc* is required for the normal morphogenesis of polarized outgrowths such as epidermal hairs, sensory bristles, arista laterals, and sensory neuron dendrites (Geng et al. 2000; He and Adler 2002; Emoto et al. 2004). Furthermore, mutations in *Mob1* were isolated in a behavioral screen for genes involved in long-term memory (Dubnau et al. 2003).

Another unique class of edited target genes is involved in actin nucleation and organization, represented by *spire* (*spir*), *Ataxin 2* (*Atx2*), and *CG32809*. *spir* belongs to the posterior group of genes and is required for properly specifying the axis in the developing oocyte and embryo (Manseau and Schupbach 1989). *spir* mutants have a defect in microtubule plus-end orientation during oogenesis (Wellington et al. 1999). Recently, the gene product,

FIGURE 2. Structure and editing of *boss*. (*A*) The transmembrane protein bride of sevenless (boss) is portrayed in the background and is predicted to pass through the lipid bilayer seven times with a large extracellular domain. An enlargement of the region found to be edited is in the boxed foreground. Threonines 529 and 533 lie at the junction between the extracellular and the first transmembrane domains, which, when edited by ADAR, are recoded to alanines. Representation of boss was visualized with TMRPres2D (Spyropoulos et al. 2004), a tool used for modeling transmembrane proteins. (*B*) Sequence chromatograms of the edited region of *boss*. The *upper* panel is a sequence trace from RT-PCR products of *boss* from wild-type animals that shows a mixed signal of A and G at threonine 529 when compared to the trace derived from the control genomic DNA (*lower* panel). Threonine 533 appears to be completely edited and displays a pure G signal in the wild-type trace when compared to the genomic trace. The larval chromatogram in the *middle* panel displays an intermediate level of editing at these two positions, suggesting that editing of boss is developmentally regulated. The open box at the *top* is the protein translation with the edited threonine codons indicated.

TABLE 1. New targets of ADAR editing

Gene class	Symbol	cDNA	Protein	Residue change[a]	3′-UTR[b]	Molecular function
Vesicular traffic	*Rab26*	GH21984	CG7605-PA	K365R		GTPase (Yoshie et al. 2000)
	Rlip	GH01995	CG11622-PA	I229V, E230G, K233E, E254G, K265R	2478, 2480, 2482, 2486, 2740	Ral GTPase activator (Jullien-Flores et al. 2000)
	rab3-GEF	HL01222	CG5627-PA	Q2022R, S2054G	1306	Rab guanyl-nucleotide exchange factor (Lloyd et al. 2000)
	endoA	GH12907	CG14296-PA	K129R, K137E		Promotes synaptic vesicle budding (Verstreken et al. 2002)
	α-Adaptin	RH30202	CG4260-PA	T207A		Component of endocytosis, subunit of AP-2 (Gonzalez-Gaitan and Jackle 1997)
	syd	GH19969	CG8110-PA	S983G		Kinesin-dependent axonal transport (Bowman et al. 2000)
Ion homeostasis	*Cpn*	GH08002	CG4795-PB	S402G		Ca^{+2} sequestration (Ballinger et al. 1993; Martin et al. 1993)
	Nckx30C	HL01989, GH04818	CG18860-PC	K365R		K^+-dependent Na^+, Ca^{2+} antiporter (Haug-Collet et al. 1999)
	CG1090	GH23040	CG1090-PA	L357L, S358G, L387L		K^+-dependent Na^+, Ca^{2+} antiporter (Webel et al. 2002)
	Atpα	GH23483	CG5670-PD	Y390C		Na^+, K^+ exchanging ATPase (Palladino et al.2003)
	CG32699	HL01250	CG32699-PA	I175M		Ca^{+2} binding, acyltransferase activity[c]
Signal transduction	*Mob1*	RH70633	CG11711-PD	N91D		Activator of Trc kinase (He et al. 2005)
	boss	GH10049	CG8285-PA	T529A, T533A		G-protein-coupled receptor (Reinke and Zipursky 1988; Kramer et al. 1991)
Ion channel	*SK*	GH16664	CG10706-PD	Y377C	2788	K^+ channel (Kohler et al. 1996)
	CG31116	GH23529	CG31116-PA	K232R, T259A, K268R, E269G		Cl^- channel (Jentsch et al. 2005a)
Cytoskeletal components	*spir*	GH13327	CG10076-PC	K339R		Actin nucleation factor (Quinlan et al. 2005)
	Atx2	GH01409	CG5166-PA	K320R, K337R		Regulator of actin filament formation (Satterfield et al. 2002)
	CG32245	GH04632, GH25458	CG32245-PB	R296R, N297D	2225, 2248, 2249, 2656	Structural constituent of cytoskeleton[c]
Other	*CG32809*	GH23439	CG32809-PB	K179R		ATP binding[c]
	retm	GH05975	CG9528-PA	Q245R		Phosphatidylinositol transporter[c]
Unknown	*CG1552*	GH14443	CG1552-PA	K121R		None
	CG31531	GH25780	CG31531-PB	K679E		None
	CG3556	GH17087	CG3556-PA	I572V		None
	CG9801	GH23026	CG9801-PA	S345G		None
	l(1)G0196	GH02989	CG14616-PC	Q1148R, S1172G, Q1176R		None[d]
	CG12001	HL01040	CG12001-PA	I325V		None
	CG30079	HL05615	CG30079-PA	I127M, T303A, Q343R, Q358R, S360G		None

