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Expression of the *cabut-RB* isoform during embryonic development in *Drosophila melanogaster.*

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Introduction

cabut (*cbt*) is a *Drosophila* gene encoding a transcription factor, which is involved in several developmental processes such as embryonic dorsal closure (Muñoz-Descalzo *et al*., 2005) and epithelial regeneration (Blanco *et al*., 2010), ecdysone response (Beckstead *et al*., 2005), neuroendocrine cell remodeling (Zhao *et al*., 2008), circadian rhythm (Kadener *et al*., 2007), axon guidance and synaptogenesis (Kraut *et al.*, 2001; Mindorff *et al.*, 2005), pole cell formation (Yatsu *et al.*, 2008), cell growth (Guertin *et al*., 2006), autophagic cell death (Gorski *et al*., 2003), and cell cycle (A.J. Katzaroff and B.A. Edgar, personal communication). The *cbt* gene contains two exons separated by an intron, and two different mRNA products have been associated to this gene, *cbt-RA* and *cbt-RB* (Figure 1A). *cbt-RA* and *cbt-RB* mRNAs encode two Cbt protein isoforms, Cbt-RA (428 amino acids) and Cbt-RB (347 amino acids), respectively, both containing a serine-rich region at the amino terminus and three classical zinc finger domains C_2H_2-type at the carboxy terminal region. The Cbt-RB isoform, however, lacks 81 residues in the amino terminal region. It has been recently shown that *cbt* mRNAs are maternally contributed, since they are present in unfertilized and early embryos (Yatsu *et al.*, 2008; Belacortu *et al.*, 2010). During germ band retraction and dorsal closure, this gene is mainly expressed in epidermal cells, yolk nuclei, amnioserosa, hindgut, and anal pads (Muñoz-Descalzo *et al.*, 2005; Belacortu *et al.*, 2010). Although previous analyses suggested that the *cbt-RB* isoform is probably an artifact, expression analyses in microarrays (Arbeitman *et al*., 2002) as well as the presence of new expressed sequence tags (EST) in *Drosophila* cDNA libraries from S2 cells and embryos indicate that *cbt-RB* mRNAs are probably expressed. To determine whether *cbt-RB* transcripts are indeed present in *Drosophila* embryos, different experiments have

been performed. Our results confirm that the *cbt-RB* isoform is transcribed during embryogenesis in *Drosophila melanogaster*.

Material and Methods

Total RNAs were isolated from either 0-9 h or 6-9 h w^- embryos using the mirVana miRNA insolation kit (Ambion) following the supplier's instructions. RT-PCR and Real Time quantitative RT-PCR (qPCR) experiments were performed with those RNAs treated with DNase (Invitrogen). In the RT-PCR experiments, the primers used to amplify *cbt-RA* and *cbt-RB* specific fragments were 5′-CCTCGGACATTGTTGGCG-3′ and 5′-GGATACCTTGCTACCTTC-3′, and 5′-TGACCAGCATTGACCCAC-3′ and 5, TGTCACATTTCTGGCTCG-3′, respectively. In these experiments, primers designed to amplify *cbt* promoter regions (called Prom 1-2 and Prom 3-4, see Belacortu *et al.*, 2010) were also used to exclude DNA contamination in the RNA extracts. In the qPCR experiments, the primers used to amplify *cbt-RA* and *cbt-RB* specific fragments were 5′-GCCAAAAGCTAGGCAAACAG-3′ and 5′-GGAAAGGGTTAGCGTCATCA-3′, and 5′-GGAAAGGGTTAGCGTCATCA-3′ and 5′-TCCACAGTGGGTGGTACTGA-3′, respectively. In both experiments, amplification reactions without adding cDNA were carried out as negative controls.

Whole mount *in situ* hybridizations in *Drosophila* embryos were performed as described (Tautz and Pfeifle, 1989). Antisense RNA probes were generated with the DIG RNA labeling kit SP6/T7 (Roche). The *cbt-RB* expression pattern was determined in *OrR* embryos using the TOPO-Intron-Cbt construct, which contains a region specific for the *cbt-RB* isoform including a part of the *cbt* intron. Sense probes generated in parallel with the same templates were used as negative controls.

Results and Discussion

In order to determine whether *cbt-RB* transcripts are present during *Drosophila* embryogenesis, RT-PCR assays were performed by using total RNA extracts from 0-9 h wild-type embryos. After DNase treatment, specific primers to amplify the *cbt-RB* isoform were used (Figure 1A). Moreover, specific primers to amplify the *cbt-RA* isoform and *cbt* promoter regions were also used as positive control and to exclude the possibility of genomic DNA contamination, respectively (Figure 1A, and data not shown). Our results showed that a 673-pb fragment was obtained when using the *cbt-RB* specific primers (Figure 1B). Subsequent sequencing of the amplified fragment indicated that it corresponds to the *cbt-RB* isoform (data not shown). To confirm these results, qPCR experiments were also performed to detect the *cbt-RB* isoform using total RNA extracts from 6-9 h wild-type embryos and a different set of primers (see Figure 1A). Again, a *cbt-RB* specific fragment was obtained in these experiments, which were performed in triplicate (Figure 1C). *cbt-RA* specific primers were also used as positive control of the amplification reaction (Figure 1C). Our results showed that the *cbt-RB* fragment was amplified as efficiently as the one corresponding to *cbt-RA* (see the amplification plot shown in Figure 1C). No amplification products were detected either in the agarosa gel or in the amplification plot in the negative control experiments, in which no cDNA was added to the reaction mixture (Figure 1C).

