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dADAR, a *Drosophila* double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing

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ABSTRACT

We have identified a homolog of the ADAR (adenosine deaminases that act on RNA) class of RNA editases from *Drosophila*, *dADAR*. The *dADAR* locus has been localized to the 2B6–7 region of the X chromosome and the complete genomic sequence organization is reported here. *dADAR* is most homologous to the mammalian RNA editing enzyme ADAR2, the enzyme that specifically edits the Q/R site in the pre-mRNA encoding the glutamate receptor subunit GluR-B. Partially purified dADAR expressed in *Pichia pastoris* has robust nonspecific A-to-I deaminase activity on synthetic dsRNA substrates. Transcripts of the *dADAR* locus originate from two regulated promoters. In addition, alternative splicing generates at least four major dADAR isoforms that differ at their amino-termini as well as altering the spacing between their dsRNA binding motifs. *dADAR* is expressed in the developing nervous system, making it a candidate for the editase that acts on *para* voltage-gated Na^{+} channel transcripts in the central nervous system. Surprisingly, *dADAR* itself undergoes developmentally regulated RNA editing that changes a conserved residue in the catalytic domain. Taken together, these findings show that both transcription and processing of *dADAR* transcripts are under strict developmental control and suggest that the process of RNA editing in *Drosophila* is dynamically regulated.

Keywords: ADAR; inosine; ion channel; nervous system

INTRODUCTION

RNA editing involving the conversion of adenosine (A) to inosine (I) in pre-mRNA is a posttranscriptional process catalyzed by proteins of the ADAR (adenosine deaminases that act on RNA) family (Rueter & Emeson, 1998). ADARs require duplex RNA secondary structure in their target substrates. There are two types of A-to-I RNA editing, promiscuous and specific (reviewed in Bass, 1997). Viral RNA genomes provided the first reported instance of promiscuous A-to-I RNA editing where extensive conversion of A to G (guanosine) was observed (Cattaneo et al., 1988). Since I base pairs with cytidine, G replaces I when cDNAs are generated. Mechanistically, cRNA intermediates of the replication process or transcripts base pair with complete complementarity to the viral genomic RNA. When the substrates for ADARs are extensive duplexes, up to 50% of adenosines are converted to inosine. Such promiscuous editing has been proposed to have roles in gene regulation, viral defense (Bass & Weintraub, 1988) and in biased hypermutation and persistent infection of certain viruses (Bass, 1997). Promiscuous editing is also seen in vivo when naturally occurring antisense transcripts are generated, as in the case of the *Xenopus* bFGF gene (Saccomanno & Bass, 1999) and in polyoma virus (Kumar & Carmichael, 1997). Supporting a role in gene regulation is the finding that extensively modified early transcripts from polyoma are almost exclusively retained in the nucleus at late times in infection, thus preventing translation, a critical feature of the polyoma life cycle (Kumar & Carmichael, 1997). Another potential role in mRNA regulation via extensive editing was recently proposed in *C. elegans* where sev-

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eral transcripts containing extended 3′ noncoding hairpin structures are extensively modified within hairpin regions (Morse & Bass, 1999).

ADARs can also edit specific adenosines in mRNA precursors (pre-mRNA). Surprisingly, most of these examples are restricted to genes encoding ligand-gated ion channels or receptor genes expressed in the nervous system and result in only one or a few adenosines deaminated per transcript (Rueter & Emeson, 1998). Because the translation machinery has been shown to interpret I as G (Basilio et al., 1962), modifications introduced by an ADAR are predicted to change the coding potential of the edited transcripts. This was first shown for pre-mRNAs encoding subunits of the glutamate-gated ion channel receptors (GluRs). The GluR-B transcripts have numerous distinct RNA editing sites that, when edited, have profound consequences for channel function (reviewed in Seeburg et al., 1998). For instance, editing that results in the conversion of a glutamine codon (CAG) to a arginine codon (CGG) at the Q/R site alters the Ca^{2+} permeability of channels containing the edited subunit (Kohler et al., 1993). The physiological significance of editing at the Q/R site is demonstrated by the neurological dysfunction and postnatal lethality associated with mutant mice expressing an editing incompetent allele of GluR-B (Brusa et al., 1995; Feldmeyer et al., 1999). Other examples where A-to-I RNA editing has been shown to perform the role of diversifying protein isoforms encoded by a single genetic locus are the mammalian serotonin receptor (5-HT_{2C}R) (Burns et al., 1997) and the squid potassium channel (sqKv2) (Patton et al., 1997). Specific editing of the pre-mRNA encoding an invertebrate voltage-gated Na^+ channel, the product of the *Drosophila para* locus, has recently been demonstrated (Hanrahan et al., 1999; Reenan et al., 2000).

Mechanistically, specific editing presents a more complex secondary structure requirement in the RNA substrate than the promiscuous editing that occurs on extended perfect duplexes. The best characterized specifically edited substrates are the regions of GluR subunit transcripts spanning the Q/R site. It was first shown for the GluR-B pre-mRNA that specific editing of the exonic adenosine at the Q/R site requires *cis*-acting sequences several hundred nucleotides away in the intron downstream of the editing site (Higuchi et al., 1993; Egebjerg et al., 1994). The intron region necessary for editing has extensive complementarity to and base pairs with the exonic region surrounding the editing site and was termed an editing-site complementary sequence (ECS). Further studies of the Q/R site in transcripts of the kainate GluR-5 and -6 paralogs showed that these complementary intronic sequences can be over 1,900 nt away (Herb et al., 1996). Mutagenic studies of the GluR Q/R site suggest that the primary determinant of specificity is in the local duplex structure surrounding the edited adenosine (Maas et al., 1996). Recent work suggests that long RNA duplexes in which many adenosines are converted can be rendered into more specific substrates by duplex interruptions, such as loops, supporting the role of local structural elements in directing ADAR specificity (Lehmann & Bass, 1999).

Both ADAR1 and ADAR2 can edit specific adenosines and have promiscuous editing activity on extended double-stranded (ds) RNA. Enzymes homologous to ADARs but having no proven A-to-I deaminase activity are tentatively classed as ADAR-like. Such a mammalian homolog of ADAR2, called RED2, has been identified but has not been shown to edit any known substrate (Melcher et al., 1996a). Recently a related family of enzymes, the ADATs (adenosine deaminases acting on tRNAs), has been identified that convert A-to-I at specific positions in tRNAs (Gerber et al., 1998; Gerber & Keller, 1999; Keegan et al., 1999; Keller et al., 1999; Maas et al., 1999).

Inosine has been shown to be present in mRNAs from numerous tissues and to occur at tissue-specific levels in rat (Paul & Bass, 1998). ADAR activities are ubiquitous in nature and have been detected in invertebrate (Skeiky & Iatrou, 1991; Bass, 1993) and vertebrate animals and are expressed in a wide variety of primary tissues and cells within organisms (Wagner et al., 1990). Moreover, ADAR activity has been detected in *Drosophila* extracts (Casey & Gerin, 1995; M. O'Connell, unpubl. observation). We report here the cloning and characterization of a *Drosophila* dsRNA-dependent adenosine deaminase, *dADAR*. Recombinant dADAR was overexpressed in the yeast *Pichia pastoris*, and has robust A-to-I editing activity on extended dsRNA. *dADAR* is expressed in the nervous system of *Drosophila* embryos making it a candidate enzyme responsible for the editing of pre-mRNAs encoding the *para* voltage-gated Na^+ channel that is expressed exclusively in the nervous system (Hong & Ganetzky, 1994). The *dADAR* locus is subject to both transcriptional and posttranscriptional regulation. Two promoters, one specific to the adult stage, generate transcripts with different 5′ untranslated regions. *dADAR* transcripts undergo developmentally regulated alternative splicing at numerous sites, generating four potential alternative in-frame translational start sites and altering the spacing of dsRNA binding motifs (DRBMs). Surprisingly, *dADAR* transcripts themselves are the target of a very specific and highly regulated RNA editing event that alters a conserved amino acid in the deaminase domain portion of the protein.

RESULTS

Cloning and genomic organization of *dADAR*

ADARs have common structural motifs including DRBMs and an adenosine deaminase catalytic domain

(Fig. 1A). We compared the amino-acid sequences of mammalian ADARs as well as an ADAR-like *Caenorhabditis elegans* homolog (Hough et al., 1999) to design degenerate PCR primers (Fig. 1B). The catalytic domain is the most highly conserved region of ADARs. Therefore, PCR was performed on a *Drosophila* whole adult cDNA library using degenerate primers that would amplify products encoding either the adenosine deaminase domain or a region encompassing both the deaminase domain and ADAR1 or ADAR2 type DRBMs (see Materials and methods). A PCR product was obtained with a degenerate primer pair spanning the deaminase domain. Cloning and sequence analysis confirmed that the product had significant homology to the adenosine deaminase domain of ADARs; however, it appeared not to have significantly higher homology to ADAR2 (32/62 amino acid identity) than to ADAR1 (30/62 amino acid identity). Gene-specific primers from within this cloned deaminase domain region and degenerate primers for either ADAR1 or ADAR2-type DRBMs were used in an additional round of amplification. Products were obtained with ADAR2-specific degenerate primers. Cloning and sequence analysis confirmed that the product had a high degree of homology to mammalian ADAR2 and we have designated this gene *dADAR*. In situ hybridization to *Drosophila* polytene chromosomes revealed a single band localizing to the 2B6–7 region of the X chromo-