[a]Corresponds to the amino acid position of the indicated protein.
[b]Corresponds to the nucleotide position of the indicated cDNA clone.
[c]Inferred function based on Gene Ontology annotation.
[d]Animals with mutant alleles cause lethality during first and second instar larval stages.

Spir, has been shown to nucleate actin filaments in a unique way and may be a novel link between actin organization and intracellular vesicle transport (Kerkhoff et al. 2001; Quinlan et al. 2005). The amino acid change in Spir introduced by editing lies between two important conserved domains, the FYVE domain and the Spir box, which have been shown to be involved in its localization on intracellular membranes and possible association with Rab11, respectively. Whether this ADAR-modified residue in Spir alters its ability to nucleate actin filaments has yet to

be shown. Another target of ADAR in this class, *Atx2,* is not implicated in actin nucleation or polymerization, but rather as a possible regulator of specific mRNAs that function to mediate the formation of actin filaments. *Atx2* is the *Drosophila* homolog of the human polyglutamine disease gene *SCA2*, which is responsible for the dominantly inherited neurodegenerative disorder spinocerebellar ataxia type 2 (Huynh et al. 2000; Satterfield et al. 2002). In *Drosophila*, loss-of-function mutants of *Atx2* suggest that its principal function is to regulate the formation or bundling of actin filaments by regulating a subset of RNA transcripts that encode mediators of actin filament formation. Studies of the *SCA2* homolog, ATX-2, in *C. elegans*, demonstrate that it forms a complex with the poly(A)-binding protein PAB-1 and functions in translational regulation in the development of the germline (Ciosk et al. 2004). The last member in this class is *CG32245* and is placed here only through its associated GO functional attribute of "structural constituent of cytoskeleton."

There are two genes that comprise the "other" class, *CG32809* and *realtime* (*retm*). *CG32809* encodes a novel protein that is classified by GO as a proton-transporting ATPase. It also contains a partial Actin interacting protein 3 domain (AIP3, pfam03915) identified using the CD-Search engine at the National Center for Biotechnology Information (NCBI) (Marchler-Bauer and Bryant 2004). However, the K179R substitution due to editing of this transcript does not lie near the AIP3 domain. The gene *retm* has an assigned function as a phosphatidylinositol transporter whose protein contains SEC14 (cd00170), MSF1 (pfam04707), and CRAL_TRIO_N (pfam03765) domains. *retm* belongs to a novel family of mitochondrial proteins, is expressed in the developing midgut and central nervous system during embryogenesis, and is subcellularly localized to mitochondria (Dee and Moffat 2005).

The largest class of genes identified has no assigned function in the GO database and contains seven members (Table 1). Although this class does not have any associated GO attributes, mutations do exist for one of its members. Mutations in *CG14616* result in lethality during the first and second instar larval stages (Drysdale and Crosby 2005).

