Int. J. Dev. Biol. 55: 975-979
doi: 10.1387/ijdb.103279fs

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Effect of the *transformer-2* gene of *Anastrepha* on the somatic sexual development of *Drosophila*

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ABSTRACT The *transformer-2* gene is involved in sex determination in tephritid flies (Tephritidae). It is required for the auto-regulation of the *transformer* gene (the memory device for sex determination in these insects) and for the female-specific splicing of *doublesex* pre-mRNA, the last gene in the sex determination gene cascade. The present manuscript addressed the question of the functional conservation of the tephritid *Anastrepha* Tra2 protein to direct sexual development in *Drosophila* (Drosophilidae). To express this protein in *Drosophila,* the GAL4-UAS system was used. The *Anastrepha* Tra2 protein supplies *tra-2* function in *Drosophila:* this protein would form a complex with the endogenous *Drosophila* Tra protein to promote the female-specific splicing of the *Drosophila doublesex* pre-mRNA. The feminisation produced by the *Anastrepha* Tra2 protein was, however, partial.

KEY WORDS: *Anastrepha, Drosophila, sex determination, transformer-2*

The characterisation of sex determination genes in *Drosophila Melanogaster* has shown that the product of a gene controls the sex-specific splicing of the pre-mRNA from the downstream gene in the genetic cascade (reviewed in Sánchez *et al.,* 2005). *Sex-lethal (Sxl)* is at the top of this cascade and acts as the memory device for female sexual development via its auto-regulatory function: its product controls the splicing of its own pre-mRNA (Bell *et al.,* 1991). In addition, Sxl controls the splicing of the pre-mRNA from the downstream gene *transformer (tra)* (Boggs *et al.,* 1987; Belote *et al.,* 1989). The Tra product and the product of the constitutive gene *transformer-2 (tra-2)* (Goralski *et al.,* 1989; Amrein *et al.,* 1990) control the sex-specific splicing of the pre-mRNA of the gene *doublesex (dsx)*, which is transcribed in both sexes (Burtis and Baker 1989). In females, the Tra-Tra2 complex binds to the female-specific exon (see Fig. 2A) and directs the splicing of the *dsx* pre-mRNA according to the female mode, giving rise to the female DsxF protein that promotes female sexual development. In males, in which no functional Tra protein is available, the *dsx* pre-mRNA follows the default male mode of splicing, giving rise to the mature *dsxM* mRNA, which produces male DsxM protein (Burtis and Baker 1989; Hoshijima *et al.,* 1991; Hedley and Maniatis 1991; Ryner and Baker 1991; Tian and Maniatis 1993; Hertel *et al.,* 1996). This promotes male sexual development.

Genes homologous to the sex determination genes of *D. melanogaster* have been sought in other insects (reviewed in Sánchez 2008; Verhulst *et al.,* 2010; Gempe and Beye 2010). In the tephritid fruit flies, the *Sxl* gene is not regulated in a sex-specific fashion (Saccone *et al.,* 1998; Lagos *et al.,* 2005) so that in the tephritids, *Sxl* does not appear to play the key discriminating role in sex determination that it plays in *Drosophila*. As in the drosophilids, the tephritid *tra* (Pane *et al.,* 2002; Lagos *et al.,* 2007; Ruiz *et al.,* 2007) and *tra-2* (Salvemini *et al.,* 2009; Sarno *et al.,* 2010) genes are expressed in both sexes. The *tra* primary transcript shows sex-specific alternative splicing. However, whereas in the drosophilids *Sxl* regulates *tra*, in the tephritids this gene appears to have an auto-regulatory function that produces functional Tra protein specifically in females (Pane *et al.,* 2002; Lagos *et al.,* 2007; Ruiz *et al.,* 2007) so that it plays the key regulatory memory device for sex determination (Pane *et al.,* 2002). A similar role for *tra* has been observed in the dipterans *Musca domestica (Muscidae)* (Hediger *et al.,* 2010) and in *Lucilia cuprina (Calliphoridae)* (Concha and Scott 2009). The injection of the respective *tra-2* dsRNA into *Musca* (Burghardt *et al.,* 2005), *Ceratitis* (Salvemini *et al.,* 2009) and *Anastrepha* (Sarno *et al.,* 2010) results in the destruction of endogenous *tra-2* function in these species and the subsequent male-specific splicing of the endogenous *tra* and *dsx* pre-mRNAs, leading to the transformation of genotypically female embryos into adult pseudomales. This highlights the role of *tra-2* in *Musca*, *Ceratitis* and *Anastrepha* sex determination.