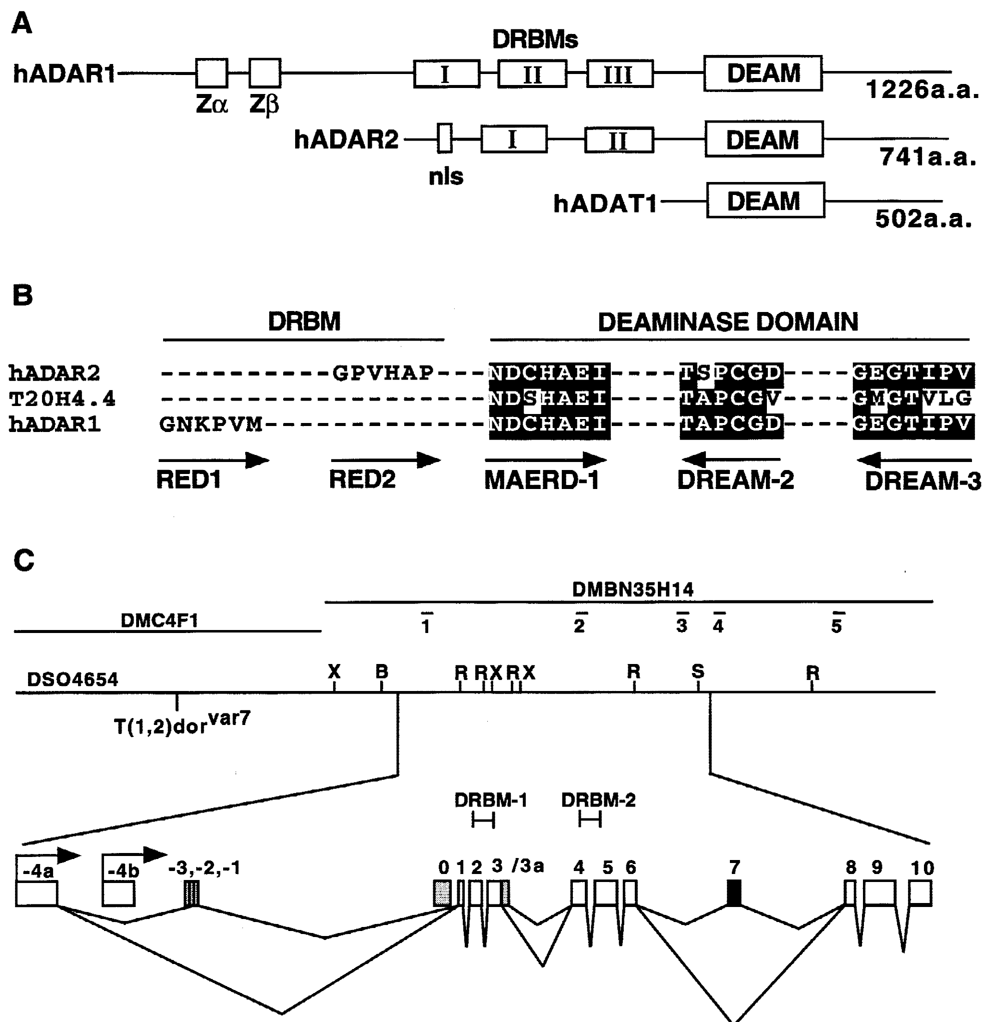

FIGURE 1. Genomic location and organization of *dADAR*. **A**: Domain organization of the ADAR1, ADAR2, and ADAT class of editases. Zα and Zβ are Z-DNA binding domains. DRBM: double-strand RNA binding motif. DEAM: deaminase domain. nls: nuclear localization signal. Roman numerals refer to the different DRBMs. **B**: Amino-acid sequence alignments of ADAR1 and ADAR2 classes of editase and the *C. elegans* ceT20H4.4 ADAR-like homolog in the regions where degenerate PCR primers were designed. **C**: Compiled genomic sequence organization and transcription unit of the *dADAR* locus. Genomic sequence was generated from the P1 bacteriophage DS04654. Overlap with European *Drosophila* Genome Project (EDGP) cosmid clone DMC4F1 and BDGP bacterial artificial chromosome (BAC) clone DMBN35H14 are shown. In the restriction map of DS04654 X: *Xho*I, B: *Bam*HI, R: *Eco*RI, S: *Sal*I. Numbers above the P1 clone refer to sequence tag sites (STS) found in DS04654 (1,2,3,4,5). Open boxes represent constitutive exons, gray boxes are alternative exons. The black box is exon 7, where RNA editing occurs.

some (data not shown). Bacteriophage P1 clones from this region were obtained (see Materials and methods) and PCR was used to identify the presence of this gene. Thirty kilobases of genomic sequence around the gene were generated to define and orient the transcription unit (Fig. 1C). The *dADAR* locus lies approximately 180 kb centromere proximal to the *Broad Complex* (*BrC*) and about 100 kb distal to the *armadillo* locus. Also, the 5′ most transcription start sites for *dADAR* are about 7 kb centromere proximal to the known translocation breakpoint T(1,2)dor^{var7}.

cDNA analysis of *dADAR*

To obtain full-length cDNA clones of the *dADAR* gene, a modification of RACE was used to obtain both 5′ and 3′ ends of cDNAs (see Materials and methods). Full-length cDNA clones revealed a number of interesting regulatory features concerning the *dADAR* locus (Figs. 1C and 2). First, *dADAR* transcripts begin with two different alternative 5′ exons that are untranslated and have heterogeneous start sites. No consensus TATA box was found upstream of the start sites for exon −4a-containing transcripts, but a number of transcripts begin at consensus eukaryotic transcription initiator sequences Py Py A_{+1} N T/A Py (Lo & Smale, 1996). Developmental studies (see below) also indicate that the exon −4a- and −4b-containing transcripts originate from alternative promoters. Secondly, several alternative splice forms were found in the 5′ end of the cDNAs and were predicted to generate alternative starting methionines (alternative exons −3, −2, −1, and 0, Fig. 2). Translation was predicted to begin either in exon −2, −1, 0, or 1, depending on exon utilization. The sequences surrounding the exon −2 and 1 starting ATGs presented the strongest flanking consensus sequences (Cavener, 1987). Developmental data indicated that the exon −1- and 1-containing forms were the most common, suggesting that these starting ATGs were the most frequently utilized start sites (see below). However, the less frequent exon −2 and 0 putative start sites shared homology at the amino-acid level (exon −2 = MKFDS, exon 0 = MKFEC) and thus may represent important *dADAR* isoforms.

Alternative splicing was observed between the two DRBMs of *dADAR* (± exon 3a) and this splicing alters the distance between these important structural motifs (Figs. 2 and 3). In addition, the distal 5′ splice donor site of exon 3a is a rare nonconsensus splice donor site (GCAAG vs. GTAAG), the implications of which will be addressed in the discussion.

The predicted dADAR protein is approximately the same size as the mammalian ADAR2 homologs (dADAR = 670 amino acids, ratADAR2 = 711 amino acids, ratRED2 = 746 amino acids). Overall, dADAR has 43% identity to rADAR2 and 35% identity to RED2. Within the deaminase domain, however, the homology rises to 46% identity for hADAR1 and 67% identity for hADAR2 enzymes. Residues important for coordinating zinc in the catalytic domain are also absolutely conserved (Betts et al., 1994; Kim et al., 1994, Lai et al., 1995) (Fig. 3). In contrast, comparison with a tRNA-specific adenosine deaminase, dADAT1, revealed only 17% identity overall and 28% identity within the catalytic domain (Keegan et al., 1999).

dADAR encodes a dsRNA-specific adenosine deaminase

The *dADAR* cDNA clone pSR1–9 was subcloned into the *P. pastoris* expression vector pSK-FLIS6 to express a fusion protein with a FLAG epitope at the amino terminus and six histidine residues at the carboxy terminus. The full-length *dADAR* produced from this construct starts at the exon 1 ATG and includes exon 3a. The recombinant fusion protein was purified by chromatography of a crude extract of *Pichia* expressing dADAR over a Ni^{2+}-NTA column and assayed for its ability to convert adenosine to inosine in dsRNA (Fig. 4A). *Pichia* does not contain any endogenous dsRNA adenosine deaminase activity (Gerber et al., 1998). The load fraction contained RNases that degraded the dsRNA substrate before it could be deaminated and assayed. Therefore, more activity is observed with 1 μL of the crude extract than with 6.25 μL. Immunoblot analysis was also performed on input and eluate fractions from the Ni^{2+}-NTA column with FLAG monoclonal antisera and revealed a band of approximately 80 kDa that comigrated with dsRNA adenosine deaminase activity (Fig. 4B). This band was very faint in the load fraction. This may be due to protein degradation, as many bands of lower molecular weight were observed that cross-react with the antibody.

It is also possible that the protein was not highly expressed as very little was recovered from the FLAG affinity column. The peak fraction from the Ni^{2+}-NTA column was chromatographed over a FLAG affinity matrix and assayed for dsRNA adenosine deaminase activity. A protein of ~80 kDa was detected when approximately 400 μL of the peak fractions from the FLAG affinity matrix were TCA precipitated and electrophoresed on an SDS-polyacrylamide gel that was silver stained (Fig. 4C). The yield of recombinant dADAR protein from *Pichia* was very low and it was not possible to determine the specific activity.

