

Dissecting the splicing mechanism of the *Drosophila* editing enzyme; *dADAR*

Roberto Marcucci¹, Maurizio Romano^{1,2}, Fabian Feiguin¹,
Mary A. O'Connell³ and Francisco E. Baralle^{1,*}

¹International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012, ²Department of Physiology and Pathology, University of Trieste, Via A. Fleming 22, 34127, Trieste, Italy and ³MRC Human Genetics Unit Western General Hospital, Edinburgh EH4 2X U, UK

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ABSTRACT

In *Drosophila melanogaster*, the expression of adenosine deaminase acting on RNA is regulated by transcription and alternative splicing so that at least four different isoforms are generated that have a tissue-specific splicing pattern. Even though *dAdar* has been extensively studied, the complete adult expression pattern has yet to be elucidated. In the present study, we investigate mature transcripts of *dAdar* arising from different promoters. Two predominant isoforms of *dAdar* are expressed in gonads and *dAdar* is transcribed from both the embryonic and the adult promoters. Furthermore, full-length transcripts containing the alternatively spliced exon-1 are expressed in a tissue-specific manner. The splicing factor B52/SRp55 binds within the alternative spliced exon 3a and plays a role in this alternative splicing event.

Drosophila adenosine deaminase acting on RNA (*dAdar*) is an RNA editing enzyme that catalyzes the hydrolytic deamination of adenosine to inosine. Inosine base pairs with cytosine and is translated as if it were guanosine. Therefore, RNA editing can alter the codon composition and thereby increase the coding capacity of the RNA (1). Alternative splicing and RNA editing are the main pathways which increase proteome complexity post-transcriptionally. Editing can alter the protein coding sequence, thereby affecting the function of the protein or affect the splicing of pre-mRNA (2–4). It can also influence its own protein expression such as in the negative autoregulation of rodent ADAR2 (4) and *Drosophila Adar* (5). Moreover, the loss of *Adar* activity has been

shown to be important for normal behavior both in *Caenorhabditis elegans* and *Drosophila* (6) and to be critical for mouse development (7,8).

Drosophila Adar lies at the tip of the X chromosome within a region that resides in an ecdysone inducible chromosomal puff. Enhanced transcription from the 4A promoter was observed during pupal development and proposed to be due to an increase in ecdysone levels (9). In adult flies, the ovary and the supporting cells of the oocyte were shown to be the major steroidogenic tissue. (10).

There are multiple promoters for the *dAdar* gene. Expression occurs from the 4A promoter during all the stages of development, whereas 4B is only actively transcribed from the pupal stage and in adults. The mRNAs that are expressed encode a complex combination of alternative spliced isoforms (9). There are two mutually excluded alternatively spliced exons, -4a, and -4b at the 5'-end, in addition to alternatively spliced exons -1 and exon 3a. Exon 3a arises from the extension of exon 3 due to the selection of a distal non-canonical GC at the 5' splice site. Inclusion or exclusion of exon 3a changes the spacing between the dsRNA binding domain of *dADAR* resulting in altered binding capability on the dsRNA targets (11). In addition, transcripts including 3a exon were shown *in vivo* to lack self-editing of exon 7 that lies within the deaminase domain. By editing its own transcript, *dADAR* generates an enzyme that is catalytically less active (5). The biological function of alternative exon-1 has yet to be determined.

Members of SR family of proteins have been shown to be required for splice site selection. Here, we identify a binding site for B52/SRp55 within exon 3a of *dAdar* and demonstrate its role in splicing this exon. In *Drosophila*, B52/SRp55 was shown to control eye development and to regulate the alternative splicing of many transcripts involved in brain organogenesis (12,13).

*To whom correspondence should be addressed. Tel: +39 040 3757337; Fax: +39 040 3757361; Email: baralle@icgeb.org

To date, all known transcripts that are edited by ADARs have been detected in the CNS (14). Although editing in *Drosophila* occurs primarily in adult nervous system, ADAR is expressed in other organs and the complete adult expression pattern has not been defined. In this article, we investigate the expression pattern and the alternative splicing of *dAdar* in the gonads. *dAdar* is expressed from both the early and late promoter and there is a tissue-specific inclusion of exon-1.

EXPERIMENTAL PROCEDURES

Primers

dAdar Exon 2 sense
5'-TACACGCACCTCTATTCACG-3'
dAdar Exon 4 antisense
5'-TGACCGTCAACAGTAATAGC-3'
dAdar Exon -4a sense
5'-TCAATAATTAGAGCAAAAAG-3'
dAdar Exon -4b sense
5'-GTGTGTAGTGCACCTTTGGCCAGCC-3'
rp 49 sense
5'-GCGGGTGCCTTGTTCGATCC-3'
rp 49 antisense
5'-CCAAGGACTTCATCCGCCACC-3'
egl Exon 2 sense
5'-TGACGTTTCGTCTTCTTCCTGG-3'
egl Exon 4 antisense
5'-ATCCGTCATGTTCTCGCAGCC-3'
B52/SRp55 Binding site WT sense 5'-GAATTAATAC
GACTCACTATAGGGAGACTACCTCGCTT
GAACAACCCACGTTTTGCATGAGTCAG-3'
B52/SRp55 binding site WT as 5'-TCTGACTCATGCAA
AACGTGGGTTGTTCAAGCGAGGTAG-3'
B52/SRp55 Binding site MUT sense 5'-GAATTAATACG
ACTCACTATAGGGAGACTACCTCGCTTGTACAT
CTCACTTTTTTTCATCAGTCAGA-3'
B52/SRp55 Binding site MUT antisense
5'-TCTGACTCATGAAAAAGTGAGATGT ACAAG
CGAGGTAG-3'
T7 B52/SRp55 dsRNA sense 5'-GAATTAATACGACTC
ACTATAGGGAGACATCAAAAATGGCTACGGCT-3'
T7 B52/SRp55 dsRNA as 5'-GAATTAATACGACTC A
CTATAGGGAGAAGCTCGGTGTCATCCAACCTT-3'