Abbreviations used in this paper: Tra-2, transformer-2.

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Accepted: 15 February 2011. *Final, author-corrected PDF published online:* 13 December 2011.

ISSN: Online 1696-3547, Print 0214-6282

The present paper studied whether the *Anastrepha* Tra2 protein shows conserved sex-determination function in *Drosophila*; i.e., whether *Anastrepha* Tra2 is able to substitute the endogenous *Drosophila* Tra2 protein in the control of sex determination in this species.

Results and Discussion

The rationale of the experiment was to express the transgenic AoTra2 protein in *Drosophila* XX pseudomales lacking the *tra-2* gene function and checked whether these pseudomales showed feminisation. The GAL4-UAS system was used to express the AoTra2 protein in *Drosophila*.

The systemic expression of AoTra2 with the ubiquitous-expression *da-GAL4* or *hs-GAL4* drivers was found to be lethal to both male and female flies. The same lethality has been observed in *Drosophila* males and females that ectopically express their own Tra2 protein (Qi *et al.,* 2006). Therefore, the *rn-GAL4* local expression driver was used. This driver expresses GAL4 in agreement with the expression domain of the gene *rotund (rn)*, which is expressed in imaginal discs as well as in the embryonic and larval central nervous systems (CNS) (St. Pierre *et al.,* 2002). The expression of *rn* in the tarsal region of the foreleg imaginal disc commences during the early third larval instar, but is no longer evident in the late third instar, though in the *rn-GAL4* line persists the tarsal expression (St. Pierre *et al.,* 2002). This *rn-GAL4* driver allowed the expression of the AoTra2 protein in the foreleg basitarsus, a well-characterised sexually dimorphic region of *Drosophila*. The expression of AoTra2 by the *rn-GAL4* driver was also lethal to both males and females. This might be due to the expression of AoTra2 in the embryonic and/or larval CNS, as mentioned above.

To prevent embryonic lethality a strategy was followed that allowed the temporal control of AoTra2 expression under the *rn-GAL4* driver. For this purpose the GAL4/GAL80 system was used. The GAL80 protein inhibits GAL4 function. GAL80 is temperature sensitive, with 18°C the most permissive temperature and 29°C the most restrictive (McGuire *et al.,* 2003). *Drosophila* XX pseudomales mutant for *tra-2* and carrying *Aotra-2-UAS* together with *rn-GAL4* and *Tub-GAL80ts* were produced. The cross producing these pseudomales was performed at 18°C, and several two-days egg collections were made. Three days later each collection was transferred to 29°C environment. By this time the larvae had hatched, which were maintained at this temperature for the rest of their development. This treatment eliminated the embryonic lethality caused by the expression of the AoTra2 protein. Even under these conditions, the males and females expressing the AoTra2 protein were lethally affected, probably because of an excess of this transgenic protein affecting the development of the larval CNS.

Finally, since at 25°C GAL80^{ts} retains some function (McGuire *et al.,* 2003), it was reasoned that if XX pseudomales mutant for *tra-2* and carrying *Aotra-2-UAS*, *rn-GAL4* and *Tub-GAL80ts* can develop at that temperature, the GAL4 from the *rn-GAL4* driver ought not to be completely inhibited. Consequently a certain amount of AoTra2 protein ought to be produced (less than at 29°C) but which might not be lethal. Thus, these pseudomales might be able to survive to adulthood. This was the case. Fig. 1 shows the effect of expressing the AoTra2 protein on the sexually dimorphic development of the foreleg basitarsus in XX pseudomales and in their brother XY males, both mutant for *tra-2* and carrying *Aotra2-UAS*, *rn-GAL4* and *Tub-GAL80ts*. The foreleg basitarsus contained several transversal rows, the last one forming the sex comb structure (SC) in males and in XX pseudomales mutants for *tra-2* (Fig. 1C). This is composed of dark, thick bristles, and is rotated to lie parallel to the proximal-distal leg axis. Females lack the sex comb (Fig. 1B). A significant reduction ($P<0.0001$, one-way ANOVA) was seen in the number of bristles forming the male sex comb structure in the foreleg basitarsus of XX pseudomales raised at 25°C (expressing the AoTra2 protein) compared to those raised at 18°C (no expression of the AoTra2 protein) (Fig. 1A). Examples are shown in Fig. 1 D,E. In some cases, no sex comb-like bristles were found, yet the last transverse row (LTR) was partially or completely rotated