Developmental expression and alternative splicing profile of *dADAR*

Quantitative RT-PCR analysis was performed using primers specific for the −4a or −4b exons and a primer in constitutive exon 4, which is downstream of all of the sites of alternative splicing. Ribosomal protein 49 (*rp49*) expression was used as an internal control (see Ma-

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      2 3                  2  5            2                                   2
*     * *  *  *       ** ** **  **    *    **                           *      **
TGCTCACATCGCTAGATTCCGAGCAACAAAAAAGCGACGAAGGGTCATTGTTATTTTTCTAATGCAATGTAATGCAGCAAATGTGCAGATTT  93
GAACAAGTGTAACGCGATTTATGTTTAATCCGCATCGAGGAAACCAAATCGAAGTAAACGCGCGGCCAGAGAAAAGAGCAGCACCGCACCGAA  186
GATAAAACAAACAAGCATTGATTTGTTTGTCCGGCCATGTCAGTGTGTGTGTCACAAGGGCTTGCAAGTGTGTGCGTGTGTCCGTGCGTGTAC  279
GTGGGTGCTTTTTTTTTCTCCCGAGTGCCGCCAAGTGAAATATGCAATTGCACGCCACTGCAGTTGCAGCCACACAAATCCTATCCTAATCG  372
ACGAAACAGCGAAGCGAGGAGGCTAGAATCGGCTGCCCAACGAAAAGAGCAGAACCGTTAACAAATGACACTCTGCCGATACAGCGAAGTAAA  465
                      (-4a)   (-3) [1383]                                (-2)
AATCAATAATTAGAGCAAAAAGgtatg...tagTTCATCATCATAGTCCTTTAAATCATAAATCACCATTAAAAtagCACAACAGATTTCACC  1930
                             (-1)                                        (-1)    (0) [2631]
AAGATATATAAAGTACATGAAATTCGATTCcagAGTCATGTTAAACAGCGCTAATAACAATTCTCCCCAGCACgtaag...tagAAGTGCATA  4643
                M  K  F  D  S  R  V  M  L  N  S  A  N  N  N  S  P  Q  H                       12
            (start -2)          (start -1)
ATTCAATTCGGCAATATTTGATATATATGGTAATATCAAATGAAGAACCGTGTCAATGATACTTGCTTTGGTGCCATAAAAAGCAAGTCCTCT  4736
                                                                                         (0)
AGAAGACGCGAGTTACTACATGCCTACACACAAAACGGGTCCTTAAAATGAAGTTTGAATGCTTTTCGTTGTACTGTACAGTGTTAAAAgtaa  4829
                                                 M  K  F  E  C  F  S  L  Y  C  T  V  L  K
    (1) [51]                                   (start 0)
a...tagCCGGTGAGTGCACCATCCGATATCAACATGAATGGCTATAACCGAAAATTGCCACAAAAACGTGGCTATGAGATGCCAAAATACTC  4962
         P  V  S  A  P  S  D  I  N  M  N  G  Y  N  R  K  L  P  Q  K  R  G  Y  E  M  P  K  Y  S   41
   (1)      (2) [115]               (start +1)
TGgtaag...tagATCCAAAAAAGAAAATGTGCAAGGAGCGCATTCCCCAGCCGAAGAACACGGTGGCCATGCTGAATGAGCTAAGACATGGA  5159
 D                 P  K  K  K  M  C  K  E  R  I  P  Q  P  K  N  T  V  A  M  L  N  E  L  R  H  G   68
                                                                          (2)    (3) [129]
CTGATTTACAAATTGGAGTCACAGACTGGTCCGGTACACGCACCTCTATTCACGATATCCGTGGAGgtaag...cagGTCGATGGACAGAAAT  5370
L  I  Y  K  L  E  S  Q  T  G  P  V  H  A  P  L  F  T  I  S  V  E                 V  D  G  Q  K  Y  96
ACTTGGGCCAGGGCCGTAGTAAAAAAGTTGCACGCATCGAAGCAGCAGCAACTGCACTGCGCAGCTTTATACAGTTTAAGGATGGAGCAGTTC  5463
  L  G  Q  G  R  S  K  K  V  A  R  I  E  A  A  A  T  A  L  R  S  F  I  Q  F  K  D  G  A  V  L  127
                                                                  (3)
TGTCGCCTCTGAAGCCGGCGGGCAACTTGGACTTTACCAGCGATGAACATCTTGAAAATGgtattGAAAATTTGTCCAGTTCAAAAATGTTTG  5556
  S  P  L  K  P  A  G  N  L  D  F  T  S  D  E  H  L  E  N  G  I  E  N  L  S  S  S  K  M  F  E  158
                                                                                 (3a)    (4)[682]
AGATCATTCAGACGATGTTGACTGAAAAGCTATCCAACCCTACCTCGCTTGAACAACCCACGTTTTGCATGAGTCAGAgcaag...tagATGT  6320
  I  I  Q  T  M  L  T  E  K  L  S  N  P  T  S  L  E  Q  P  T  F  C  M  S  Q  N              V  185
CAGCAAAAGTGCTATTACTGTTGACGGTCAGAAGAAGGTTCCAGATAAGGGTCCTGTCATGCTCCTCTACGAATTATTTAATGACGTTAATTT  6413
 S  K  S  A  I  T  V  D  G  Q  K  K  V  P  D  K  G  P  V  M  L  L  Y  E  L  F  N  D  V  N  F  216
                                                                                            (4)
CGAATGCATTAATATTGACGGCGCCCAGAACAATTGTCGCTTCAAAATGACCGTCACAATCAACGAAAAGAAGTTCGATGGAACAGgtatg..  6504
 E  C  I  N  I  D  G  A  Q  N  N  C  R  F  K  M  T  V  T  I  N  E  K  K  F  D  G  T  G          245
  (5) [86]
.aagGTCCTTCCAAAAAGACGGCGAAAAATGCGGCAGCTAAGGCGGCACTTGCTTCGTTATGCAATATTTCCTACAGTCCAATGGTGGTGCCA  6674
        P  S  K  K  T  A  K  N  A  A  A  K  A  A  L  A  S  L  C  N  I  S  Y  S  P  M  V  V  P  274
CAGAAGAACGTACCCCTGCCAATCGACGACAAGTCGTCATCGATGGAGTTGCCTCAGATACACGCGGATACGATTGGTCGGTTGGTCTTAGAA  6767
Q  K  N  V  P  L  P  I  D  D  K  S  S  S  M  E  L  P  Q  I  H  A  D  T  I  G  R  L  V  L  E   305
AAGTTCATGGAAGTAATCAAGGGCCAGGAGGCTTACTCGCGTCGAAAGGTATTAGCGGGCATTGTAATGACTGAAAACATGAATTTTTGTGAA  6860
K  F  M  E  V  I  K  G  Q  E  A  Y  S  R  R  K  V  L  A  G  I  V  M  T  E  N  M  N  F  C  E   336
        (5)      (6) [63]
GCCAAAgtgag...cagGTTATTTCAGTTTCGACGGGCACCAAGTGTGTCAGCGGTGAGCATATGAGTGTGAACGGAGCTGTCCTAAATGATT  7005
A  K                 V  I  S  V  S  T  G  T  K  C  V  S  G  E  H  M  S  V  N  G  A  V  L  N  D  S 364
                                                                                 (6)     (7) [892]
CCCATGCTGAAATAGTCTCCAGGCGTTGTCTTCTCAAATATTTATATGCACAGCTGGACCTTCAGTGCAATCAGGgtata...tagCCACAGC  7979
  H  A  E  I  V  S  R  R  C  L  L  K  Y  L  Y  A  Q  L  D  L  Q  C  N  Q  A              T  A  391
ATATCAGTCGATTTTCGTGAGGAATACTGATGGGCAATACCCTTATAAACTAAAATCCGGGGTACATTTCCATTTGTATATAAATACAGCACC  8072
 Y  Q  S  I  F  V  R  N  T  D  G  Q  Y  P  Y  K  L  K  S  G  V  H  F  H  L  Y  I  N  T  A  P  422
                                                                          (7)    (8) [1051]
TTGTGGGGATGCACGGATATTTAGTCCTCACGAAAACGACACTGGTGTTGATAAACATCCAAATAGgttgg...cagAAAAGCTCGTGGCCAG  9205
 C  G  D  A  R  I  F  S  P  H  E  N  D  T  G  V  D  K  H  P  N  R                 K  A  R  G  Q  449
                                                                                         (8)
CTGCGCACCAAAATCGAGTCCGGTGAGGGGACGATTCCAGTGAAAAGCAGTGATGGTATCCAGACGTGGGATGGCGTATTGCAGgtaac...c  9360
L  R  T  K  I  E  S  G  E  G  T  I  P  V  K  S  S  D  G  I  Q  T  W  D  G  V  L  Q              477
(9) [73]
agGGCCAACGCCTGCTAACAATGTCGTGCTCGGATAAAATTGCCCGTTGGAACATCGTGGGCATCCAAGGCTCACTATTGTCTTCCATAATTG  9453
  G  Q  R  L  L  T  M  S  C  S  D  K  I  A  R  W  N  I  V  G  I  Q  G  S  L  L  S  S  I  I  E  508
AACCGGTGTACCTGCATTCGATTGTGCTGGGAAGCCTGTTGCATCCGGAGCACATGTACCGCGCAGTTTGCGGCCGAATTGAGAAGTCCATTC  9546
  P  V  Y  L  H  S  I  V  L  G  S  L  L  H  P  E  H  M  Y  R  A  V  C  G  R  I  E  K  S  I  Q  539
AAGGCCTGCCACCGCCGTACCATCTGAACAAGCCGCGCCTGGCACTAGTCACTTCGGCCGAGCCGCGGAATCAGGCCAAGGCTCCCAACTTCG  9639
  G  L  P  P  P  Y  H  L  N  K  P  R  L  A  L  V  T  S  A  E  P  R  N  Q  A  K  A  P  N  F  G  570
GGATCAATTGGACCATTGGGGACACCGAGCTGGAAGTGGTGAACTCGCTAACCGGTCGAACCATTGGCGGCCAGGTGTCTCGCATCACAAAGC  9732
  I  N  W  T  I  G  D  T  E  L  E  V  V  N  S  L  T  G  R  T  I  G  G  Q  V  S  R  I  T  K  Q  601
AGGCGTTTTTCGTTAAGTATGGATTTCTAATGGCAAACTTGCCCGGTATTTTAGTCCGCAAAGTAACCACTGACTATGGGCAAACCAAAGCTA  9825
  A  F  F  V  K  Y  G  F  L  M  A  N  L  P  G  I  L  V  R  K  V  T  T  D  Y  G  Q  T  K  A  N  632
                      (9)   (10) [67]