Preparation of RNA templates for pull-down and dsRNA experiments

RNA templates for pull-down experiments were made by annealing the oligonucleotides listed above, that encode a T7 polymerase sequence followed by either the wild-type or mutant binding site for B52/SRp55. The annealed oligos were then transcribed with T7 RNA Polymerase (Stratagene) as previously described (15). Two specific oligonucleotides also containing a T7 polymerase sequence were used to amplify 426 bp of B52/SRp55 with cDNA from S2 cells for the dsRNA experiment. The amplified product was then purified and ~5 µg of DNA was transcribed with T7 Megascript kit (Ambion). The two RNA strands were annealed in 100 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol by incubating

at 65°C for 20 min followed by a slow cooling to room temperature.

Drosophila stock

All RT-PCR experiments were performed with *Oregon R* flies, obtained from the Bloomington Stock Center. The stock was maintained on standard cornmeal-agar medium at 25°C. Ovaries, oocytes and testes were meticulously dissected in 1× PBS for the light microscopy studies (see Supplementary material), and images were taken with the stereo microscope (Leica 12MZ 125).

Cell culture, transfections and RNAi experiment

Drosophila S2 cell lines were cultured in Schneider's *Drosophila* media (GIBCO) with glutamax (Invitrogen) supplemented with 10% fetal bovine serum and gentamicin (100 mg/ml) at room temperature. For the RNAi experiments, 15 µg of dsRNA specific for B52/SRp55 were transfected into S2 cells that were plated at 1 × 10⁶/well in six-well culture dishes with Fugene 6 Transfection Reagent (Roche). The cells were maintained in culture for 3 days before RNA was extracted.

RNA extraction and RT-PCR

Total RNA was prepared with TRI Reagent (Sigma Aldrich) from cultured S2 cells or from homogenized samples that had been dissected from flies. cDNA was synthesized with First Strand cDNA kit (Amersham Pharmacia) with random-hexamer primers. PCR products were separated by electrophoresis on an agarose gel and visualized by staining with ethidium bromide. To amplify the alternative spliced region within exon 3/3a, RT-PCR was performed with *dAdar* exon 2 as sense primer, and *dAdar* exon 4 as antisense primer. To amplify the *dAdar* -4a/-4b spliced region, primer specific for either exon -4a or -4b were used as sense primer and exon 4 as antisense.

The PCR conditions were the following: 94°C for 3 min for the initial denaturation, 94°C for 30 s, 55°C for 30 s, 72°C for 50 s for 35 cycles and 72°C for 7 min for the final extension. The PCRs were optimized to be in the exponential phase of amplification. The results of the transfections represent at least three independent experiments. The relative amount of different splice variants was quantified by optical densitometry with ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2004). Data were expressed as percentage of mean and SD. The significance of differences was determined by Student's t-test and *P* < 0.05 was considered to be statistically significant.

RNA affinity purification and western blot analysis

RNA affinity purification was performed as previously described (16). The immunoblots were hybridized with mouse anti-SR antibody 1H4 (Zymed Laboratories) and revealed with chemiluminescent detection of HRP (ECL detection kit; Amersham-Pharmacia) followed by exposure to X-ray film (Kodak X-OMAT AR).

RESULTS

Alternative splicing of exon 3/3a of *dAdar* in tagmata

Exon 3 of *dAdar* undergoes alternative splicing due to the presence of two competing 5' donor splice sites (5'ss), a strong proximal consensus GT, followed by a distal non-canonical GC. The consequence of differential 5'ss choice is that two isoforms are produced. Selection of the proximal donor site results in the generation of a short isoform, whereas the selection of the distal site produces the longer transcript that extends exon 3 and generates the alternative exon 3a isoform (Figure 1a).

RT-PCR analysis was performed to determine if there is expression of *dAdar* transcripts containing exon 3a in the tagmata of flies. Total RNA was prepared from the head, thorax, leg and abdomen of adult male and female *Oregon R* flies. RT-PCR analysis was performed with specific primers in exon 2 and 4 that were located close to the region of interest (Figure 1b). As shown in Figure 1b, the head, thorax and leg of both male and female displayed the same splicing profile. In particular, the spliced variant that includes exon 3a was expressed at a lower level in comparison to the main transcript that excludes it (Figure 1b, lanes 1-6). However, in both male and female abdomens the opposite was observed and expression of the transcript containing 3a was the major isoform

(Figure 1b, lanes 7 and 8). Therefore, these results demonstrate that the alternative splicing of exon 3/3a of *dAdar* in the tagmata is regulated.

The adult abdomen has a different embryological origin from the rest of the body as it is derived from nests of histoblasts located in the embryonic and larval epidermis. This is also the compartment where the gonads are located. Therefore, we investigated the alternative splicing of *Adar* within the abdomen. Abdomens were microdissected and the testis and ovary were isolated. By RT-PCR analysis, we found the same splicing pattern as previously observed for the whole abdomen of the flies (Figure 1c, lanes 1 and 3). The expression pattern of *dAdar* exon 3/3a transcripts from the body of flies without the testis or ovaries was analyzed as a control. As it is clearly shown in Figure 1c, the gonads have a specific splicing profile that is distinct from the bodies, suggesting that *dAdar* transcripts containing exon 3a are mainly expressed in *Drosophila* gonads.

Alternative splicing of *dAdar* in *Drosophila* gonads

Transcripts of *dAdar* originate from either an embryonic or an adult promoter and this generates mRNAs with two mutually excluded exons, -4a and -4b at the 5'-end, together with a complex combination of spliced isoforms.