Functional consequences of editing targets

Three notable targets of ADAR are the integral membrane-spanning proteins encoded by the genes *boss*, *CG31116*, and *SK*. The G-protein-coupled receptor boss is unique in that it acts as a ligand to determine the fate of the R7 photoreceptor cell in the *Drosophila* compound eye by a specific inductive interaction between the boss-bearing R8 photoreceptor neuron and the R7 precursor cell (Fortini et al. 1992; Hart et al. 1993). Interestingly, the edited sites convert threonines 529 and 533 to alanines, which lie at the junction of the large extracellular domain and the first transmembrane domain (Fig. 2A). At the larval stage, the T533 codon

FIGURE 3. (Legend on next page)

is a mix of signals, whereas in the adult, the T533 codon contains a pure G signal, indicating this is likely the predominant form of boss in the adult fly (Fig. 2B). Furthermore, this suggests that editing of boss at these sites is developmentally regulated. These substitutions may have an effect on the way in which the R8 photoreceptor cell presents boss to neighboring R7 cells, potentially modifying the action of boss at later stages in eye development.

The gene *CG31116* encodes a chloride channel belonging to the highly conserved ClC chloride channel family. In humans, mutations of ClC family members are associated with and underlying myotonia, Dent's disease, Bartter syndrome, osteoporosis, neurodegeneration, and possibly epilepsy (Jentsch et al. 2005b). Some ion channels, such as K^+ channels, possess structurally distinct elements that perform the selective conduction of ions (filtering) and the actual opening or closing of the pore (gating) (Jiang et al. 2002). Crystallographic and electrophysiological studies of the anionic ClC channels from *Escherichia coli* and *Salmonella typhimurium* elegantly show that the processes of gating and filtering are closely tied (Dutzler et al. 2002) and that the side-chain carboxyl group of a glutamate residue serves as the gate of the pore lying within the selectivity filter (Fig. 3A,B). Obstructing the pore with its side chain closes the pore, while binding of a Cl^- ion opens it. We have identified four edited sites, two of which affect residues in the selectivity filter (Fig. 3C). One of these residues is the highly conserved glutamate residue whose side chain acts as the anionic gate. We have found this glutamate codon to be edited in ~10% of the adult head transcripts to encode a *Gly* residue, which interestingly is a residue that does not contain the carboxyl side chain capable of closing the ion pore and would be consistent with an open pore conformation. Electrophysiologic and crystallographic studies testing channel function support this observation. Converting the glutamate residue in the *Torpedo* ray ClC-0 channel to Ala, Gln, or Val causes the channel gate to stay open (Dutzler et al. 2003). Moreover, *CG31116* is expressed in the ventral nerve cord, lateral cord glia, and the ventral midline, consistent with a neuronal function (Kearney et al. 2004).

Another striking example of putative functional consequences of RNA editing on protein function is the transcript for the gene *SK*. This gene encodes a voltage-independent ion channel that is activated by submicromolar concentrations of intracellular calcium. These channels are high-affinity calcium sensors that transduce fluctuations in calcium concentrations into changes in membrane potential. SK channels are not gated by direct calcium binding, but are heteromeric complexes with calmodulin (CaM), where gating is mediated by binding of calcium to calmodulin and subsequent conformational alterations in the channel protein (Xia et al. 1998). The edited form of the SK transcript results in the tyrosine at position 377 being converted to a cysteine. This Y377C substitution is directly within the highly conserved calmodulin-binding domain (CaMBD) (Fig. 3D). The crystal structure of the CaMBD from rat SK2 complexed with apoCalmodulin reveals that the key region of CaMBD involved in apoCaM binding consists of 10 residues, which form an α-helix that binds only the C lobe of CaM (Schumacher et al. 2001). Interestingly, the structure suggests a van der Waals contact between the side chain of Y435 and residues in the E and H α-helix of CaM (Fig. 3E). Tyrosine 435 in the rat SK2 channel is the cognate tyrosine at position 377 that is edited in the *Drosophila* SK channel. Whether this Y377C substitution has a functional role in the ability of calmodulin to bind to the channel and therefore affect the transductive capacity of calmodulin remains to be tested.