ACGTTAAGGACTATCAGgtgag...tagATCGCAAAGCTAGAATTGTTCTCTGCATTCAAGCGAGAAGACCTTGGCAGCTGGCTGAAGAAACC  9974
  V  K  D  Y  Q                I  A  K  L  E  L  F  S  A  F  K  R  E  D  L  G  S  W  L  K  K  P  659
CATTGAACAAGACGAGTTCGGTCTTGCCGAAtgaCTGCACTCAGCACTATTATATAATTTAAAAAAGACAACAGCAACGGCAAGCAATTAAT  10067
 I  E  Q  D  E  F  G  L  A  E                                                                 669
AGAATTAGACGACATAGATTTTATGACAAATTCTGATATAGAATGCAAATTATATTGGGTGCAAGCACATCGCTATGATATACCACTTCATAT  10160
GATT
```

FIGURE 2. DNA sequence and predicted amino acid sequence of *dADAR.* Sequence of the *dADAR* transcription unit showing exons and intron/exon junctions. All exon boundaries are labeled by numbers in parentheses at the consensus 3′ acceptor site (NAG) and 5′ splice donor site (GTRAG) with the following exceptions: Exon −4a is a 5′ UTR exon and thus does not have an acceptor site at its 5′ limit; exons −3 and −2 are defined by competing 3′ acceptor sites and thus are numbered at their 5′ ends only; and exon 3a inclusion depends upon utilization of alternative 5′ donor sites and is thus labeled only at its 3′ end. Intron sizes are indicated in brackets 3′ to the acceptor site for a particular intron. Underlined amino acid sequences represent DRBMs. Potential alternative starting methionines are indicated in bold. Asterisks indicate the start sites of cloned cDNAs with number of independent cDNAs listed above. Numbering of nucleotide (plain) and amino acid (bold) sequences on the right refer to GenBank accession number AF208535.

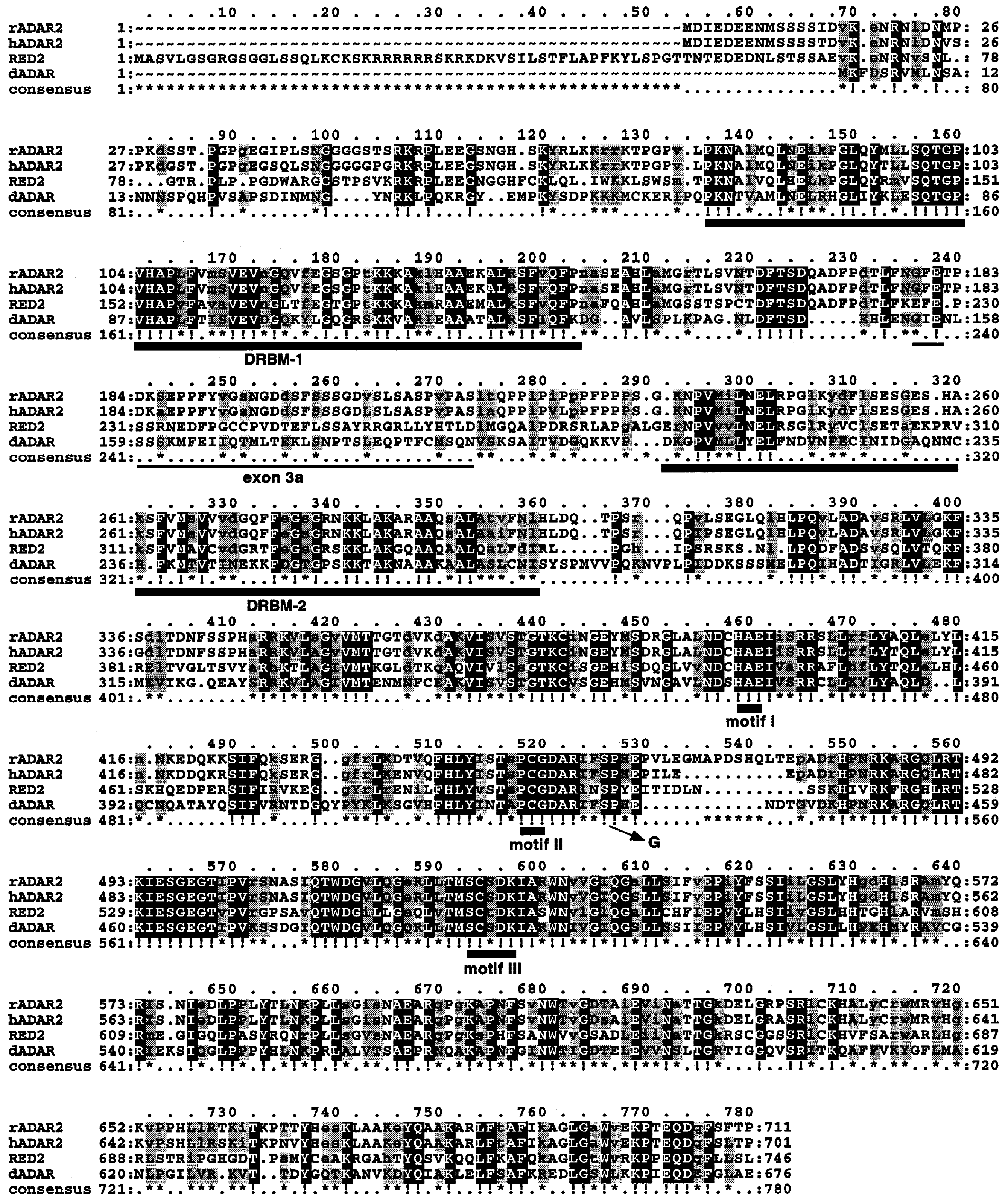

FIGURE 3. Amino acid sequence alignment of *dADAR* and known ADARs. dADAR protein sequence alignment of the exon −2-, −1-, and 3a-containing (longest) alternative splice form. Bold lines indicate the positions of DRBMs (1–2) and deaminase domain motifs (I–III) and the thin line indicates the position of alternative exon 3a. Arrow indicates the position of the S/G editing site. The sequences are rat ADAR2 (rADAR2), human ADAR2 (hADAR2), the ADAR-like homolog rat RED2, and dADAR. Black boxes represent identity across all homologs and gray boxes represent identity (upper case) or similarity (lower case) to dADAR in at least 75% of the homologs. The consensus line symbols are as follows: !: identity across all sequences, *: high degree of identity or similarity to dADAR, .: lack of conservation with dADAR.

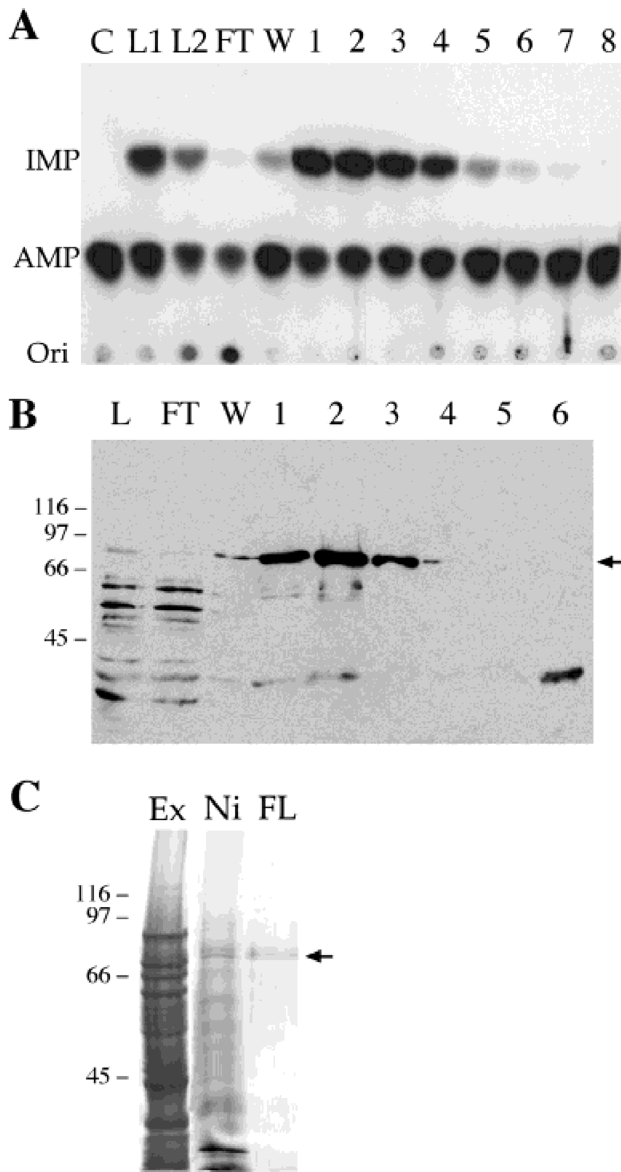

FIGURE 4. Recombinant dADAR possesses A-to-I editing activity on extended dsRNA. **A**: Fractions eluted from a Ni^{2+} NTA column were incubated with dsRNA labeled with α-P^{32}-ATP for 1 h at 37 °C. After digestion with P1 nuclease, the products were separated by thin layer chromatography. The fastest migrating spots were IMP, then AMP, and the origin is at the bottom. The lanes are as follows: C: negative control in which dsRNA was incubated without extract; L1: load fraction, 1 μL; L2: load fraction, 6.25 μL; FT: column flow through fraction, 6.25 μL; W: column wash fraction, 6.25 μL; 1–8: fractions 1–8 eluted from the Ni^{2+} NTA column, 6.25 μL. **B**: Immunoblot analysis with anti-FLAG serum (1:1,000) of fractions eluted from the Ni^{2+} NTA column. An arrow indicates a band of 80 kDa that fractionates with the deaminase activity. The load (L), flow-through (FT), and wash (W) fractions each contain 2 μL, whereas lanes 1–6 contain 7.5 μL of the indicated fraction eluted from the Ni^{2+} NTA column. Molecular weight masses in kilodaltons are indicated on the left. **C**: Fractions from the different affinity purification steps were electrophoresed on a 10% SDS polyacrylamide gel and stained with silver. The lanes are as follows: Ex: −1–2 μL of the extract that was loaded onto the Ni^{2+} NTA column; Ni: 2–18 μL of a pool of the peak activity eluted from the Ni^{2+} NTA column; FL: 3–400 μL of the peak of activity eluted from the FLAG affinity column. Molecular weight masses are indicated on the left.

terials and methods). Expression from the −4a promoter was detected in all stages (Fig. 5A). Also, −4a transcripts appeared to reproducibly increase in the pupal stage ($n = 3$). This may reflect an increased role for *dADAR* during morphogenesis that is consistent with the fact that the *dADAR* locus resides in an ecdysone inducible chromosomal puff (see Discussion). The major transcripts detected by PCR were subcloned and sequenced and corresponded to the four forms generated by inclusion or exclusion of alternative exons −1 and 3a and lacking the other alternative exons. Thus,

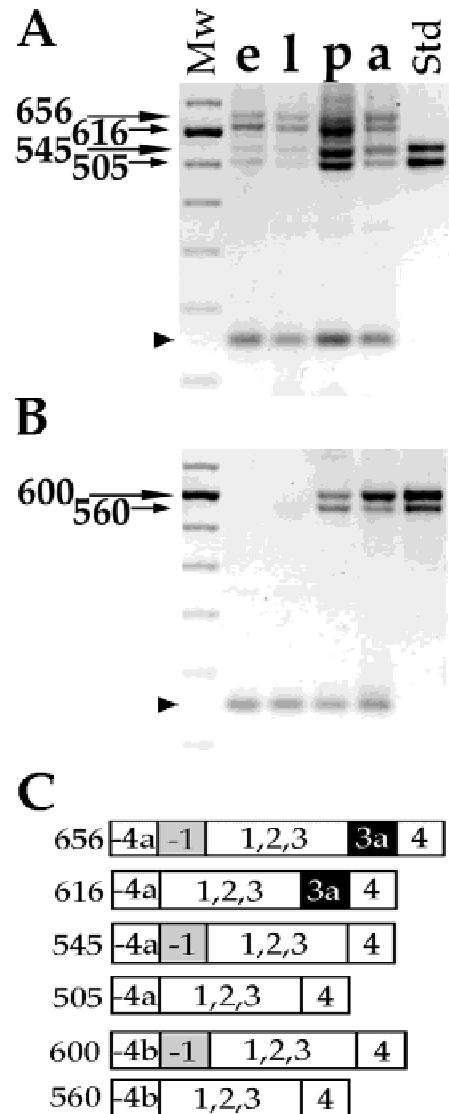

FIGURE 5. Developmental expression profile of *dADAR*: Promoters and alternative splice-forms. **A**: Quantitative PCR analysis of embryonic (e), larval (l), pupal (p), and adult (a) *dADAR* transcripts from the −4a promoter. **B**: Similar analysis for the −4b promoter. Arrowheads mark the position of *rp49* internal control bands. Standard (std) lanes contain cloned and sequence-verified fragments corresponding to the −4a and −4b products ± exon −1. The product sizes in **A** and **B** are indicated by arrows and refer to the indicated alternative splice form sizes in **C**. **C**: Alternative splice products are indicated by exon composition and size (in base pairs) next to each splice form.

four protein isoforms could be generated starting at the exon −1 or exon 1 ATGs and either including or excluding exon 3a, which alters the spacing between DRBMs. We did not determine the frequencies of inclusion of alternative exons −3, −2, or 0 as they occur at lower levels.

Expression of transcripts including exon −4b was found to be more highly regulated than that of transcripts containing exon −4a (Fig. 5B). Two major exon −4b-containing transcripts were detectable in pupae and adults. Cloning and sequence analysis identified these two products as either including or excluding the −1 exon and lacking the other alternative exons. Unlike −4a transcripts, all −4b transcripts lacked alternative exon 3a.

The *dADAR* expression pattern was determined by in situ hybridization to whole *Drosophila* embryos of various developmental stages. Although a low level of nonspecific staining was seen in early stage 13 embryos (end of germ-band retraction; data not shown), specific staining was seen by late stage 13 after condensation of the ventral nerve cord occurs. Staining then proceeded to intensify within the central nervous system, reaching high levels in the embryonic ventral nerve cord and brain by stage 16 (Fig. 6).

dADAR itself undergoes RNA editing