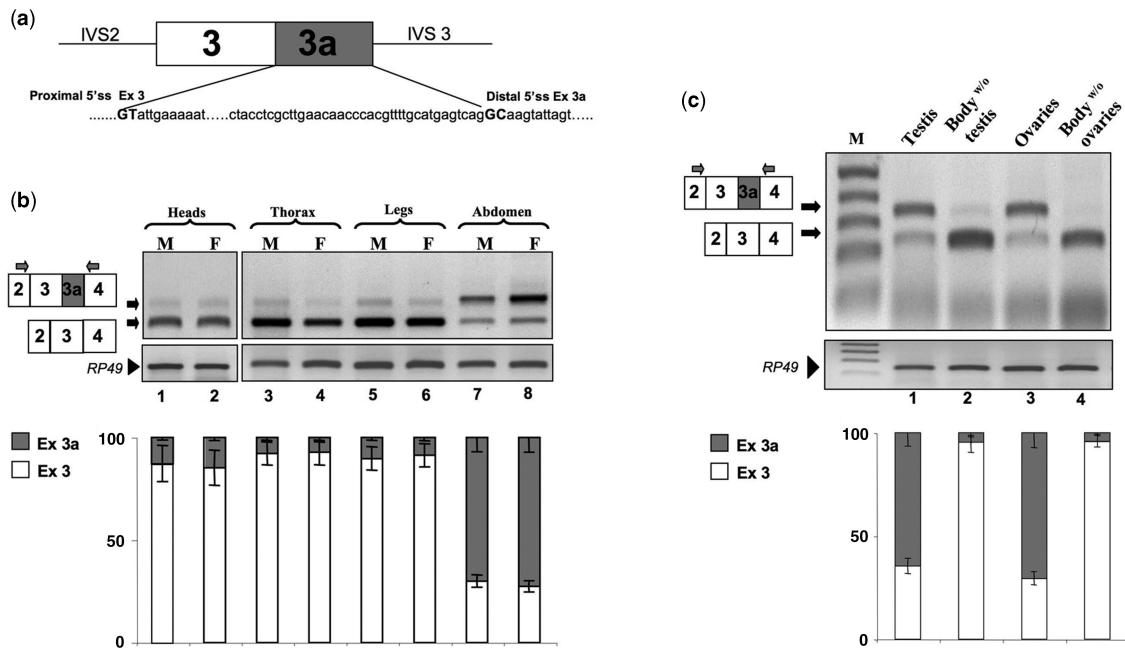


Figure 1. RT-PCR splicing pattern and tagmata expression of *dAdar* exon 3/3a. (a) Schematic representation of the *D. melanogaster Adar* (CG12598) transcription unit in the region of alternative spliced exon 3/3a. Introns are shown as lines and exons as boxes. The alternative spliced exon 3a is represented as a gray box. The dinucleotides of the proximal and distal 5'ss are in bold and some of the intervening sequence of exon 3a is shown. (b) RT-PCR analysis of the alternative splicing pattern of *dAdar* exon 3/3a from heads, thorax, legs and abdomens of *Oregon R* males and females adult flies (M, F). The gray arrows show the relative location of the primers used in the RT-PCR reaction. The two black arrows indicate the alternative spliced products generated in the RT-PCR reaction, either including or excluding exon 3a. In the lower panel, a black arrowheads mark the position of *rp49* that is used as an internal control. (c) Detection by RT-PCR of the splicing pattern of *dAdar* exon 3/3a expressed in testis and ovaries (lanes 1 and 3, respectively). Lanes 2 and 4 represent the controls obtained from the male fly bodies without testis and the female bodies without ovaries. Expected band size for the longer transcript that includes exon 3a was 346 bp and for the splice product excluding 3a was 235 bp. Lane M is the 1 Kb plus DNA marker. The stacked column charts below the gels show the relative percentage of amount of exon 3a/3 spliced isoforms quantified by optical densitometry. Gray columns indicate the exon 3a percentile, while white columns indicate the exon 3 percentile. Percentage was measured from three independent experiments; average with SD is shown.

Four major isoforms were shown to be expressed from the early promoter (4A) that correspond to either the inclusion or exclusion of the alternative exons -1 and 3a. However, from the late promoter (4B), only two isoforms are transcribed. By alternative splicing exon -1 is either included or excluded, whereas exon 3a is always skipped (Figure 2) (9).

We have analyzed the splice site selection and tissue-specific expression of the two mutually excluded alternatively spliced exons, -4a -4b in the testis and ovaries. Promoter-specific transcripts were amplified with primers annealing specifically to the end of either exon -4b or -4a (Figure 3). RT-PCR analysis of exon -4b containing transcripts from the testis and ovaries displayed no major differences to their respective body controls either including or excluding exon -1 (Figure 3a, upper panel, lanes 1–4). However, transcripts containing both exon -4a and exon 3a (Figure 3a, lower panel, lanes 5 and 6) were enriched in the testis and ovaries. The alternative exon -1 was expressed in a tissue-specific manner being more abundant in the ovaries than in the testis (Figure 3a, lanes 5 and 6). Direct sequencing of the RT-PCR products confirmed the exon composition. As expected, the bodies of the flies previously used as controls express almost exclusively the two isoforms lacking exon 3a (Figure 3a, lanes 7 and 8).