Editing in *D. melanogaster* is thought to be a gene sparing strategy. That is, a different form of the same protein is used at different times in the animal's life cycle to perform similar functions and is facilitated by the differential activities of ADAR throughout development (Keegan et al. 2005). Our observations, including the example of

FIGURE 3. Structure and editing of ClC and SK ion channels. (*A*) Schematic of a ClC chloride channel conductance filter showing the closed and open conformations. The chloride ion is represented by red spheres, and the glutamate side chain is shown in red. Notice in the closed configuration the glutamate 148 side chain is occupying the ion binding site, while in the open configuration, it has moved out of the site and is replaced by a Cl^- ion. Reprinted (with permission from AAAS © 2003) from Dutzler et al. (2003). (*B*) Ribbon structure of the ClC conductance filter depicted in the closed configuration with a chloride ion shown as a red sphere with the side chain of the edited glutamate 148 colored red. The amino acids at the ends of the α-helices, including the glutamate 148 at the end of helix F, are brought together near the membrane center to form the ion-binding site, shown in light blue. The vestibules toward the interior and exterior of the membrane are depicted as hatched domains. Reprinted (with permission from Macmillan Publishers Ltd. © 2002, http://www.nature.com/nature/index.html) from Dutzler et al. (2002). (*C*) Sequence alignment of the edited portion of ClC channels. Residues from the Cl^- selectivity filter are represented by the gray shaded boxes, and identical residues are indicated with an asterisk. The four residues whose codons are edited in *D. melanogaster* are indicated by black boxes. The α-helices are depicted as open boxes below the alignment. The amino acid sequences and GenBank numbers are *Mus musculus* ClC2, NP_034030.1; *Oryctolagus cuniculus* ClC2, AAB05937.1; *Homo sapiens* ClC2, AAB34722.2; *D. melanogaster* GH23529p, AAM50193.1; *D. melanogaster* CG31116-PA, AAF54701.3; *Anopheles gambiae*, XP_312021.2; *S. typhimurium*, AE008704. The sequence alignments were performed with CLUSTALX (Thompson et al. 1997). (*D*) Sequence alignment of the Calmodulin binding domain of SK channels. The tyrosine 377 residue whose codon is edited in *D. melanogaster* is indicated by a black box. Identical residues are indicated with an asterisk, and the Calmodulin binding domain is depicted as an open box *below* the alignment. The amino acid sequences and GenBank numbers are *D. melanogaster* CG10706-PD, NP_726987.1; *D. melanogaster* GH16664p, AAM50183.1; *A. gambiae*, XP_314474.2; *M. musculus* SK1, AAK48900.1; *H. sapiens* SK1, AAH75037.3; *H. sapiens* SK2, AAP45946.1. The sequence alignments were performed with CLUSTALX (Thompson et al. 1997). (*E*) Ribbon structure of the rat SK2 calmodulin binding domain (colored in light blue) complexed with the apoCalmodulin C lobe (in purple). Notice that tyrosine 435 (colored in red) of SK2, which is the highly conserved tyrosine 377 in *D. melanogaster*, is involved in the interaction with apoCalmodulin. Reprinted (with permission from Elsevier © 2004) from Schumacher et al. (2004).