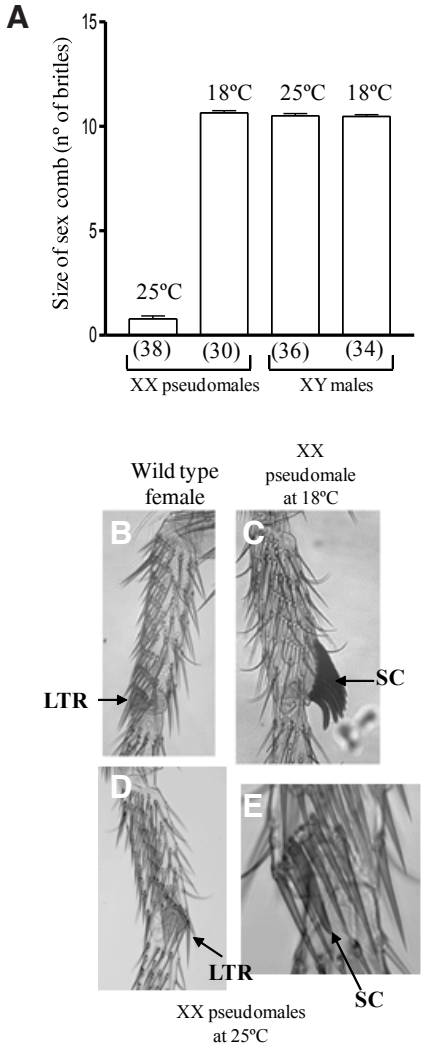

Fig. 1. Effect of the *Aotra2* transgene on the somatic development of *Drosophila*. (A) *Size (number of bristles) of the sex comb in the* Aotra2 *transgenic* Drosophila *flies. The bars in the histograms represent the 95% confidence limits. The sample size is given in parenthesis underneath the histogram. XX pseudomales:* ywTDAo#1/w; tra-2B/Df(2R)trix,tra-2[-]; Tub-GAL80ts,w+/ rn-GAL4,w[+]. *XY males:* ywTDAo#1/Y; tra-2B/Df(2R) trix,tra-2[-]; Tub-GAL80ts,w[+]/ rn-GAL4,w[+]*(Cross I in Materials and Methods).* **(B)** *Foreleg basitarsus of wild type female. LTR stands for last transversal row.* **(C)** *Foreleg basitarsus of XX pseudomale mutant for* tra-2 *at 18°C. SC stands for sex comb.* **(D,E)** *Foreleg basitarsus of XX pseudomales mutant for* tra-2 *at 25°C.*

resembling the location of the sex comb (Fig. 1D). In other cases, a sex comb-like bristle was found between the female-like bristles that form the last transversal row (arrow in Fig. 1E). The sex comb size of pseudomales raised at 18°C was the same as the sex comb of their XY brothers whether raised at 18 or 25°C (Fig. 1A).

The presence of endogenous *Drosophila* DsxF protein is expected to cause this reversion of the male towards the female phenotype. The analysis of the *Drosophila dsx* pre-mRNA splicing in transgenic *Drosophila* XX pseudomales mutant for *tra-2* and expressing the AoTra2 protein confirmed this expectation. The inducible *hs-GAL4* driver was used to express the *Aotra2* transgene. XX pseudomales *yw/w; Df(2R)trix,tra2[-]/tra-2B; Aotra2/hs-GAL4* were produced at 25°C. After the hatching of the adults the flies were divided into two populations; one was maintained at 25°C (control flies) and one subjected to heat-shock pulses (experimental flies) to induce the expression of the *Aotra2* transgene. At 25°C, the transgenic lines did not express the female *dsx* mRNA isoform (Fig. 2B). After the heat shocks, however, these transgenic lines expressed the female *dsx* mRNA isoform (Fig. 2B). Two amplicons could be detected. The smaller one (646 bp) corresponded to the female *dsxF* mRNA. The larger amplicon (758 bp) was to be expected if the intron 3 were retained (Fig. 2B). The cloning and sequencing of both fragments confirmed these suppositions. These results were not the consequence of the heat-shocks since their brothers (males *yw/Y; Df(2R)trix,tra2[-]/tra-2B; Aotra2/hs-GAL4*) expressing the *Aotra2* transgene did not express the female *dsx* mRNA isoform (Fig. 2C). Negative controls for all these PCR reactions produced no amplicons (see Materials and methods). Thus, the *Anastrepha* Tra2 protein is able to promote the female-specific splicing of the *Drosophila dsx* pre-mRNA.