In summary, these results demonstrate that in ovaries there is a coordinated inclusion of both exon -1 and 3a when the upstream -4a exon is chosen.

dAdar exon -4a containing transcripts are developmentally expressed in *Drosophila* ovaries

The *dAdar* gene lies in an ecdysone inducible chromosomal puff and an increase in *dAdar* transcript levels is

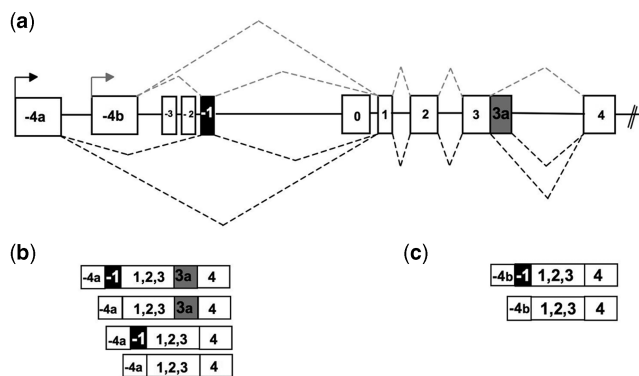


Figure 2. Schematic organization of the *dAdar* gene from the transcriptional start sites to the end of exon 4. (a) Schematic representation of the *dAdar* gene encompassing the region from the two promoter to the end of exon 4. In the article, to avoid confusion, we refer to the promoter with capital letters (4A and 4B), whereas -4a and -4b are the mutually excluded exons. Exons are shown as boxes and introns as black lines. The black and gray arrowheads mark the position of the embryonic (4A) and adult (4B) transcriptional start sites. The black and gray dashed lines indicate the pattern of alternative splicing that arise from the mutually excluded alternative spliced exons -4a and -4b. The two other alternative spliced exon (-1 and 3a) are indicated by a black and gray box respectively. (b and c) Diagrams of the alternative splicing composition of the main spliced variants that arise from the embryonic and adult promoters.

observed during the pupal stage (9). Moreover, significant levels of ecdysone are found in adult females and the nurse cells that are the supporting cells of the oocyte, are the major steroidogenic tissue (10).

Light microscopy images of *Drosophila* ovaries at days 1, 3, 5 and 10, show that in the ovary there is an increase in the number of mature oocytes and consequently the number of nurse cells from eclosion through to day 10 (Supplementary Figure S1). Therefore, exon 3a could be differently expressed in the adult females during this developmental stage in the ovary. To test this hypothesis, we microdissected the ovary and testis and analyzed the splicing pattern of exon 3/3a by RT-PCR. This time-course analysis revealed a significant increase in the spliced variants containing exon 3a in the gonads by day 3 (Figure 3b). This increased inclusion of the exon 3a in ovaries and testis suggests an enhanced activity from the early -4A promoter. Indeed a time-dependent increase in the length of transcripts encoding exon 3a was observed in the ovary but not in the testis (Figure 3c). In contrast, we did not observe any substantial change in the splicing pattern when we analyzed the transcripts that arise from the adult 4B promoter (Supplementary Figure S2). These experiments suggest that promoter choice dictates splice site preference in ovaries.

Expression of *dAdar* spliced isoforms 3/3a during oocyte development

Transcripts of *dAdat1*, a specific tRNA editing enzyme was shown to be produced by nurse cells and then loaded into the egg before fertilization (17). To determine if *dAdar* behaves in a similar manner its expression was analyzed during oocyte development.

Each ovary is composed of 16 ovarioles, in which the developing oocyte starts its differentiation in the germarium to generate an unfertilized egg after maturation. The ovary was dissected and RNA isolated from the germarium that included the earliest stages of the oocytes development; stages 4–8, stages 10 and 11 as well as mature unfertilized eggs (Supplementary Figure S3).

Expression of the *Egalitarian* gene was analyzed as a positive control (Figure 4a, lower panel), as it is expressed from the earliest stages of oogenesis and is required for oocyte specification in the germarium (18). RT-PCR analysis revealed that transcripts that both include and exclude 3a are expressed from early stages of oocyte formation and are detected during oocyte development (Figure 4a, upper panel). Furthermore exon -4a was also detected during these development stages of oogenesis (Figure 4b).

Exon 3a contains a binding site for the SR proteins B52/SRp55

B52 is the SRp55 homologue in *D. melanogaster*. B52/SRp55 is an essential splicing factor required for *Drosophila* development and it is known to regulate the splicing mechanism of many transcripts involved in brain organogenesis (12,13). The RNA binding consensus sequence for B52/SRp55 has been previously identified (19). The predicted secondary structure for the B52/SRp55 binding site is a hairpin structure with a guanosine