TABLE 2. Primer pairs for RT-PCR

Gene	Sense primer/antisense primers for RT-PCR
CG10076	5′-CAGAGCAACCTGGACGAGA-3′/5′-TTCGCATCTTGGTATAGCA-3′
CG10706	5′-CTCTAATGACAATATGTCCGGG-3′/5′-TGTGTTTGCATTATCCATTAGT-3′
CG1090	5′-CTGGTTTCCTGGTACGTGGT-3′/5′-TATTACGTTGACGTGCGAGC-3′
CG11622	5′-AGTGCTGGCACTTGGGTATC-3′/5′-ATTGCACTTGGGCAATTGTT-3′
CG11711	5′-GAGTTCTCCTCCATTAGCCAA-3′/5′-AGTCCAAACCAGCTGGAAG-3′
CG12001	5′-TTTCTTGACCTGTTGGAGGC-3′/5′-TGTCGTAGCCAAATCCCTTC-3′
CG14296	5′-GATCAACAAGGCCAACCAGT-3′/5′-TGGCTCCAAAAAGTTCTGCT-3′
CG14616	5′-CTAAACGAGGAGCTGGCATC-3′/5′-TTGCTAAAACCACCGAAACC-3′
CG1552	5′-GCCTCGTCCTTTTGAGCCT-3′/5′-TGAGGAGCGAGTTATTGGG-3′
CG18860	5′-AACTCGACCACCACGAAAAC-3′/5′-GATCAGCACCAGCAAACTGA-3′
CG30079_1	5′-TGCGAAACCCTCCAGAAAC-3′/5′-CGGTGAGGGATACGTTTGCT-3′
CG30079_2	5′-CGCGTTACCAGCCCATCC-3′/5′-TGATCTGGGGTATGGATAGCG-3′
CG31116	5′-GATCTGACGTCACAGCCTTTTG-3′/5′-TGGAATGATGTGACAAGTTTAC-3′
CG31531	5′-GCGGTCAACCAAATTGAA-3′/5′-CTGGGTAGGAATCGGAGT-3′
CG32245	5′-TATCTTCCGTTTGGGTCGTC-3′/5′-CGTCATACGCTCGCTGAGTA-3′
CG32699	5′-CAAATGCAATACATGACGGC-3′/5′-GGCGTAATACTCCAGGTCCA-3′
CG32809	5′-GAAAACTCTGGAACGACCG-3′/5′-TTCCGTTCTGAAGAGTACA-3′
CG3556	5′-CCGAAATGGATCCCAGATT-3′/5′-TTGATTACGCCATTATTAT-3′
CG4260	5′-TTCCAACGAAATACCCAAGC-3′/5′-GTAGTTTTGCAGCAATCGCA-3′
CG4795	5′-GTGCTTCGACTGAGCCCC-3′/5′-GCCGTAGTTGGTGGAGTA-3′
CG5166	5′-ACCGCAGCTATCGCATGT-3′/5′-CGCCCATGTGAGATTGGT-3′
CG5627	5′-GGCCATTTCACGTAGCGG-3′/5′-CACAGCCAGGGGATACAGA-3′
CG5670	5′-GGCTGGACGCTGTCATCTT-3′/5′-CGGCAATCTTCTTATTACG-3′
CG7605	5′-GAGTACGCGCAGGAGGAC-3′/5′-CGGGCTTATTGTTGTCTT-3′
CG8110	5′-AATCGCTGAACCCCATACTG-3′/5′-GAGGCAACGTATCGAATGGT-3′
CG8285	5′-CCCATGATACGGCTGAGAT-3′/5′-TGCTCACCCACGTATTCAA-3′
CG9528	5′-GATCAGCAGCATAGCATCTTG-3′/5′-CGGCGGATTCATTGATCTTCT-3′
CG9801	5′-TGCGACTTCGCATATGGT-3′/5′-CTCAGCTCTGGCTTGTTG-3′
CG11622_UTR	5′-CCTCCTTGGCCTCGGGAT-3′/5′-CGCCGTTTAACAGTAATTATAGTTTGAA-3′
CG5627_UTR	5′-ATCGATGTCATCATCATCCG-3′/5′-GGACTTACAAATGTTTTAACAATTTTGAA-3′
CG10706_UTR	5′-AACGGCCATCACATTTCTGT-3′/5′-CCAATGATATTTCCCCATTCC-3′
CG32245_UTR1	5′-CCGCAGCAGCAAGATCAA-3′/5′-AGATCCCTCGTTTCTTTCTTGA-3′
CG32245_UTR2	5′-TTGAGTTACTATCCTTTAGGAAATGCT-3′/5′-TGTCAACAGTTCATTGAATATTTTGG-3′
CG32245_UTR3	5′-AACCAGTTTTGAAAGTCTTGAATTG-3′/5′-AATTAAGGTGTCAATTGGTAAAACAGT-3′
CG32245_UTR4	5′-AATACAACTGTTAATCTGTTTCCACTG-3′/5′-GCTCTTTTGGATACCAAAAAAGTTGAAGAAAT-3′

the effect on Cl^- gating, the possible developmental alteration of the activity of the boss ligand, and the other possible functional implications of editing in the 3′ UTRs, suggest an even greater role of editing by modulating specific protein function and transcript stability. It is very likely that more targets of editing exist in *Drosophila* because the bias of our sampling method was limited to the abundant mRNAs represented in our cDNA libraries coupled with the observation that editing is not 100% efficient. In summary, this analysis has identified 38 editing targets, 27 newly discovered, which doubles the total number of editing targets in *D. melanogaster* to 55.