Finally, we were interested in studying the expression pattern of the *cbt-RB* isoform during embryogenesis. To do that, we performed whole mount *in situ* hybridizations in embryos with a specific *cbt-RB* probe (see Figure 1A). Our results indicated that, as it happened with the *cbt-RA*

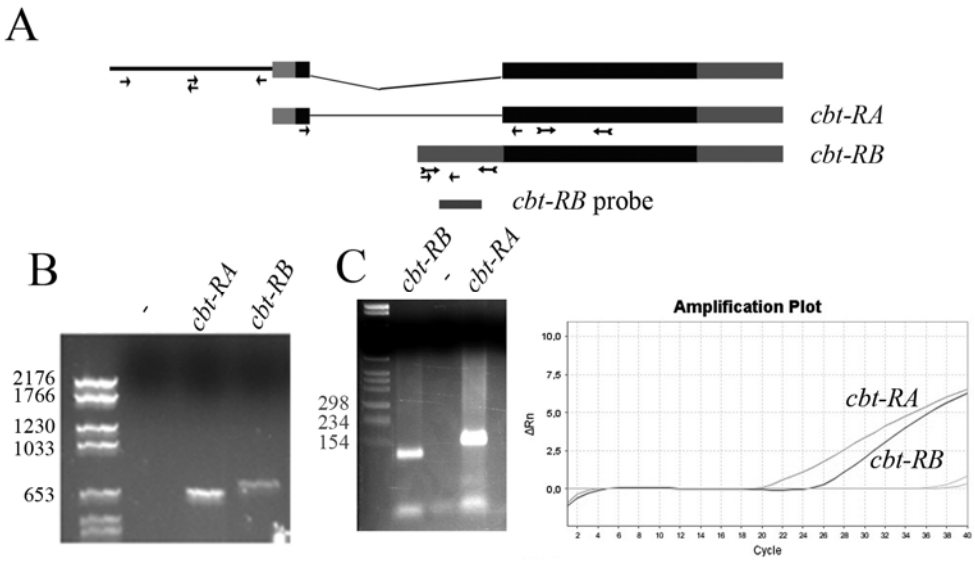

Figure 1. Structure of *cbt-RA* and *cbt-RB* transcripts, and analysis of *cbt-RB* expression by RT-PCR and qPCR. A. Genomic structure of the *cbt* gene and its putative transcripts. Coding regions are shown in black, non-coding regions, in grey. Intron is shown as an open triangle in the genomic region and as a line in the *cbt-RA* transcript. The promoter region of the *cbt* gene line is depicted as a thicker line upstream the coding region. The locations of the primers used in RT-PCR and qPCR experiments are indicated by arrows with flags and by arrows, respectively. The *cbt-RB* specific probe used in whole mount *in situ* hybridizations in embryos is also shown. B-C. 2% agarose/TBE gels showing the specific *cbt-RA* and *cbt-RB* fragments obtained in the RT-PCR and pPCR experiments using total RNA extracts either from 0-9h or 6-9 h embryos and primers shown in A. In both cases, - is the negative control in which no cDNA was added to the amplification reaction mixture. In C, the amplification efficiency of *cbt-RA* and *cbt-RB* fragments (upper and medium line in the graph) is shown in the amplification plot. When no cDNA is added to the reaction mixture, no amplification is detected (lower line in the graph).

isoform, *cbt-RB* was expressed in early embryos suggesting that it is also maternally contributed (Yatsu *et al*., 2008; Belacortu *et al*., 2010). However, *cbt-RB* transcripts were not present in germinal pole cells as is the case for *cbt-RA* mRNAs (Yatsu *et al.,* 2008; Belacortu *et al.,* 2010). During germ band retraction and dorsal closure (stages 10-13), *cbt-RB* transcripts were detected in the epidermis and, at low levels, in the yolk cell. *cbt-RB* expression was neither observed in the hindgut nor in anal pads (Figure 2). Thus, these results indicate that the *cbt-RB* isoform is indeed expressed in embryos although at lower levels than the *cbt-RA* isoform and not as ubiquitously. Interestingly, we have recently raised different antibodies able to recognize both Cbt proteins, Cbt-RA and Cbt-RB. However, we found that while the Cbt-RA protein is clearly detected in *Drosophila* embryos by immunohistochemistry and western blotting analyses, the Cbt-RB protein is not expressed (Belacortu *et al*., 2010). Taken together, our results suggest that the *cbt-RB* transcripts are probably not translated. It is likely that these transcripts are long non-coding RNAs (ncRNA) such as those derived from the *Drosophila roX1* (Meller and Rattner, 2002; Kelley, 2004) and *Pgc*

(Nakamura *et al*., 1996) genes. Other putative mRNA-like ncRNAs have been identified in *Drosophila* (Inagaki *et al*., 2005) and thousands of ncRNAs are expressed in mammals, which constitute at least 10% of total polyA RNAs (Okazaki *et al*., 2002; Numata *et al*., 2003; Ota *et al*., 2004). Although in most cases the function of these ncRNAs remains unclear, their abundance in different organisms strongly suggests that they could have important functions in different biological processes. Further analyses will be required to elucidate the putative role of the *cbt-RB* ncRNA during *Drosophila* embryogenesis.