We discovered a nucleotide position in the *dADAR* transcript in exon 7 (Fig. 1) that gave a reproducible mixed A/G signal in automated sequencing of bulk *dADAR* RT-PCR product (Fig. 7A). Cloning and sequencing of *dADAR* transcripts showed that cDNAs also contained either A or G at the same position. Because inosine base pairs preferentially with cytosine, an A-to-G transition occurs in the course of synthesis of double-stranded cDNA when A-to-I editing occurs in pre-mRNA. Sequencing of PCR products generated from genomic DNA gave exclusively an A signal at the same position. Also, P1 bacteriophage genomic clones containing the *dADAR* locus had A at this position. Similar analyses were performed on genomic DNAs and RT-PCR products of related *Drosophila* species (Fig. 7B). *D. simulans* is estimated to be 2.5 million years diverged from *D. melanogaster* (Powell, 1997). In each case, RT-PCR products revealed a mixed A/G signal at the same location in the related species whereas sequence of the corresponding PCR products generated from genomic DNA gave a pure A signal. Thus, the A-to-G changes seen in cDNAs of *dADAR*, at variance with the genomically encoded A at this position, bear all the hallmarks of ADAR mediated A-to-I RNA editing seen in other natural mRNA substrates. The A that is edited occurs in the first position of a serine (S) codon and would convert the coding potential to that of glycine (G) (AGT to GGT). This amino acid change occurs in the deaminase domain of *dADAR*, which is a highly conserved

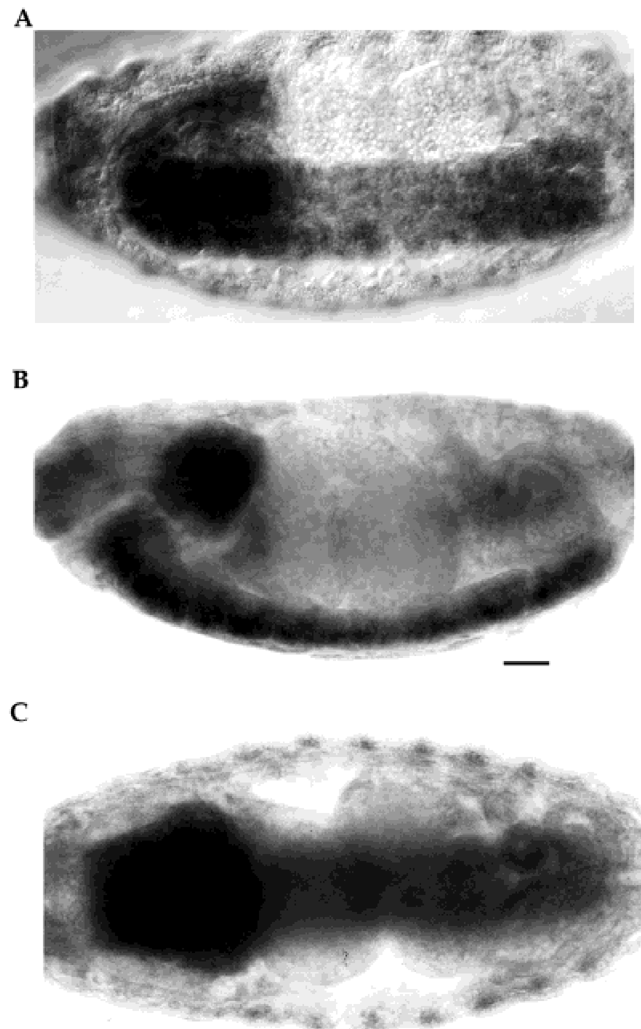

FIGURE 6. Embryonic expression pattern of *dADAR* by in situ hybridization. *Drosophila* wild-type stage 16 embryos were hybridized with a *dADAR* probe and whole mounted (see Materials and methods). Ventral (**A**), lateral (**B**), and dorsal (**C**) views are shown. Strongest expression is seen in the ventral nerve cord (**A**) and brain (**B**). Lower levels of expression can be seen in the peripheral nervous system in each segment (**C**). Scale bar for **A**--**C** indicates 25 μm.

portion of the dADAR protein. The S/G site is six amino acids carboxy-terminal to the first cysteine in motif II of the deaminase domain that is thought to chelate a zinc ion at the active site (Betts et al., 1994; Kim et al., 1994; Lai et al., 1995) (Fig. 7C).

The editing of the pre-mRNA encoding dADAR was found to occur in a highly developmentally regulated fashion. cDNAs were isolated from several developmental stages and the extent of RNA editing was determined (see Materials and methods, Fig. 8). Editing at the S/G site of *dADAR* was low in embryonic and pupal cDNAs (1.0% and 3.8%, respectively) and increased dramatically upon eclosion. Editing increased more than 40-fold from embryo to adult. Although the analysis of editing from adult flies revealed that editing

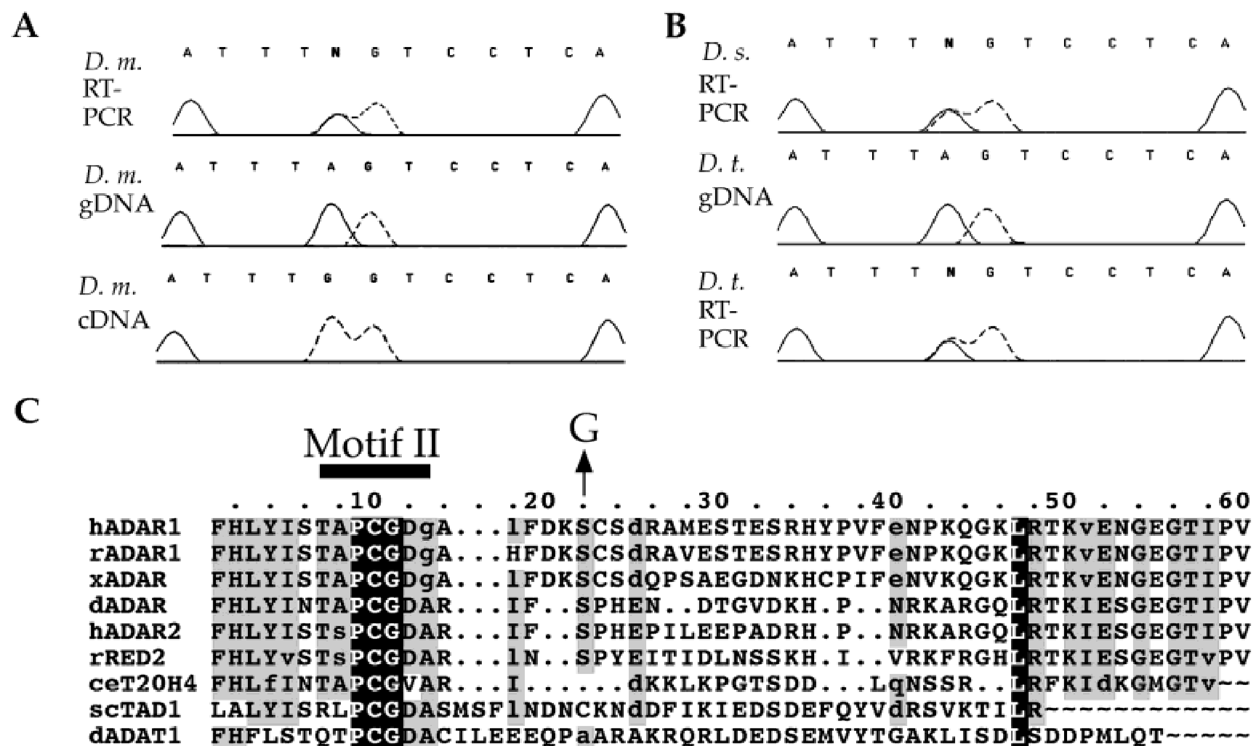

FIGURE 7. RNA editing of *dADAR*. **A**: PCR products were amplified from *D. melanogaster* (*D.m*) RNA (RT-PCR), genomic DNA (gDNA), and cloned cDNA (cDNA) and subjected to automated sequencing. The region around the edited adenosine is shown and a mixed sequence signal is indicated by N. **B**: Similar analyses to **A** were performed on *D. simulans* (*D.s*) and *D. takahashi* (*D.t*) RNA (RT-PCR) and *D. takahashi* gDNA (gDNA). **C**: Sequence alignment of the ADAR1 (hADAR1, rADAR1, xADAR), ADAR2 (dADAR, hADAR2), and ADAT (scTAD1p, dADAT1) classes of editase in the region of the dADAR S/G editing site (indicated by arrow). rRED2 and ceT20H4.4 are ADAR-like homologs that have not been shown to possess ADAR activity. Motif II in the catalytic domain is indicated by a black bar.

occurred in 41 ± 6% of all transcripts, we found that the 3a-containing transcripts are essentially unedited (no 3a-containing cDNAs were edited out of 100 analyzed). So, editing of *dADAR* pre-mRNA occurs in a temporal- and transcript-specific manner.

DISCUSSION

Adenosine deaminases that act on RNA have been found throughout the animal kingdom (Bass, 1997). One role for these enzymes has been proposed in viral defense, through promiscuous modification of dsRNA intermediates and subsequent mutation of viral genomes (Bass & Weintraub, 1988). Another role has been that of antisense regulation of gene expression through the nuclear retention of highly modified mRNAs (Kumar & Carmichael, 1997). ADARs can also perform highly specific editing of select positions within pre-mRNAs. Despite the paucity of examples of specific RNA editing by ADARs, it is clear that mRNAs from many tissues contain inosine at detectable levels (Paul & Bass, 1998) and that enzymes that perform specific A-to-I editing are also ubiquitous.

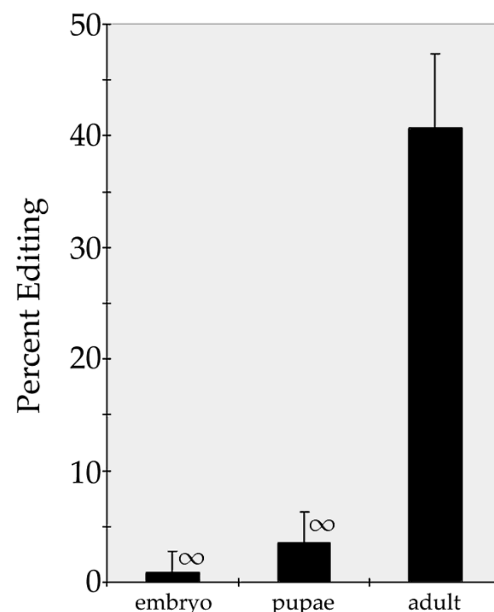

FIGURE 8. Developmental profile of *dADAR* editing. Quantitation of RNA editing was performed by cloning and restriction analysis of partial *dADAR* cDNAs (see Materials and methods). Clones were obtained from embryos, pupae, and adults. Total clones assayed were: embryo: $n = 98$; pupae: $n = 133$; and adult: $n = 140$. ∞: significantly different from adult, $p < 0.001$ by Student's *t*-test.

The *Drosophila dADAR* locus

We report here the cloning and analysis of a *Drosophila* homolog of the ADAR class of enzymes, named *dADAR*. We have shown that the recombinant dADAR protein can convert A-to-I on synthetic dsRNA substrates. We have mapped the *dADAR* locus cytogenetically, and cloned and sequenced the entire transcription unit. *dADAR* is subject to an array of transcriptional and posttranscriptional regulatory mechanisms. *dADAR* expression is driven by two promoters in temporally distinct patterns. Likewise, alternative promoters are also utilized by mammalian ADAR1; one promoter is constitutive and the other is interferon (IFN) inducible (George & Samuel, 1999a, 1999b; Patterson & Samuel, 1995). The IFN inducible ADAR1 is larger and localized to both the nucleus and cytoplasm, whereas constitutive ADAR1 is nuclear (Patterson et al., 1995).

Additional diversity is generated by alternative splicing of *dADAR* transcripts. Alternative exons that contain different translation start sites are utilized in two abundant splice forms (exon -1 and exon 1) that probably generate proteins with alternative amino termini. Another alternative splice choice of *dADAR* (exon 3a) alters the spacing between DRBMs. By comparison, both mammalian ADAR1 and ADAR2 also undergo alternative splicing to generate ADAR isoforms with differential activities on specific substrates (Gerber et al., 1997; Lai et al., 1997; Liu & Samuel, 1999).

Developmental expression of *dADAR*

The expression pattern of *dADAR* was determined by in situ hybridization to *Drosophila* embryos and shown to be largely nervous system specific. In fact, the temporal and spatial expression patterns of *dADAR* overlap substantially with that reported for transcripts of the *para* locus (Hong & Ganetzky, 1994). The *para* locus encodes the α-subunit of the major voltage-gated Na^+ channel of the *Drosophila* nervous system and *para* transcripts have been shown to possess four A-to-I RNA editing sites (Hanrahan et al., 1999; Reenan et al., 2000). In addition, the pre-mRNA encoding the *Drosophila* calcium channel *Dmca1A* has been reported to undergo RNA editing (Smith et al., 1996) and these reports have been substantially confirmed (M.A. O'Connell and M.J. Palladino, unpubl. results). *Dmca1* is also expressed in the nervous system of late stage embryos (Smith et al., 1996).