MATERIALS AND METHODS

Identification of putative ADAR editing targets

We compared the nucleotide sequence of all full-length cDNA clones derived from libraries of adult head tissue to the Release 4 genome sequence and to the Release 4.1 computed gene models. Using Sim4 (Florea et al. 1998), we recorded all A>G substitutions, then manually inspected each clone for sequence quality discrepancies and Sim4 artifacts using Consed Autofinish (Gordon et al. 2001). This produced a curated list of 108 genes that were chosen as candidates for experimental testing.

Validation of targets

We used a similar method for RNA isolation and RT-PCR described previously (Stapleton et al. 2002a). RNA and genomic DNA were isolated at the same time using the Trizol reagent (Invitrogen) from adult heads from the isogenic strain *y^1; cn^1 bw^1 sp^1*. For the *boss* analysis, we isolated RNA from 0–24-h embryos, a mixed stage of larvae, and pupae from the isogenic strain. RT-PCR amplicons of the *boss* region from embryo and pupal RNA were negative, but positive for the larva RNA. RNA preps were digested with RNAase-free DNAase I (Roche) and tested by PCR to ensure the absence of contaminating genomic DNA. We designed gene-specific primers for the RT-PCR and the genomic PCR amplifications using Primer3 (Rozen and Skaletsky 2000) and the primer-picking feature in Consed Autofinish. The