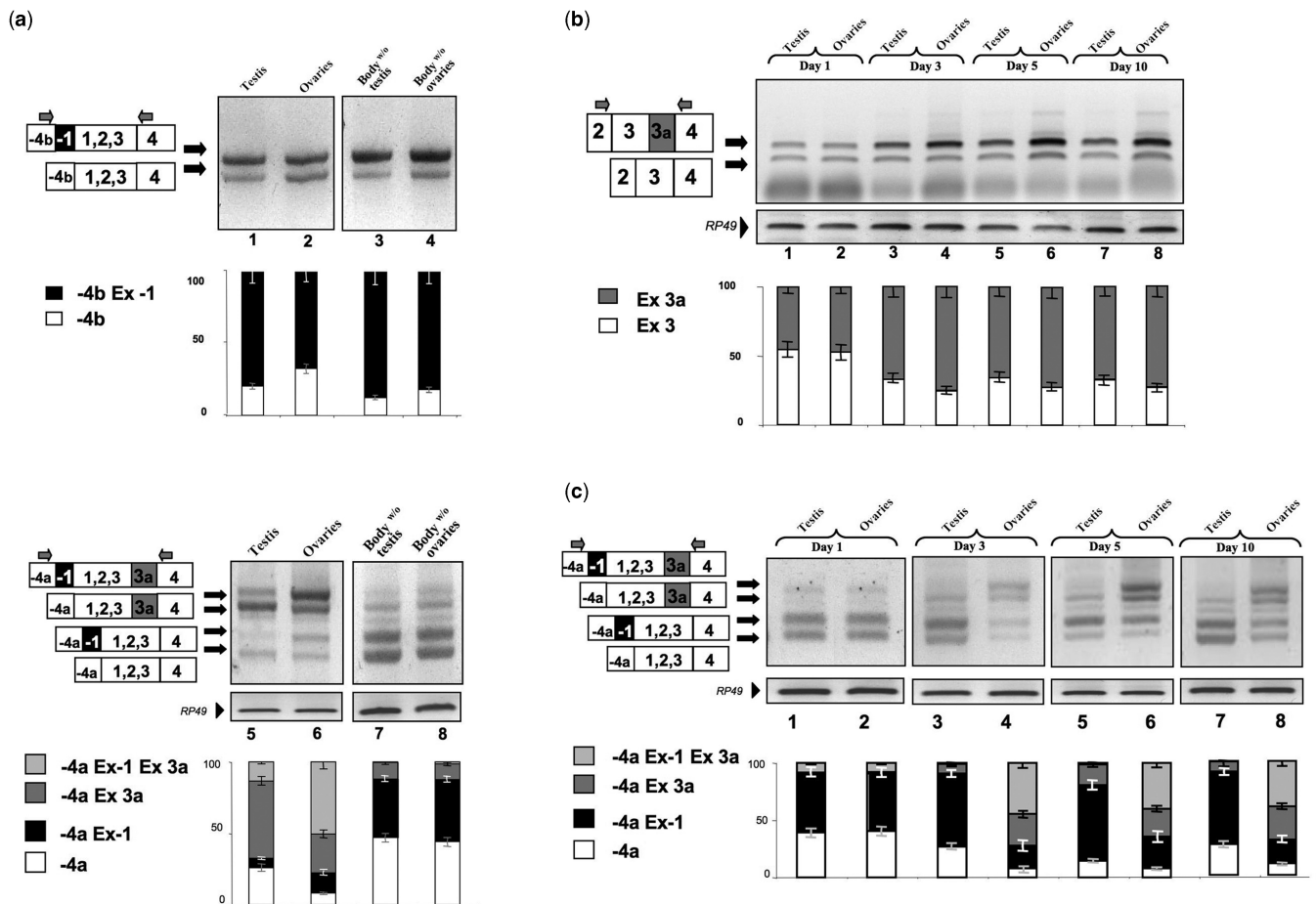


Figure 3. Promoter choice of *dAdar* and analysis of alternative splice pattern in gonads. (a) In the upper panel, RT-PCR analysis of the adult -4b alternative splicing pattern that is expressed in the testis and ovary (lanes 1 and 2) and the corresponding controls (lanes 3 and 4) is shown. In the lower panel is the RT-PCR of the -4a alternative splicing pattern expressed in testis and ovary (lanes 5 and 6), and the corresponding controls (lanes 7 and 8). A diagram of the alternative spliced isoforms are shown on the left with their exon composition and the black and gray boxes indicate the alternative spliced exons -1 and 3a. Exon -1 is expressed in a tissue-specific manner and is found in the ovary. The splicing pattern of *dAdar* transcripts in testis and ovary (lanes 5–8) is the inverse to their controls (lanes 7 and 8). The gray arrows indicate the relative location of the primers used in the RT-PCR. The expected band size for the -4b-Ex4 transcripts is 615 bp and 575 bp, respectively. The expected band size for the -4a-Ex4 transcripts is 608 bp for the full length, 568 bp (excluding exon-1) 497bp (excluding exon 3a) and 457bp (excluding exon-1 and exon3a). (b) A time course performed by RT-PCR of the expression pattern of *dAdar* exon3/3a from male and female gonads. (c) A time course of the alternative splicing pattern of -4a exon from male and female gonads analyzed by RT-PCR. Black arrowheads indicate the position of the constitutively expressed *rp49* rRNA used as an internal control in the lower panels of (a), (b) and (c). The stacked column charts below the gels show the relative percentage of the spliced isoforms that was quantified by optical densitometry. On the left of each column chart, a color key indicates the different alternative spliced isoforms. The quantification was performed on three independent experiments and the average with SD is shown.

residue at the +8 position that is crucial to maintain its structure (19). We found that this motif with the guanosine residue almost perfectly matched a sequence within exon 3a of *dAdar* (Figure 5a). The predicted secondary structure for the putative B52/SRp55 binding site within *dAdar* exon 3a suggests that its binding motif lies in a loop and the +8 guanosine residue mimics its important role in maintaining the hairpin structure (Figure 5b).

To assess whether the B52/SRp55 is able to interact with this putative binding motif, we performed an RNA affinity purification followed by western blot analysis. The substrates used were either in vitro transcribed *dAdar* RNA containing exon 3a or as control mutated *dAdar* exon 3a where the binding site for both B52/SRp55 and

the +8G nucleotide were disrupted by point mutations (Figure 5a). These RNA templates were bound to adipic acid dehydrazide agarose beads by covalent conjugation. After incubation with *Drosophila* Schneider's S2 extract, the RNA protein complexes were separated on an SDS-PAGE gel and then subjected to western blot analysis with the antibody 1H4 that recognizes the phosphorylated epitope on SR proteins. As a loading control for this experiment, an aliquot of the pulled down RNA protein complexes was visualized by staining with Coomassie Blue (Figure 5c, left panel). As shown in Figure 5c, right panel, only the RNA containing the wild-type *dAdar* exon 3a sequence pulled down a protein that migrated at the same molecular weight as B52/SRp55 in the S2 extract. Therefore, B52/SRp55 could regulate the