Transcriptional analysis of *dADAR* revealed that the two promoters are differentially regulated. The promoter driving -4a expression produces transcripts that are detectable in all developmental stages tested. The four most abundant alternative splice forms made from this promoter are the combinations $\pm$ exon -1 and $\pm$ exon 3a (Fig. 5A). A reproducible increase is seen in *dADAR* transcript levels during the pupal stage. This is consistent with the fact that *dADAR* is in an ecdysone inducible puff region near the *BrC*, which encodes transcription factors that are primary response genes to the hormone ecdysone. *dADAR* may be directly ecdysone regulated by virtue of its location or it may be a secondary response gene that is regulated by genes such as the *BrC*. Transcription from the -4b promoter is also highly regulated, being detected exclusively in the pupal and adult stages. Two forms of *dADAR* were detected from this promoter, the $\pm$ exon -1 forms; the -4b promoter produces transcripts that never include alternative exon 3a. As mentioned earlier, the 5′ donor site that is utilized to include exon 3a is a nonconsensus splice signal that may be suboptimal. Many regulated splicing events are associated with weak splice consensus signals that are activated in a specific manner by splicing enhancer elements and the proteins that bind them (Hertel et al., 1997). Recent results suggest that specific splicing enhancers may be modulated dramatically by the choice of promoter suggesting that transcription machinery is involved in recruiting splicing factors (Cramer et al., 1997, 1999). Thus, the association of the presence of *dADAR* alternative exon 3a with transcription from a specific promoter may represent such a case of transcription/splicing coupling.

dADAR is edited and encodes a dsRNA-dependent adenosine deaminase

We discovered in the course of sequence analysis that a region of the *dADAR* transcript has either A or G at a particular nucleotide position in cDNAs. Because most specific A-to-I editing sites have been discovered as this type of sequence discrepancy and there are a number of trivial nonediting explanations for this result, we proceeded along several lines of inquiry to gather further evidence for RNA editing. Automated sequence analysis of bulk RT-PCR product was shown to produce a mixed A/G signal, whereas sequence of PCR product from genomic DNA of the same flies gives only an A signal (Fig. 7A). In addition, we were curious to determine whether this phenomenon was evolutionarily conserved. In two relatives of *D. melanogaster* we observed the same process in adult animals (Fig. 7B). Also, the developmental regulation and transcript specificity of *dADAR* editing rule out any polymerase-based artifact or polymorphism (see below).

The editing of this A in pre-mRNA of *dADAR* is predicted to result in a serine (S) to glycine (G) substitution because of a change in coding potential (AGT to GGT). This S/G change occurs in a highly conserved portion of the dADAR catalytic domain between the two cysteines in motifs II and III of the deaminase domain that are thought to chelate a zinc ion at the active site (Mian et al., 1998). It is interesting that this region between motif II and III is where most diversity is seen between

the ADARs and between ADARs and ADATs (Gerber et al., 1998; Gerber & Keller, 1999; Keller et al., 1999; Maas et al., 1999). In mammalian ADAR2, alternative splicing introduces 40 amino acids in this region due to inclusion of an expressed Alu cassette (Gerber et al., 1997). This change does not dramatically affect ADAR2 specificity but does affect catalytic activity of the isoforms on specific substrates in vitro. By analogy, *dADAR* S/G change would not be predicted to abrogate catalytic function, but rather to change some aspect of enzymatic function such as rate of catalysis or substrate specificity. Interestingly, rADAR2 is capable of editing its own transcript within an intron thereby generating a splice acceptor site. The change causes alternative splicing to take place and has been shown to cause down-regulation of rADAR2 expression that could serve an autoregulatory function or prevent ADAR hyperactivity (Rueter et al., 1999).

The developmental and transcript specificity of *dADAR* editing is very intriguing. dADAR probably edits its own transcript. Despite extensive attempts and searches to discover other ADAR homologs in *Drosophila*, no other ADAR homolog has been identified (R.A. Reenan and M.J. Palladino, unpubl. observation). The editing of *dADAR* was almost exclusively adult specific. Similarly, *dADAR* transcripts arising from the −4b promoter are predominantly adult specific. One simple explanation for the developmental regulation of *dADAR* editing is that in certain tissues, dADAR modifies its own transcript when the enzyme concentration, and hence the editing activity, reaches a critical level. Thus, the specific role of the −4b adult-specific promoter could be to increase dADAR enzymatic activity in certain tissues, whereupon *dADAR* transcript self-editing takes place. Autoregulation then could serve to further enhance, reduce, or otherwise modify the activity of dADAR. A similar mechanism could lead to the activation of other non-*dADAR* regulated adult-specific editing sites in *Drosophila*. It is worth noting that two editing sites of the *para* locus are edited in a developmental pattern that is similar to the *dADAR* S/G site, predominantly in adulthood (Hanrahan et al., 2000). In support of such a model is the observation that overexpression of mammalian ADAR2 causes modification of sites not normally modified in vivo (Rueter et al., 1999).

ADAR substrate specificity has been shown to be faithfully reproduced in vitro without the necessity for cofactors (Rueter et al., 1995; Yang et al., 1995; Dabiri et al., 1996; Maas et al., 1996; Melcher et al., 1996b; Polson et al., 1996; Gerber et al., 1997; O'Connell et al., 1997; Yang et al., 1997). Moreover, enzymes from different species have been shown to reproduce specific in vivo editing patterns on defined substrates, reducing the likelihood that species-specific cofactors are necessary for specific editing (Casey & Gerin, 1995; Hurst et al., 1995). Therefore, models that modulate RNA editing levels by either changes in enzyme concentration or generation of substrate-specific isoforms are attractive.

One intriguing result is the lack of editing seen in exon 3a containing *dADAR* transcripts. The simplest explanation of this result is that the exon 3a-containing form may be expressed tissue specifically as are most alternatively spliced transcripts (Chabot, 1996) and to the exclusion of all exon 3a-lacking forms. As mentioned earlier, the exon 3a-containing form alters the DRBM spacing. Thus, the exon 3a-containing form of dADAR may be incapable of editing the *dADAR* transcript or may never reach high enough levels such that self-editing rarely or never takes place. We are pursuing in vivo overexpression of the various *dADAR* isoforms as well as in vitro experiments with recombinant dADAR and specific substrates to test these hypotheses.