Whereas the expression of *Anastrepha* Tra2 protein in the XX pseudomales produced their feminisation, its expression in their XY normal brothers mutant for *tra-2* did not affect their normal male development. This different effect is explained by the presence of Tra protein in the XX pseudomales and its absence in XY normal males. It should be remembered that the female-specific *dsx* pre-mRNA splicing mode requires the binding of the Tra-Tra2 complex to the female-specific exon 4 (see Fig. 2A). Therefore, the *Anastrepha* Tra2 protein supplies *tra-2* function in *Drosophila*: this protein would form a complex with the endogenous *Drosophila* Tra protein to promote the female-specific splicing of the *Drosophila dsx* pre-mRNA.

The feminisation produced by the *Anastrepha* Tra2 protein was, however, partial, indicating that the function of this protein in *Drosophila* was incomplete. There are two possible explanations for this. It might be due to an insufficient quantity of *Anastrepha* Tra2 protein being produced in the *Drosophila* transgenic flies; it was necessary to restrict the amount of *Anastrepha* Tra2 protein that was expressed since the production of any greater amount is lethal. Alternatively, the interaction between the endogenous *Drosophila* Tra protein and the transgenic *Anastrepha* Tra2 protein might be affected such that the *Drosophila*Tra-*Anastrepha*Tra2 complex is less efficient than the *Drosophila*Tra-Tra2 complex at inducing the female-specific splicing of the endogenous *Drosophila dsx* pre-mRNA, as it has been suggested for the interaction between the *Anastrepha* Tra and the *Drosophila* Tra2 proteins (Ruiz and Sánchez 2010). In this context, it is worth mentioning firstly the high degree of divergence