TABLE 3. Primer pairs for genomic PCR

Gene	Sense primer/antisense primers for genomic PCR
CG10076	5′-TAAGAGAAGTCCTGCGGAGC-3′/5′-ATCAACGACTTGGGGAACTG-3′
CG10706	5′-GCAGTTGACGAAACGGGTAT-3′/5′-ATTTCCCCATTCCAAAGACC-3′
CG1090	5′-TCAGGAAAACATGGGCTACC-3′/5′-TATTACGTTGACGTGCGAGC-3′
CG11622	5′-CCCACAAGGTCAAGGAGAAA-3′/5′-ATTGCACTTGGGCAATTGTT-3′
CG11711	5′-GCCACTCCTTACCGACAGAA-3′/5′-AGCGTTTAGTTCCCCCAGTT-3′
CG12001	5′-GCAATGCATTTGGTTTGATG-3′/5′-AGCTACCATACTTCCGCCCT-3′
CG14296	5′-GATCAACAAGGCCAACCAGT-3′/5′-CCACGAATCTCATCGTCCTT-3′
CG14616	5′-GAGACATCGCTCCTACAGCC-3′/5′-CCGGAAGAGGAGACCGTTAT-3′
CG1552	5′-TTGGTTCACAAATCGCAGAA-3′/5′-CACCCTAACCCAACCTAGCA-3′
CG18860	5′-CTCCCGATTAAAGCCATCAA-3′/5′-CTAGCTTCCTGCTAATGCCG-3′
CG30079_1	5′-AGAGCCCGTCAGTGAAGTGT-3′/5′-AAGGAGTGCGAAGAGAACGA-3′
CG30079_2	5′-TGCTGCTCTTGATGTTCGAC-3′/5′-ACACTTCACTGACGGGCTCT-3′
CG31116	5′-CCGCCAACATTTCAGAGTTT-3′/5′-GCACCTCCAATCTGAGAACC-3′
CG31531	5′-ATTGCGGGACATGGAATTTA-3′/5′-TTAAAAATCTCCACACGCCC-3′
CG32245	5′-CGGCTATAAAGTGCACAGCA-3′/5′-GCTCGACGAAGTCGCTAGAT-3′
CG32699	5′-CAGCCAGAGAAGGCGTAATC-3′/5′-CAAATGCAATACATGACGGC-3′
CG32809	5′-AAGCCCTACATCCTCACCCT-3′/5′-ATCATGTCGGAGGTGGAAAC-3′
CG3556	5′-GGATCCCAGGTAAAACAGCA-3′/5′-ACCATTAGCTCGTCCACACC-3′
CG4260	5′-GTAGTTTTGCAGCAATCGCA-3′/5′-TTCCAACGAAATACCCAAGC-3′
CG4795	5′-TGTTGTAACTTCGACAGGCG-3′/5′-ACCTGTTGTTGCAGAAACCC-3′
CG5166	5′-GGTGACTTCCAGTTGAGGGA-3′/5′-TTGCGAGTTACCCTGGTAGG-3′
CG5627	5′-TACACCGCCACAGTATACGC-3′/5′-CACAGCCAGGGGATACAGAT-3′
CG5670	5′-GATCCACCATTTCATCCACC-3′/5′-GTTCCATGCACTTGAGCAGA-3′
CG7605	5′-TGTGGAGCTGTCCTTCACAG-3′/5′-ATCGGGAGGATATGGCTCTT-3′
CG8110	5′-TACGTACGGCTCAATGTCCA-3′/5′-ACTGGAGACCAAGGATCGTG-3′
CG8285	5′-AGATTGCGTCGCAGAACTTT-3′/5′-GGGTATTCAGAGTCTGGGCA-3′
CG9528	5′-AAGTGTTCCACTACCACGGC-3′/5′-TGAAGCAGTACACCCAGACG-3′
CG9801	5′-TTAGTTCCCGTACTCCGTGC-3′/5′-ATGGTGTCAACTGGGGTGAT-3′
CG11622_UTR	5′-TGGGCATGGAACTAAAGGAG-3′/5′-AGATTTCTTTGGGGCTTTGC-3′
CG5627_UTR	5′-CTTGTCATCATCCACATCGC-3′/5′-ACATGGATTAGGAAAGCCCC-3′
CG10706_UTR	5′-GCAGTTGACGAAACGGGTAT-3′/5′-CCAATGATATTTCCCCATTCC-3′
CG32245_UTR1	5′-CCGCAGCAGCAAGATCAA-3′/5′-AGATCCCTCGTTTCTTTCTTGA-3′
CG32245_UTR2	5′-TTGAGTTACTATCCTTTAGGAAATGCT-3′/5′-TGTCAACAGTTCATTGAATATTTTGG-3′
CG32245_UTR3	5′-AACCAGTTTTGAAAGTCTTGAATTG-3′/5′-AATTAAGGTGTCAATTGGTAAAACAGT-3′
CG32245_UTR4	5′-AATACAACTGTTAATCTGTTTCCACTG-3′/5′-GCTCTTTTGGATACCAAAAAAGTTGAAGAAAT-3′

sense/antisense primer pair sequences for regions of the 27 validated genes are shown in Tables 2 and 3

Previously, we cloned PCR products from multiple RT reactions in order to evaluate the potential for editing. However, in this report, we directly sequenced the PCR products. For sequencing, PCR products were treated with 0.3 μL of Exonuclease I (USB), 0.5 μL of Shrimp Alkaline Phosphatase (Roche), and 0.5 μL of 1 M Tris (ph 8.0) in a final reaction volume of 75 μL and incubated for 45 min at 37°C and then heat-inactivated for 10 min at 65°C. The same sense and antisense primers used for RT-PCR and genomic PCR were used as sequencing primers in a 10-μL reaction containing 1 μL of ABI Big Dye III terminator mix (Applied Biosystems). Sequencing reactions were precipitated according to the manufacturer's protocol and loaded onto an ABI Prism 3730 DNA Analyzer. Sequence data were assembled and analyzed with the Phred/Phrap suite of programs and visualized with Consed. Chromatogram trace peaks at positions chosen for interrogation that displayed an unambiguous mixture of A and G signals or a pure G signal were recorded as being a positive target of ADAR. For the RT-PCR amplicons that were ambiguous or weak, we cloned PCR products from two independent RT-PCR reactions and picked 48 clones each for sequence analysis.

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