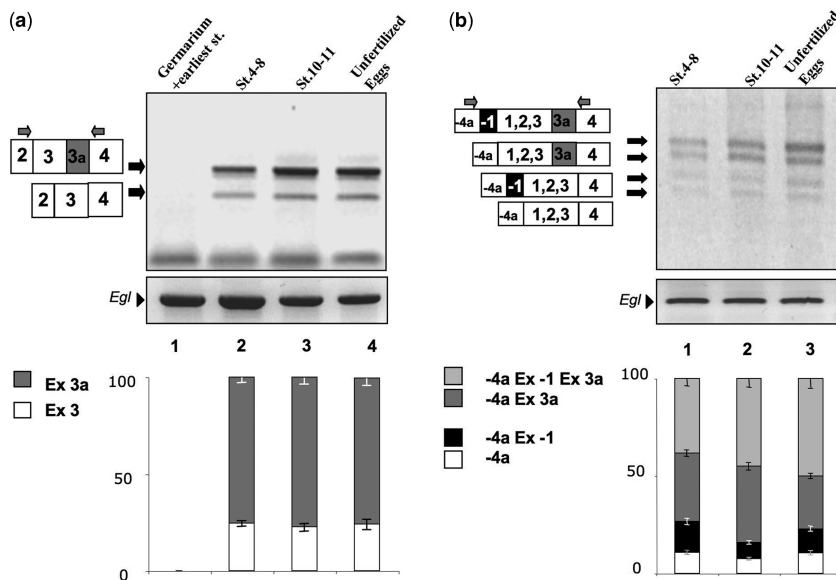


Figure 4. *dAdar* expression during the oocyte development. In (a and b) RT-PCR of *dAdar* in oocytes undergoing maturation to unfertilized mature eggs is shown. The alternative spliced region exon 3/3a and transcripts originating from the 4A promoter were amplified. The gray arrows indicate the relative location of the primers used in the RT-PCR reactions. St.4-8 and St.10-11 are the oocytes stages from which total RNA was prepared and RT-PCR analysis was performed. Black arrowheads indicate the mRNA of the constitutively expressed *egalitarian* gene (*Egl*) which was used as an internal control. The stacked column charts below the gels show the relative percentage of the spliced isoforms quantified by optical densitometry. On the left site of each column chart, the color key indicates the different alternative spliced isoforms. The quantification was performed on three independent experiments and the average with SD is shown.