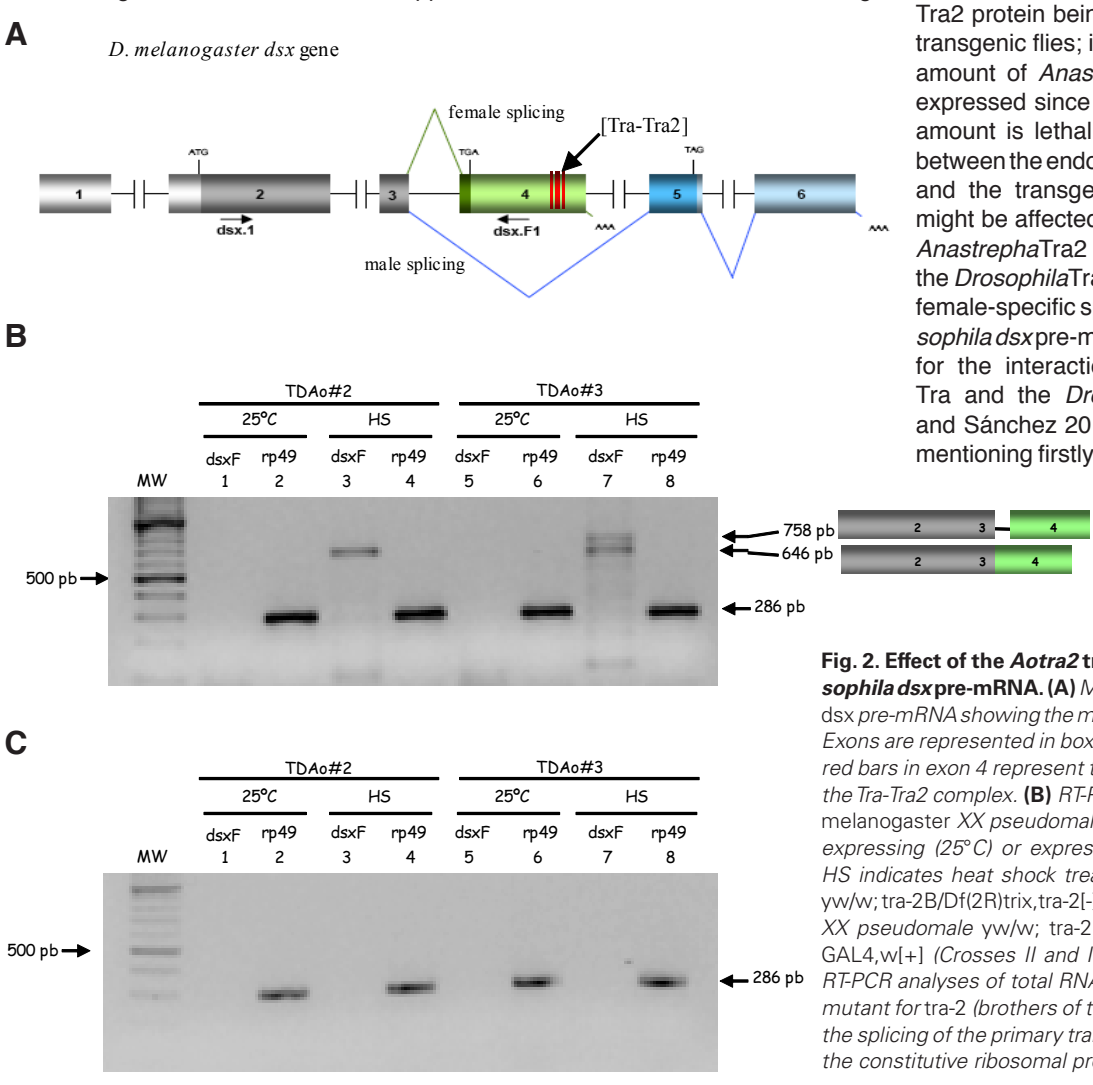

Fig. 2. Effect of the *Aotra2* transgene on the splicing of *Drosophila dsx* pre-mRNA. (A) *Molecular organisation of* Drosophila dsx *pre-mRNA showing the male and the female splicing pattern. Exons are represented in boxes and introns by dotted lines. The red bars in exon 4 represent the specific binding sequences for the Tra-Tra2 complex.* **(B)** *RT-PCR analyses of total RNA from* D. melanogaster *XX pseudomales mutant for* tra-2 *and either not expressing (25°C) or expressing (HS) the* Aotra-2 *transgene. HS indicates heat shock treatment.* TDAo#2*: XX pseudomale* yw/w; tra-2B/Df(2R)trix,tra-2[-];TDAo#2/hs-GAL4,w[+]; TDAo#3*: XX pseudomale* yw/w; tra-2B/Df(2R)trix,tra-2[-]; TDAo#3 / hs-GAL4,w[+] *(Crosses II and III in Materials and Methods).* **(C)** *RT-PCR analyses of total RNA from* D. melanogaster *XY males mutant for* tra-2 *(brothers of the XX pseudomales). As a control, the splicing of the primary transcript of gene* rp49 *that codes for the constitutive ribosomal protein 49 was monitored.*

between the *Anastrepha* and the *Drosophila* Tra2 proteins (Sarno *et al.,* 2010); secondly, the incomplete feminisation of *Drosophila* XX pseudomales mutant for *tra* and expressing the *Ceratitis* Tra protein (Pane *et al.,* 2005); and thirdly, the incomplete feminisation of *Drosophila* XX pseudomales mutant for *tra-2* and expressing either the human (Dauwalder *et al.,* 1996) or the *Sciara* (Martin *et al.,* 2011) *tra-2* ortholog, whereas *Drosophila virilis tra-2* gene can fully replace the endogenous *tra-2* function of *Drosophila melanogaster* for normal female sexual development (Chandler *et al.,* 1997). Collectively, these results suggest that the interaction between the Tra and Tra2 proteins of different species might be impeded as a consequence of changes accumulated in these proteins after the drosophilids separated from the other dipteran phylogenetic lineages, thus suggesting that the Tra and Tra2 proteins co-evolved to exert their functions in sex determination.

Materials and Methods

Flies and crosses

Drosophila flies were cultured on standard food. For the description of the mutant alleles and GAL4 constructs see Lindsley and Zimm (1992) and FlyBase. The crosses to produce the flies were:
Cross I: Females *ywTDAo#1; tra-2B/CyO·Cy; Tub-GAL80ts[w+]/MKRS,Sb* and males *w/Y; Df(2R)trix,tra-2[-]/CyO·Cy; rn-GAL4[w+]/MKRS,Sb*
Cross II: Females *yw; tra-2B/CyO·Cy; TDAo#2[w+]/MKRS,Sb* and males *w/Y; Df(2R)trix,tra-2[-]/CyO·Cy; hs-GAL4[ry+]/MKRS,Sb*
Cross III: Females *yw; tra-2B/CyO·Cy; TDAo#3[w+]/MKRS,Sb* and males *w/Y; Df(2R)trix,tra-2[-]/CyO·Cy; hs-GAL4[ry+]/MKRS,Sb*

Morphological analysis

Flies used for the analysis of adult forelegs were kept in a mixture of ethanol:glycerol (3:1) for several days. They were then macerated in 10% KOH at 60°C for 15 min, thoroughly washed with water and mounted in Faure's solution for inspection under a compound microscope.