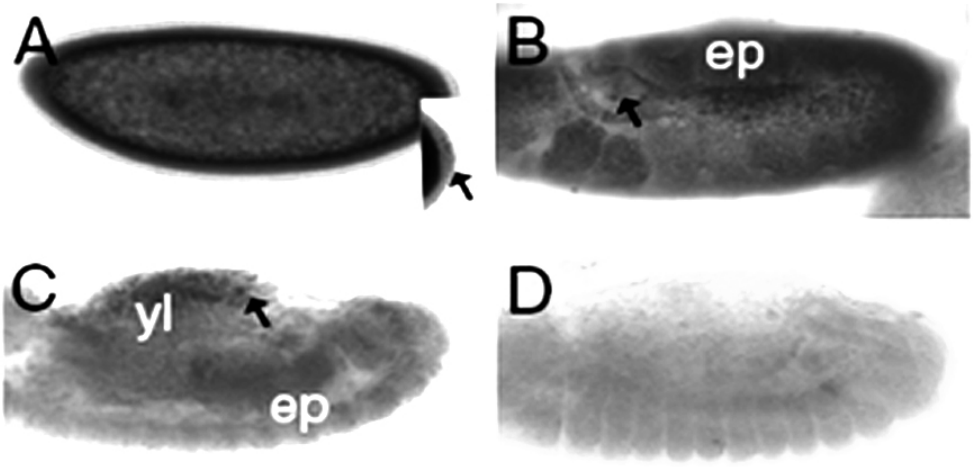

Figure 2. *cbt-RB* expression during embryonic development. A-D. Whole mount *in situ* hybridization of wild-type *Drosophila* embryos with digoxigenin-labeled (A-C) antisense and (D) sense *cbt-RB* riboprobes (as a negative control). A. Cellular blastoderm embryo (stage 2-3) showing ubiquitous *cbt-RB* expression in the yolk. No expression is detected in pole cells (indicated by an arrow). B-C. Embryos during band germ retraction (stage 10) and dorsal closure (stage 13), respectively. *cbt-RB* expression is detected in yolk nuclei (yl, indicated by an arrow) and in the lateral epidermis (ep). D. Stage 13 embryo hybridized with a *cbt-RB* sense probe. In all cases, anterior is to the left and dorsal is up.

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Morphological variation of the aedeagus in *Drosophila buzzatii* (Diptera, Drosophilidae).

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Introduction

The insect's aedeagus, which is the intromittent organ of the male genitalia, presents rapid and divergent evolution in relation to other morphological characters and is, therefore, a useful organ for taxonomic studies. Like any other phenotypic character, it shows geographic variation that may be related to environmental factors (Tatsuta and Akimoto, 1998; Kelly *et al.* 2000; Jennions and Kelly, 2002; Franco *et al.*, 2008). In the *Drosophila* genus and particularly in the *D. repleta* species group, the aedeagus morphology of its members has been considered the most important diagnostic character for species identification (Vilela, 1983). The *Drosophila buzzatii* cluster (*D. repleta* group: *D. buzzatii* complex) is a monophyletic group of seven cactophilic species of South America (Manfrin and Sene, 2006), and the male genitalia is a key trait that allows to distinguish its sibling species (Vilela, 1983; Silva and Sene, 1991).

One of these species, *Drosophila buzzatii*, has a wide geographical distribution, being found in Caatinga Domain Brazil until the Chaco Domain (Barker *et al.*, 1985). Although *D. buzzatii* has been extensively studied by cytogenetic and molecular markers (Baimai *et al.*, 1983; Ruiz *et al.*, 1984; Barker *et al.* 1985; Figueiredo and Sene, 1992; DeBrito *et al.* 2002; Khun *et al.*, 2003), there is a lack of information about the morphological diversity of its natural populations.

In this context, the objective was to investigate the population differentiation of the aedeagus morphology collected from nature, from four brazilian populations of *Drosophila buzzatii* [Irecê-BA, northeastern (n = 6), Furnas-MG, southeastern (n = 7), São Simão-SP, southeastern (n = 6) and Osório-RS, southern (n = 5)].

Material and Methods

The aedeagus (Figure 1) was removed and mounted on slides according to Kaneshiro (1969). The slides were photographed with a digital camera mounted on microscope. In this study, we used elliptical Fourier analysis (Kuhl and Giardina, 1982) to parameterize the coordinates of the of aedeagi