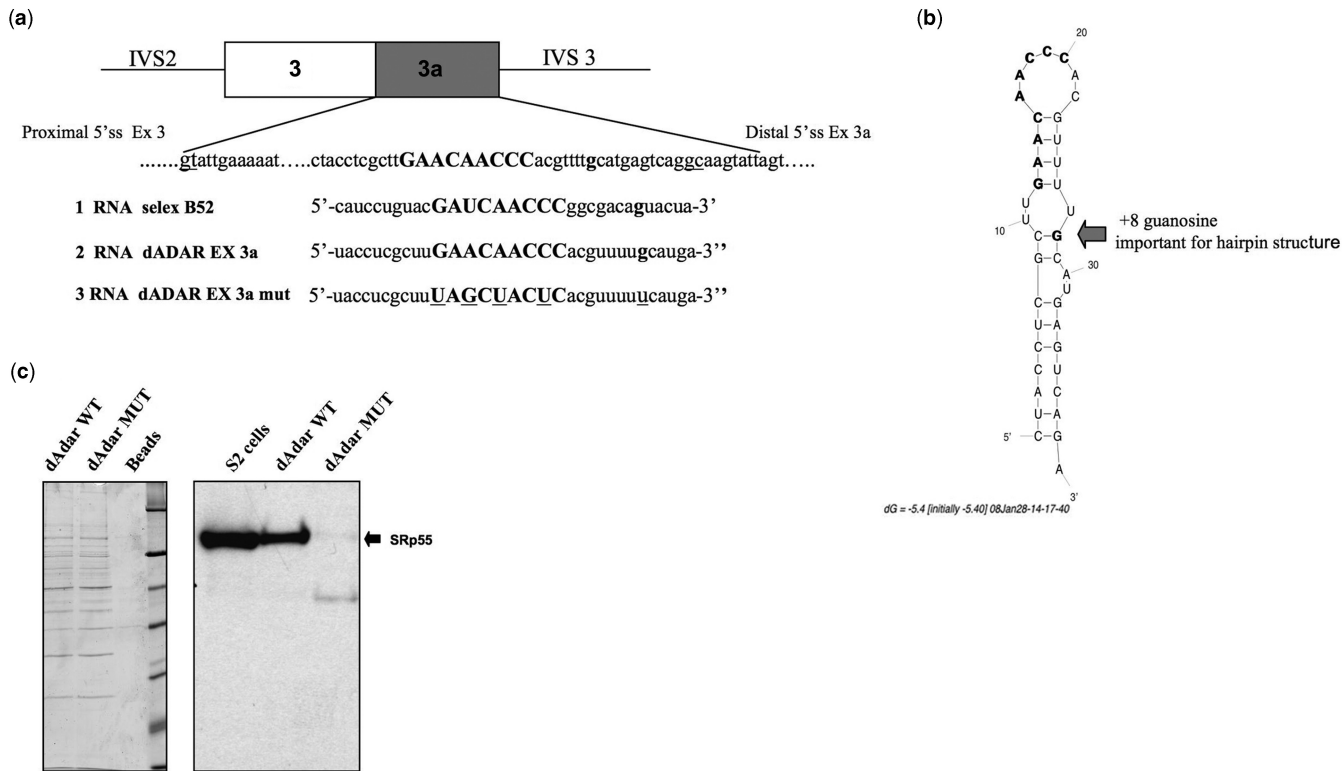


Figure 5. Exon 3a binds the alternative splicing factor B52/SRp55. (a) Schematic representation of the *dAdar* region encompassing exon 3/3a showing a partial sequence of exon 3a that includes the B52/SRp55 binding site. (1) The RNA sequence found by SELEX as a high affinity binding site for B52/SRp55. (2) The RNA sequence of *dAdar* exon 3a used for the RNA affinity technique. This RNAs contains the conserved consensus motif for B52/SRp55 and this is shown in bold. (3) (c) Left panel, RNA-affinity purification of proteins bound to RNA encoding either wild type or mutated exon 3a. Right panel, Western blot analysis of the bound proteins with 1H4 monoclonal antibody. The black arrow indicates phosphorylated B52/SRp55 that is present in S2 nuclear extract. The Beads lane is the negative control. The protein gel in the left panel is stained with Coomassie Blue to visualize the pulled down RNA protein complexes and is a loading control for the experiment.

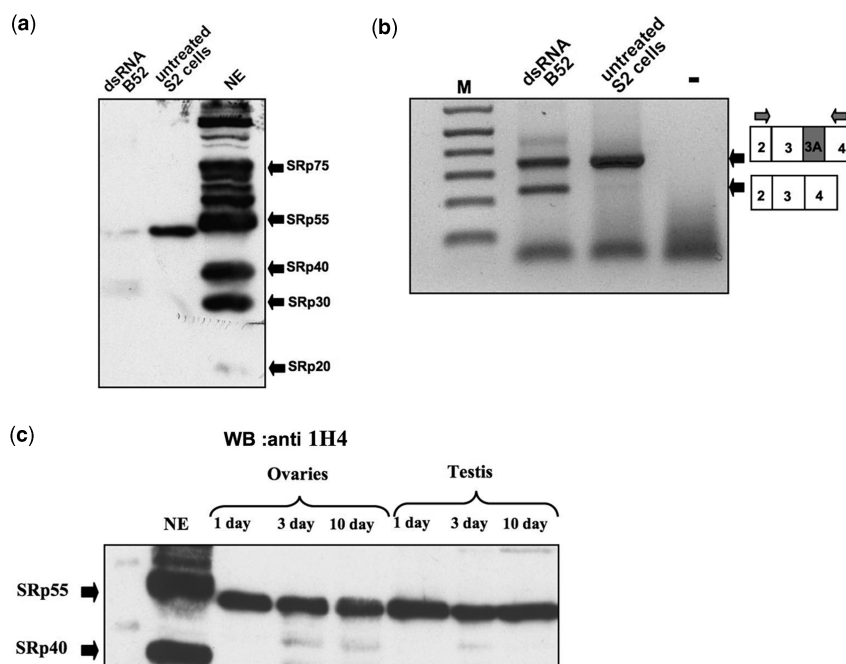


Figure 6. Regulation of the splicing of *dAdar* exon 3/3a by B52/SRp55. (a) Western blot analysis to determine the level of down regulation of endogenous B52/SRp55 protein after knockdown with dsRNA. NE indicates HeLa nuclear extract. The black arrows indicate the molecular weight of the SR proteins that are identified with the 1H4 monoclonal antibody. (b) RT-PCR analysis of *dAdar* after dsRNA knockdown of B52/SRp55 in S2 cells. M, is the 1 kb plus DNA marker and the symbol minus, indicates the negative RT-PCR control. (c) Western blot analysis of total protein extracts from female and male gonads during early development (days 1, 3 and 10) probed with 1H4 monoclonal antibody. NE indicates HeLa nuclear extract. The black arrows indicate the SR proteins that are recognized with the 1H4 monoclonal antibody.

alternative splicing of exon 3a in *Drosophila* by binding within exon 3a.

B52/SRp55 is required for the *dAdar* exon3/3a splicing regulation

To demonstrate that B52/SRp55 is important for the alternative splicing of *dAdar* exon 3/3a, we took advantage of double-stranded RNA-mediated interference for B52/SRp55 proteins in a *Drosophila* cell line (20).

Transient transfection of the specific siRNA in embryonic *Drosophila* Schneider's S2 cell line induced a strong reduction in the B52/SRp55 levels (Figure 6a). Its specificity has already been demonstrated and described in Park *et al.* (21). To determine whether the knockdown of B52/SRp55 affects the splicing of the third exon of *dAdar* gene, cells treated with dsRNA were then probed for the splicing of exon3/3a. The S2 cells endogenously express *dAdar* mRNA that includes the exon 3a. After RNAi depletion of B52/SRp55 there was an increased exclusion of the exon 3a sequences (Figure 6b). Thus, our data demonstrates that the binding of B52/SRp55 within exon 3a is required for the inclusion of this alternatively spliced exon in the mRNA.

DISCUSSION

In this article, we describe the expression, tissue specificity and the complex transcriptional/splicing regulatory mechanism of *dAdar*. As the biological role of RNA editing is

principally in the adult nervous system in *Drosophila*, to date most of the research on the expression pattern and the alternative splicing of *dAdar* has focused primarily on the brain. Indeed, the complete adult expression pattern has yet to be elucidated. The splicing pattern of the *dAdar* transcripts in the thorax and legs resembles that already characterized for the fly brain. However, *dAdar* is also expressed in the gonads where the splicing mechanism favors the inclusion of the alternative exon 3a. Interestingly, *dAdar* is expressed from both the early and adult promoters in testis and ovaries and full-length transcripts containing both alternatively spliced exons 3a and -1 are expressed in a tissue-specific manner as this transcripts is primarily expressed in the ovaries.

