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Spatial and temporal expression of *dADAR* mRNA and protein isoforms during embryogenesis in *Drosophila melanogaster* ☆

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ABSTRACT

*A*denosine *D*eaminases *A*cting on *R*NA (ADARs) function to co-transcriptionally deaminate specific (or non-specific) adenosines to inosines within pre-mRNAs, using double-stranded RNAs as substrate. In both *Drosophila* and mammals, the best-studied ADAR functions are to catalyze specific nucleotide conversions within mRNAs encoding various ligand- or voltage-gated ion channel proteins within the adult brain. In contrast, ADARs within developing fly embryos have scarcely been studied, in part because they contain little or no editase activity, raising interesting questions as to their functional significance. Quantitative RT-PCR shows that two major developmentally regulated mRNA isoform classes are produced (full-length and truncated), which arise by alternative splicing and also alternative 3′-end formation. *In situ* localization of specific *dADAR* mRNA isoforms during embryogenesis reveals that the full-length class is found primarily within the developing germ band and central nervous system, whereas the truncated isoform is mostly located in gut endothelium. Developmental Western immunoblots show that both isoform classes are expressed into protein during embryogenesis. Both the *rnp-4f* 5′-UTR unspliced isoform and the full-length *dADAR* mRNA primarily localize in the embryonic germ band and subsequently throughout the developing central nervous system. Previous studies have shown that some *rnp-4f* pre-mRNAs are extensively edited by dADAR in the adult brain. Computer predictions suggest that intron–exon pairing promotes formation of an evolutionarily conserved secondary structure in the *rnp-4f* 5′-UTR, forming a 177-nt RNA duplex resembling an editing site complementary sequence, which is shown to be associated with splicing failure and to generate a long isoform. Taken together, these observations led us to explore the possibility that interaction between *rnp-4f* pre-mRNA and nuclear full-length dADAR protein may occur during embryogenesis. In *dADAR* null mutants, *rnp-4f* 5′-UTR alternative splicing is significantly diminished, suggesting a non-catalytic role for dADAR in splicing regulation. A working model is proposed which provides a possible molecular mechanism.

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1. Introduction

*A*denosine *D*eaminases *A*cting on *R*NA (ADARs) have been described throughout the animal kingdom, where they function to co-transcriptionally deaminate specific (or non-specific) adenosines to inosines within pre-mRNAs (reviewed in Schaub and Keller, 2002; Keegan et al., 2004; Valente and Nishikura, 2005) or pre-miRNAs (reviewed in Jepson and Reenan, 2008). Mammals contain genes encoding proteins ADAR1, ADAR2, and ADAR3, whereas there is only a single *ADAR* gene in *Drosophila melanogaster*, designated *dADAR* (Palladino et al., 2000). Non-specific deamination has been shown to function as a defense mechanism against viruses producing double-stranded RNAs during their life cycle (Kumar and Carmichael, 1997), and has been described in some *Drosophila* adult brain *rnp-4f* gene transcripts (Petschek et al., 1996, 1997). Among the best-studied examples of specific deamination are mRNAs encoding mammalian (Higuchi et al., 1993) and *Drosophila* (Palladino et al., 2000) adult brain ligand- or voltage-gated ion channel proteins. It is often found that the double-stranded RNA serving as ADAR substrate in such examples arises by imperfect base-pairing between an exon and an adjacent intron *E*diting site *C*omplementary *S*equence (ECS), where editing site specificity is due to the pattern of bulges and loops in the RNA duplex (Higuchi et al., 1993; Reenan et al., 2000).

Alternative splicing in *Drosophila dADAR* pre-mRNA produces several different full-length isoforms within both embryos and

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adults (Palladino et al., 2000). Isoforms also arise by self-editing of transcripts near the catalytic site in exon 7, an event that is positively correlated with a developmental switch in editase activity that occurs in pupae and is accentuated in the adult central nervous system (CNS). In addition, a truncated set of *dADAR* mRNA isoforms has been identified which arises by differential 3′-UTR end formation and not alternative splicing (Ma et al., 2002), where the mRNA terminates in intron 6. It has been shown via *in situ* hybridization that *dADAR* mRNAs localize in the CNS in late stage embryos (Palladino et al., 2000), and in the adult brain (Ma et al., 2001).

Previous studies have been largely restricted to characterization of *dADAR* function in the adult CNS, where editase activity is very high. Very recently, the expression pattern of alternatively spliced full-length *dADAR* isoforms in some adult tissues outside the CNS has been described (Marcucci et al., 2009). The spatial and temporal expression of *dADAR* mRNA and protein isoforms during *Drosophila* embryogenesis has received very little attention. Editing activity in *Drosophila* embryos which is directed toward specific pre-mRNA substrate "hot spots" identified in the adult brain has been shown to range from very low to non-existent (Hanrahan et al., 2000; Ma et al., 2002; Keegan et al., 2005). The functional significance of *dADAR* isoforms that are produced during embryonic development remains to be determined, and may include non-catalytic roles.