If editing of *dADAR* occurs through a double-strand intermediate, like that shown for mammalian ADAR substrates, then the *dADAR* editing site may possess an ECS in noncoding regions. No obvious ECS was found in *D. melanogaster* in the introns flanking the S/G site exon. Moreover, evolutionary comparisons of *D. melanogaster* and *D. takahashi* genomic sequences also failed to identify an ECS, even though both of the species perform the S/G edit at comparable frequencies. Such an approach has identified putative ECSs for *para* editing sites (Hanarahan et al., 2000). We are pursuing further evolutionary comparisons as well as searching for putative ECSs in more distant regions of the *dADAR* transcription unit.

A hallmark of ADARs is that they have enzymatic activity on extended dsRNA (Bass et al., 1997). Each biochemically characterized ADAR to date possesses, in addition to promiscuous editing activity on extended dsRNA substrates, a characteristic specific activity on known pre-mRNA substrates. Because dADAR has a *bona fide* A-to-I deaminase activity like mammalian ADARs, we hope to determine whether dADAR in any of its various isoforms edits defined pre-mRNA substrates in *Drosophila*.

MATERIALS AND METHODS

Fly stocks

The wild-type strain used in this study is Canton S. *Drosophila simulans* (stock #14021-0251.102) and *Drosophila takahashi* (stock #14022-0311.0) were obtained from the National *Drosophila* species resource center at Bowling Green State University.

Primers

Sequence in upper case corresponds to the *dADAR* sequence (GenBank accession number AF208535). Sequence changes or additions to add or generate restriction enzyme

sites for cloning are indicated in lower case. Location of the 5′ nucleotide of the primer in relation to the reported *dADAR* genomic DNA is given in parentheses. Where degenerate primers amplified a product from the *dADAR* locus, the location of the 5′ nucleotide of the primer is given. When sequences have been added at the 5′ end, the number refers to the first 5′ *dADAR* nucleotide.

3PRIME: GGTATATCATAGCGATGTGCTTGC (10,151);
AP1: CCATCCTAATACGACTCACTATAGGGC (Clontech);
DIRT: ATTAATTGCTGGCCGTTGCTGTTG (10,067);
DREAM-2: cggatCCRCANGGNGCNGT (8,079);
DREAM-3: cggatccACXGGDATXGTXCCYTCXCC (9,246);
DREND: gccgagctcTTCGGCAAGACCGAACTCG (10,005);
DRIP1: ccggatccACAGCTGGACCTTCAGTGCAATC (7,055);
DROP: ccggatccGTCTCCAGGCGTTGTCTTCTC (7,044);
DROWN-2: CGTGAGGAATACTGATGGGCAA (8,016);
DRUP-6: cggatccTTCTTCTGACCGTCAACAGTAA (6,356);
DRUTR: ccgagctcGCAGCAAATGTGCAGATTTGAAC (75);
MAERD-1: ccgagctcAAYGAYTGYCAYGCXGARAT (6,999);
MINUS1L: ccccggatccTTAAACAGCGCTAATAACAATTCTCCCC (1,971);
MEMO-3/4(sac): gggagctcGAACATCTTGAAAATGATGTCAGC (5508);
RED-1: ccgagctcGGXCCXGTXCAYGCXCC (5,187);
RED-2: ccgagctcGGXAARAAYCCXGTXATG;
RP49us: GCGGGTGCGCTTGTTCGATCC;
RP49ds: CCAAGGACTTCATCCGCCACC;
RSP1: GACACACTTGGTGCCCGTC (6,962);
RSP2: CCAACCCTACCTCGCTTG (5,590);
RTEXON-5: GCAAGTGCCGCCTTAGCTGCCGC (6,634);
RTRP49: CTTGAGACGCAGGCGACCGTTGG;
SLIP: ccgagctCCGTTAACAAATGACACTCTGCCG (427);
SHL3: ccgagctcGTGTGTAGTGCACTTTTGGCCAGCC (1,250);
SAC3a: agggagctcGTATTGAAAATTTGTCCAGTTCA (5,524);

Cloning

dADAR was cloned by degenerate PCR using a Marathon (Clontech) ds-cDNA library as template. The library was constructed according to the manufacturer's protocol using adult Canton-S total RNA prepared using a modified LiCl/urea procedure (Auffray & Rougeon, 1980). The degenerate primers MAERD-1, DREAM-2, and DREAM-3 were designed to the conserved catalytic domain amino acid sequences NDCHAEI, TAPCG, and GEGTIPV, respectively (see Fig. 1). First-round PCR was performed using the primer combination MAERD-1 and DREAM-3 and *Taq* polymerase (Promega). Reactions were performed in a Robocycler (Stratagene) using the following parameters: denaturation: 94 °C for 45 s; annealing: 53 °C for 45 s; extension: 72 °C for 30 s; for 40 cycles. After one round of PCR, no amplification product was detectable. Second-round amplification conditions were a modification of touchdown PCR (Don et al., 1991). Second-round PCR conditions were as follows using primers MAERD-1 and DREAM-2: denaturation: 94 °C for 45 s; annealing for 45 s (cycles 1–10, starting at 63 °C and decreasing 1 °C per round to 54 °C; then continuing for 40 cycles at 54 °C); extension: 72 °C for 30 s; for 50 cycles total. A product was obtained that was subcloned into pBluescript (Stratagene) and sequenced. Based upon sequence analysis, the specific primer PROD-I was designed and PCR was performed using the primer combinations PROD-I and either degenerate primer RED-1 (designed to ADAR2-specific amino acid sequence GPVHAP) or primer RED-2 (designed to ADAR1 specific amino acid sequence GNKPVM) with the Marathon library as template. PROD-1 and RED-1 gave a robust PCR product that was cloned and sequenced. A similar approach was used to obtain full-length 5′ and 3′ ends using gene specific primers and the AP1 primer, which recognizes the adapters used to generate the cDNA library (Clontech, Marathon cDNA Amplification Kit User Manual, PT1115-1).

Initially, 30 5′ clones generated by the DRUP-6 and AP1 primers were analyzed. Alternative splicing of these 5′ ends was as follows: (1) Exon −4a-containing clones: 15 were exon −4a spliced directly to exon 1; 10 included exon −1 and then spliced to exon 1; 2 included the exon-2, −1 unit and then spliced to exon 1; 1 included the exon −3, −2, −1 unit and then spliced to exon 1; and 1 included exon 1 and exon 0 spliced to exon 1. (2) Exon −4b-containing clone; one clone had a novel 5′ end (−4b), included exon −1, and was spliced to exon 1. Further transcripts containing this 5′ end were obtained by RACE and because exon −4b is downstream of exon −4a and is never included in exon −4a-containing transcripts, it is presumed to arise from an alternative transcriptional start site.