The alternative 3a exon is only present in transcripts that originate from the 4A promoter. Therefore, a time course of the expression of exon 3/3a was performed and the number of transcripts including exon 3a was observed to increase by day 3 after eclosion. A concurrent increase in the number of oocytes and supporting nurse cells was observed in the ovaries during this time course. A direct correlation can be drawn between the use of promoter 4A and the increased level of ecdysone produced by the nurse cells. This is consistent with the *dAdar* locus being located in an ecdysone inducible puff region near the *BrC* complex, which encodes transcription factors that also respond to this steroidogenic hormone (22). This increased usage of the 4A *dAdar* promoter was also observed during the pupal stage as ecdysone regulates larval development and metamorphosis (9). In adult

females, ecdysone is also produced in the nurse cells in the ovary. Therefore, we propose that enhanced activity from the 4A promoter is a consequence of an increased level of the ecdysone produced by these nurse cells.

As transcripts of a related protein *dAdat* (the *Drosophila* t-RNA editing enzyme) were shown to be produced by the nurse cells and loaded into the egg before the fertilization (17), we wanted to determine if *dAdar* could be detected during oogenesis. Full-length transcripts of *dAdar* were detected from early stages in oocyte development through to unfertilized mature eggs. One question that needs to be addressed is whether the predominant transcripts expressed in gonads are translated into functional proteins. However, this will remain unanswered until better *dADAR* antibodies are available. In *Drosophila*, the oocyte nucleus has been shown to be transcriptionally inactive during most of stages of oogenesis (23). The embryo receives maternally provided transcriptional input from the two other cell sources in the oocyte: the nurse cells and the somatic follicle cells. Therefore, in the future it will be interesting to understand the contribution to *dAdar* expression both from the nurse cells and the somatic follicle cells.

Recently, additional roles for editing enzymes have been proposed in relation to *Alu* elements, siRNA and miRNA function. ADARs can edit noncoding RNAs and can affect the processing of miRNAs such as pri-miR-142 (24) or affect the seed sequence as occurs in miR-376 (25), so that the miRNA is redirected to silence a different set of transcripts. Therefore, it is interesting to consider that *dADAR* could interact with noncoding RNAs and retarget the silencing machinery to transcripts that are required to be inactivated during the oocyte development.

To determine which splicing factor could regulate the alternative splicing of exon 3/3a, we analyzed the nucleotide sequence of exon 3a. The B52/SRp55 binding sequence obtained by SELEX (19) is very similar to the sequence we found in exon 3a that has a hairpin configuration with the protein binding site exposed in a loop and the guanosine residue that is critical for RNA structure. We demonstrated that B52/SRp55 specifically binds within exon 3a. Depletion of B52/SRp55 in *Drosophila* S2 cell line by RNAi demonstrates that this splicing factor controls the inclusion of the exon 3a as its down-regulation promotes the exclusion of exon 3a. However, we cannot conclude that the increase level of inclusion of exon 3a present in *dAdar* transcripts in the gonads is due to an increase in B52/SRp55 protein. Indeed, in testis and ovary the expression level of B52/SRp55 does not change during the developmental stages that have been analyzed (Figure 6c). This result suggests that in *Drosophila* gonads additional splicing factors participate in the complex splicing mechanism that regulates the alternative splicing of exon 3a. These results confirm previous reports that B52/SRp55 together with other splicing factors participate in the recognition of the distal 5' splice site (20). B52/SRp55 was previously shown to control eye development and to regulate the splicing of many transcripts involved in brain organogenesis (12,13). Here, we describe a novel function for B52/SRp55 to regulate the splicing of the exon3/3a in *Adar* transcripts in testis and ovaries (26).

The inclusion or exclusion of exon 3 in *dAdar* transcripts was demonstrated to affect both the binding and editing activity of the protein (5). *In vivo* no transcripts have been isolated that contain exon 3a and are edited in the deaminase domain. However, *in vitro* transcripts including the 3a isoform were shown to have reduced self-editing in exon 7 that lies within the deaminase domain. In addition, the exon 3a containing transcript introduces an additional 38 amino acids between dsRBM 1 and dsRBM 2 that generates two distinct enzymes. The spacing between the binding domains with exon 3a is very similar to the distance between the binding domains of ADAR2, whereas when this exon is excluded the distance is reminiscent of the distance between the dsRNA binding domains found in ADAR1. This conservation in the spacing of the dsRNA binding domains suggests that it is important for function. It has also been shown that the different isoforms of *dAdar* can form heterodimers and this can change its binding capability on the dsRNA target (11). Therefore, in the gonads different isoforms of *dAdar* with different editing activity could specifically edit transcripts that are expressed there and as a consequence modify the properties of the encoded proteins.

In ovaries, the inclusion of the alternative exon-1 results in a protein being expressed that has an additional 12 amino acids at the amino-terminal. This exon introduces an alternative in-frame translational start site that is preferentially transcribed from the early promoter. It has previously been shown that the use of alternative translational starts can regulate several cytokine family proteins whose expression level has to be fine tuned to maintain their physiological activity (16,27). Therefore, the question arises could this be a form of regulation of *dAdar* in the ovary that is in addition to autoediting? This regulation would represent a coordinated translational-transcriptional control that regulates alternative splicing and therefore the activity of the editing enzyme in the ovary.

New editing targets have recently been found (28). Several of these novel edited transcripts were shown to be involved in ion homeostasis, cytoskeletal components and signal transduction pathway and they may be edited in the gonads and have a biological role there.

In the gonads, development continues throughout the lifetime of the animal and the pathway involves the coordinated division and differentiation of a large number of cells. This site of extensive adult development probably requires a fine tune regulation of *dAdar*.

It is possible that the presence of different spliced isoforms of *dAdar*, may reflect differences in *dAdar* substrate specificity. Indeed, the tissue-specific alternative splicing of *dAdar* in *Drosophila* gonads may expand the repertoire of ADAR targets beyond the brain to *Drosophila* gonads (29).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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