Production of the Aotra2 transgenic Drosophila melanogaster lines (TDAo)

For the construction of the *Aotra2* transgene, the *tra-2* ORF of *A. obliqua* was amplified by RT-PCR. The PCR reaction was performed using primers *PRtra2-1Ao* (5'AGAGTTGGAATGAGTCCACG3') and *PRtra2-2Ao* (5'CATATTTTTAATAGCGCGTACG3'). The resulting amplicon was cloned in *pGEMT-easy* (Promega), following manufacturer's instructions. The inserted fragment was then cut with EcoRI and cloned in the EcoRI site of *pUAST*. The microinjections for generating the *TDAo* (*UAS::Aotra-2* cDNA) transgenic *D. melanogaster* lines were performed by Genetic Services (Sudbury, MA, USA). To ascertain that each transgenic line was carrying the correct transgene, PCR on genomic DNA with primers *PRtra2-1Ao* and *PRtra2-2Ao* was used to amplify the whole transgene and the amplicons were cloned in *pGEMT-easy* (Promega), following manufacturer's instructions and sequenced. Three transgenic lines were produced: *TDAo#1* (with the *Aotra-2* transgene inserted in the X chromosome), and *TDAo#2* and *TDAo#3* with the *Aotra-2* transgene inserted in the third chromosome. *TDAo* stands for *pUAS::Aotra-2* cDNA.

Analysis of the splicing of dsx pre-mRNA in transgenic Drosophila Melanogaster lines

The effect of *Anastrepha* Tra2 protein on the splicing control of *Drosophila dsx* pre-mRNA was studied in transgenic *Drosophila* XX flies mutant for *tra-2* and expressing the *Anastrepha* Tra2 protein. The inducible *hs-GAL4* driver was used to express the *Aotra2* transgene. XX pseudomales of genotype *yw/w; tra-2B/Df(2R)trix,tra2-; TDAo#2/hs-GAL4* and of genotype *yw/w; tra-2B/Df(2R)trix,tra2-; TDAo#2/hs-GAL4*, and their respective XY brothers of genotype *yw/Y; tra-2B/Df(2R)trix,tra2-; TDAo#2/hs-GAL4* and of genotype *yw/Y; tra-2B/Df(2R)trix,tra2-; TDAo#2/hs-GAL4* were produced at 25°C (see Crosses II and III above). After the hatching of the adults, the XX pseudomales and their XY brothers were divided into two populations; one was maintained at 25°C (control flies) and one subjected to heat-shock pulses (experimental flies) to induce the expression of the *Aotra2* transgene. Two 3 hours heat shock pulses (37°C) per day for two consecutive days with recovery at 25°C between pulses were given to the experimental flies. The effect of transgenic AoTra2 protein on *Drosophila dsx* pre-mRNA splicing was analysed by RT-PCR on total RNA from adults, which was prepared using the Ultraspec-II RNA isolation kit (Biotecx) following the manufacturer's instructions. Reverse transcription reactions were performed with an oligo-dT. Two percent of the synthesised cDNA was amplified by PCR, and one percent of this PCR was used for a second round of amplification, in a total volume of 50 μl. The primers used were: *dsx1* (5'CATCGGGAACATCGGTGATC3') and *dsxF1* (5'AACGGCTGT-CACACATCGAC3').

The PCR conditions were 95°C, 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 20 seconds each cycle, plus an extension step at 72°C for 7 minutes. In all cases, PCR reactions with RNA samples were performed to guarantee there was no contamination with genomic DNA (negative controls of PCR reactions). The amplicons were cloned in *pGEMT-easy* (Promega), following manufacturer's instructions.

DNA sequencing

It was used an automated 377 DNA sequencer (Applied Biosystems) and the primers forward M13 (-20) and reverse M13 reverse (-24).

Acknowledgments

We thank B. Oliver, I. Guerrero, M. Calleja, F. Díaz-Benjumea, E. Sánchez-Herrero and S. Campuzano for providing Drosophila *stocks. We are grateful to F. Díaz-Benjumea for bringing our attention to the GAL-80 driver. This work was financed by grant BFU2008-00474 awarded to L. Sánchez by the* D.G.I.C.Y.T.

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