A full-length cDNA clone was generated by RT-PCR using the DRUTR and DREND primers. This cDNA was used as a probe for in situ hybridization to *Drosophila* polytene chromosomes using a standard procedure (Ashburner, 1989). A single band was seen on the X chromosome in the 2B6–7 region. P1 bacteriophage clones that were mapped to this region were obtained (Berkeley *Drosophila* Genome Project). DNA from this series of P1 clones was obtained by boiling-lysis of single *Escherichia coli* colonies and PCR was performed using the RSP1 and RSP2 primer pair. P1 clones DS04654 and DS07495 gave strong signals of the appropriate size and were also shown to hybridize to a full-length *dADAR* cDNA by Southern analysis (data not shown). P1 DS04654 was chosen for sequence analysis and >30 kb of sequence were generated from this clone by direct automated sequencing (University of Connecticut Health Center molecular core facility). Genomic sequence spanning all of the cDNAs described in this study and including all intron–exon junctions has been entered into GenBank (accession number AF208535). In the course of completing this manuscript, two sequences that overlap with our sequence were reported, EDGP cosmid clone DMC4F1 and BAC clone DMBN35H14. DMBN35H14 predicts the dADAR open reading frame and generates a protein that agrees completely with our sequence with the following exceptions. The DMBN35H14 protein begins with exon −1 and splices to exon 1. The DMBN35H14 protein does not predict alternative exon 3a. Lastly, the DMBN35H14 protein predicts translation through a 63-bp intron to include the sequence SPQPAKHCETNYNAKPILDQV. This intron extends from nucleotide positions 6,867 to 6,929 of our sequence. We have never seen inclusion of this intron in cDNAs ($n > 350$).

Quantitative RT-PCR

Whole RNA was isolated from unstaged embryos and pupae, larvae at L3 stage, and adult (mixed age and gender) *D.*

melanogaster (Canton S) by a modification of the LiCl/urea procedure (Auffray & Rougeon, 1980). Ribosomal protein 49 amplification products were used as an internal RT-PCR control (O'Connell & Rosbash, 1984; Winick et al., 1993) for total amount of RNA and efficiency of amplification. Amplification of −4a- and −4b-specific transcripts used the SLIP and DRUP6 and SHL3 and DRUP6 primer combinations, respectively. These primer combinations span all known sites of alternative splicing and specifically detect transcripts arising from the two promoters. Standard reverse transcription (RT) reactions were performed with an equimolar mix of RT-RP49 (*rp49*-specific) and RT-EXON5 (*dADAR*-specific) primers. Standard PCR reactions were performed (annealing at 59 °C and extension at 72 °C for 2.25 minutes); however, RP49US and RP49DS were added at the denaturation step of cycle 16 and aliquots were taken at 22, 25, 28, 30, 32, 34, and 36 cycles. *dADAR* and *RP49* products amplified exponentially through cycles 36 and 32, respectively (data not shown). *dADAR* products were saturating at cycle 34. RP49 products did not show saturation under these conditions. Subsaturation PCR products (cycle 32) were quantified by densitometry of ethidium stained agarose gels (Fig. 5). This procedure was performed three times with similar results.

In situ hybridization

Digoxigenin-labeled double-strand DNA probes were generated by PCR according to the manufacturer's instructions (Boehringer Mannheim). Dig-11-dUTP:dTTP was adjusted to 1:1.7. The probe corresponds to the entire predicted open reading frame and was generated by PCR with primers MINUS1 and DREND using a full-length cloned *dADAR* cDNA as template. Probes were digested with *Cfo*I, *Sau*3a, *Hae*III, and *Sau*96I restriction enzymes (Promega). Embryos were collected on apple juice-agar plates for 12 h and were prepared as described (Lehmann & Tautz, 1994). Embryos were hybridized at 44 °C for 16 h. Preabsorbed anti-digoxigenin antibody (1:2,000) and NBT/BCIP were used for probe detection (Boehringer Mannheim) (Lehmann & Tautz, 1994). Embryos were dehydrated with ethanol, cleared with xylene, and mounted in Poly-mount (Polysciences).

Analysis of in vivo RNA editing

Direct automated sequencing of RT-PCR products was performed to assess RNA editing. The same primers were used to sequence cloned DNAs or genomic PCR products and were nested with respect to those used to generate the products. Editing of *dADAR* at the S/G site introduces an *Ava*II restriction enzyme site (AGTCC to GGTCC). For quantitating editing frequencies (Fig. 7), cDNAs were cloned by RT-PCR using the indicated RNA as template and the primer combination DROP and DREND. RT-PCR products were either subjected to direct sequencing on an ABI model 371 automated sequencer (UCHC molecular core facility) or subcloned into pBlueScript (Stratagene). For each developmental stage, three independent RT-PCR reactions were performed. Error bars in Figure 8 are standard deviation.

Expression of epitope-tagged recombinant dADAR protein in *P. pastoris* and protein purification

PCR amplification was performed on the coding sequence of *dADAR* missing the first methionine and the stop codon (637 amino acids) from the cDNA clone pSR1–9 with primers containing *Nhe*I restriction sites at their 5′ termini. The *dADAR* isoform used was full length beginning in exon 1, including exon 3a, and unedited at the S/G site. The PCR product was first subcloned in the T/A cloning vector pGEM-Teasy (Promega) and a *Nhe*I fragment was subsequently subcloned into the *Spe*I site in pSK-FLIS6 to express a recombinant protein with the FLAG epitope tag (Sigma) at the N-terminus and a histidine hexamer at the C-terminus. A *Not*I digestion of this subclone was performed and used for gene replacement of the AOX1 locus in the *P. pastoris* strain GS115 (Invitrogen *Pichia* expression manual).

Small-scale liquid nitrogen extracts for expression monitoring were made from His^+ transformants. Immunoblot analysis with anti-FLAG M2 monoclonal antibody (1:5,000) (Sigma) and the conversion of adenosine to inosine in dsRNA were used to monitor the expression of recombinant dADAR. The large-scale protein preparation was performed as previously described (O'Connell et al., 1998). Extract was applied to Ni^{2+}-nitrolotriacetic acid agarose (Ni^{2+}-NTA; Qiagen) and the column was washed with 10 mM imidazole and eluted with 250 mM imidazole. Fractions were analyzed for their ability to convert A to I in dsRNA (Fig. 4A). Immunoblot analysis of the column fractions with an anti-FLAG M2 monoclonal antibody revealed a band of 80 kDa that comigrated with dsRNA adenosine deaminase activity (Fig. 4B). Fractions containing recombinant dADAR from the Ni^{2+}-NTA column were pooled and further purified by chromatography on a FLAG M2 antibody matrix (Sigma) and eluted with FLAG peptide (Sigma) as previously described (Keegan et al., 1999). Fractions were again analyzed for their ability to convert A to I in dsRNA. Four hundred microliters of the fractions containing peak activity were pooled, precipitated with trichloroacetic acid to a final concentration of 10%, electrophoresed on an 12% SDS-polyacrylamide gel, and proteins were visualized by silver staining (Fig. 4C).

dsRNA adenosine deaminase assay

The dsRNA substrate was prepared by in vitro transcription as previously described (O'Connell & Keller, 1994). dsRNA containing 200 fmol of labeled adenosine was used per assay. The assay was performed at 37 °C for 1 h with either pure enzyme or partially purified fractions as previously described (O'Connell & Keller, 1994). The specific activity of the enzyme could not be measured due to the low level of expression of dADAR in *P. pastoris*.

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