Here, we utilize RT-PCR to describe and quantify patterns of specific developmentally regulated *dADAR* mRNA isoforms, and *in situ* hybridization to describe their locations in the developing embryo. The expression of both major mRNA isoform classes is described. We utilize Western immunoblots to show for the first time that both major isoform classes are expressed into protein during embryogenesis. Our study is the first to systematically characterize the temporal and spatial expression of full-length and truncated *dADAR* mRNA and protein isoforms during *Drosophila* embryogenesis. The observation that developmentally regulated *dADAR* mRNA isoforms in embryos are expressed into proteins raises questions as to their functional significance. Preliminary results are presented which are interpreted to show that in a *dADAR* null mutant, alternative splicing in the 5′-UTR of *rnp-4f* pre-mRNA transcripts is partially diminished, suggesting a non-catalytic role for dADAR in the regulation of alternative splicing, since no editing is observed in the wild-type 5′-UTR.

2. Materials and methods

2.1. Drosophila propagation and embryo preparation

Wild-type *D. melanogaster* strains used in this study were Oregon R and Canton S, obtained from the National *Drosophila* Stock Center. Other species were obtained from the *Drosophila* Species Stock Center. A null mutant *dADAR* stock was also utilized (Ma et al., 2001). Fly culture and embryo collection methods employed have been previously described (Peters et al., 2003). Embryo developmental staging was as described by Campos-Ortega and Hartenstein (1997). Staged embryos were stored at −80 °C until used for RNA or protein isolations. Embryos for use in mRNA isoform *in situ* hybridization were dechorionated in 50% bleach solution for 5 min and processed for removal of vitelline envelopes (Rothwell and Sullivan, 2000). The final washed embryos were stored at −20 °C in 100% methanol.

2.2. Preparation of RNA and protein extracts from Drosophila developmental stages

Total cell RNA and poly(A^+) mRNA isolations were as described (Peters et al., 2003). RNA concentrations were estimated by OD_{260} spectrophotometry (NanoDrop Technologies), and stored at −80 °C. Prior to −80 °C storage, embryos for protein work were washed with 0.7% NaCl and stored in 100–200 mg aliquots. Embryos were ground with a tight-fitting Teflon pestle homogenizer on ice in extraction buffer [1X PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), 10 mM $Na_2S_2O_5$, 1 mM DTT, 1 mM PMSF, containing 1X Complete^R protease inhibitor (Roche)]. The crude lysates were centrifuged at 16,000*g* for 15 min at 4 °C to obtain clear supernatants. The supernatant was removed and stored in aliquots at −80 °C. Protein concentrations were estimated by OD_{280} spectrophotometry.

2.3. Localization of dADAR mRNA isoforms by whole embryo in situ hybridization

DNA fragments (Fig. 1) specific to *dADAR* exon-4a (primer set "A" to recognize combined isoform classes), exon 7 (primer set "D" to recognize only full-length isoforms), and intron 6 (primer

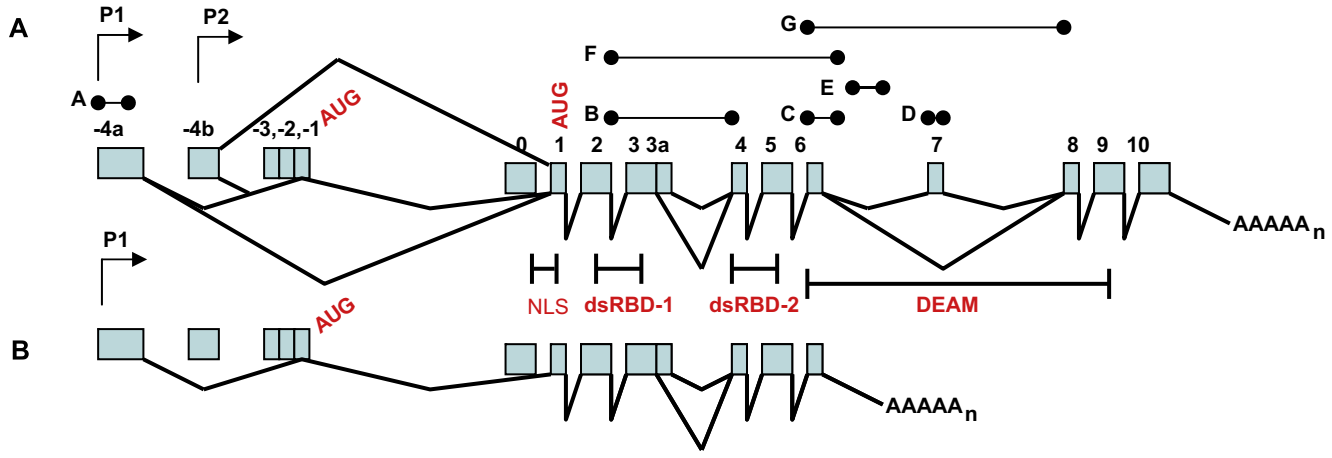

Fig. 1. *D. melanogaster dADAR* gene structure, splicing patterns, and positions of location-specific primer sets (horizontal bars, Table 1) utilized for developmental RT-PCR and *in situ* hybridizations. (A) Splicing patterns for full-length isoforms found in both embryos and adults (Palladino et al., 2000). Labels indicate positions of alternate promoters (P1, P2), alternative start codons (AUG), nuclear localization signal (NLS), double-stranded RNA-binding domains (dsRBD-1, dsRBD-2), and deaminase domain (DEAM). Utilization of promoter P2 occurs only in pupae and adults, where exon 3a is excluded from transcripts. (B) Splicing patterns for truncated isoforms found only in embryos, GenBank no. GQ168940 (designated isoform dADARe1) and GQ168941 (designated isoform dADARe2), which, respectively, exclude and include exon 3a (Ma et al., 2002). These isoforms arise due to alternative 3′-end formation within intron 6.

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