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The class 2 selenophosphate synthetase gene of *Drosophila* contains a functional mammalian-type SECIS

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Synthesis of monoselenophosphate, the selenium donor required for the synthesis of selenocysteine (Sec) is catalyzed by the enzyme selenophosphate synthetase (SPS), first described in *Escherichia coli*. SPS homologs were identified in archaea, mammals and *Drosophila*. In the latter, however, an amino acid replacement is present within the catalytic domain and lacks selenide-dependent SPS activity. We describe the identification of a novel *Drosophila* homolog, *Dsps2*. The open reading frame of *Dsps2* mRNA is interrupted by an UGA stop codon. The 3′UTR contains a mammalian-like Sec insertion sequence which causes translational readthrough in both transfected *Drosophila* cells and transgenic embryos. Thus, like vertebrates, *Drosophila* contains two SPS enzymes one with and one without Sec in its catalytic domain. Our data indicate further that the selenoprotein biosynthesis machinery is conserved between mammals and fly, promoting the use of *Drosophila* as a genetic tool to identify components and mechanistic features of the synthesis pathway.

INTRODUCTION

Selenium is a trace element present in a number of prokaryotic and eukaryotic proteins in the form of the amino acid selenocysteine (Sec) (Low and Berry, 1996; Stadtman, 1996). Biosynthesis of selenoproteins rests on the production of the selenium donor compound monoselenophosphate (MSP) from selenide and ATP, a reaction catalyzed by the enzyme selenophosphate synthetase (SPS). SPS was initially identified as the product of the gene *selD*, one of four essential selenoprotein synthesis genes (*selA–D*) of *Escherichia coli* (Leinfelder *et al.*, 1990). MSP is used to synthesize Sec from seryl-tRNA (Forchhammer and Böck, 1991). $tRNA^{Sec}$, the *selC* gene product of *E. coli* (Leinfelder *et al.*, 1988; Forchhammer and Böck, 1991), and eukaryotic homologs (Park *et al.*, 1997) recognize UGA stop codons and cause incorporation of Sec into the elongating polypeptide. This process involves protein–RNA interactions mediated by the *selB* gene product, a Sec-specific elongation factor which interacts with $tRNA^{Sec}$ and a stem loop element, termed 'Sec insertion sequence' (SECIS) (Zinoni *et al.*, 1990; Berry *et al.*, 1991). In prokaryotes, SECIS elements reside immediately downstream of the UGA (Zinoni *et al.*, 1990), whereas in archaea and eukaryotes SECIS elements reside in the 3′untranslated regions (3′UTRs) of the selenoprotein-coding mRNA (Berry *et al.*, 1991; Wilting *et al.*, 1997).

Selenoprotein synthesis *per se* is conserved in evolution (Low and Berry, 1996; Stadtman, 1996). As in *E. coli,* selenoprotein synthesis was shown to be essential in mammals, since targeted disruption of the mouse selenocysteine tRNA gene caused early embryonic lethality (Bosl *et al.*, 1997). *selD* homologs have been identified in mammals, referred to as SPS1 and SPS2, respectively (Guimaraes, 1996). Furthermore, invertebrates have been demonstrated to synthesize selenoproteins. In case of thioredoxin reductase of *Caenorhabditis elegans* incorporation of Sec involves a UGA codon (Buettner *et al.*, 1999; Gladyshev *et al.*, 1999). Its alternative decoding is mediated by a functional 3′UTR-contained SECIS element, which differs by a G replacing the conserved single-stranded A in the mammalian SECIS element (Buettner *et al.*, 1999). In addition, a *selD* homolog of *Drosophila* was identified. However, its gene product, which corresponds to mammalian SPS1 (Persson *et al.*, 1997; Alsina *et al.*, 1999), lacks selenide-dependent SPS activity due to an arginine substitution of the critical Cys (or Sec) residue in the catalytic domain of the enzyme when expressed in *E. coli* (Persson *et al.*, 1997). None the less, disruption of the gene resulted in the

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reduction of selenoprotein synthesis, providing circumstantial evidence that the gene indeed does play a role in selenoprotein synthesis (Alsina *et al.*, 1999).

Here we describe a novel *selD* homolog of *Drosophila,* termed *Dsps2*. The results show that in contrast to another invertebrate, *C. elegans*, the fly contains two *Dsps* genes of the same kind as observed in mammals. Its *Dsps2* transcript contains a UGA stop codon in the catalytic center of the enzyme, but readthrough activity is provided by a mammalian-like SECIS element which functions in both transfected cultured cells and transgenic embryos. Furthermore, mammalian SECIS elements mediate readthrough activity in *Drosophila* cells, indicating that the components for SECIS-dependent selenoprotein biosynthesis are functionally conserved between mammals and *Drosophila*.

RESULTS AND DISCUSSION

The structure of the *Dsps2* gene, as revealed by sequence comparison of cDNA and corresponding genomic DNA, is shown in Figure 1A. *Dsps2* contains four exons, spanning a genomic region of ~2 kb (Figure 1A). The locus is located on the left arm of the second chromosome in section 31D/E (Figure 1B) and codes for a single transcript of ~1.5 kb (Figure 1C).

Whole-mount *in situ* hybridization of ovaries and embryos shows that *Dsps2* transcripts are expressed during all stages of oogenesis in nurse cells (Figure 1D). They accumulate in the oocyte and remain present at high levels up to the blastoderm stage (Figure 1E). Subsequently, the maternally derived transcripts decrease (not shown) and become replaced by zygotically expressed transcripts first detected in the midgut anlagen (Figure 1F). During subsequent stages, transcripts become enriched in a variety of tissues and organs including gut and nervous system (Figure 1G).

The 1324 bp *Dsps2* cDNA includes a 48bp 5′UTR, a 1110 bp open reading frame (ORF) and a 166 bp 3′UTR including a conventional polyadenylation signal (DDBJ/EMBL/GenBank accession No. AJ278068). It codes for a 370 amino acid polypeptide with a high degree of overall similarity to the SPSs of mouse, human, *Methanococus jannaschii* and *E. coli* (Figure 2). DSPS2 contains a conserved ATP/GTP binding site required for ATP hydrolysis during monoselenophosphate production (Veres *et al.*, 1994). The residue corresponding to Cys-17 in the catalytic domain of *E. coli* SelD is encoded by a UGA codon as observed in the mammalian class 2 SPSs (Figure 2). This observation implies that as in mammals, DSPS2 is a selenoprotein (Guimaraes, 1996) and its translation involves alternative decoding of the UGA stop codon in a SECIS element-dependent manner.

Eukaryotic SECIS elements can be grouped into two distinct classes (Grundner-Culemann *et al.*, 1999). Both contain the SECIS core AUGA motif, a stem and adenosine loop as well as the critical GA or AA pairs on the 3′ side of the stem, opposite AUGA, forming non-Watson–Crick G–A, A–G tandem base pairs (Walczak *et al.*, 1996; Martin *et al.*, 1998; Grundner-Culemann *et al.*, 1999). The predicted secondary structure of *Dsps2* mRNA suggests that the 3′UTR can potentially fold into a form II SECIS element (Figure 3A).

To demonstrate SECIS function of the *Dsps2* 3′UTR, we used a reporter gene construct in which the ORFs of bacterial *LacZ* and the luciferase genes were fused via an UGA stop codon

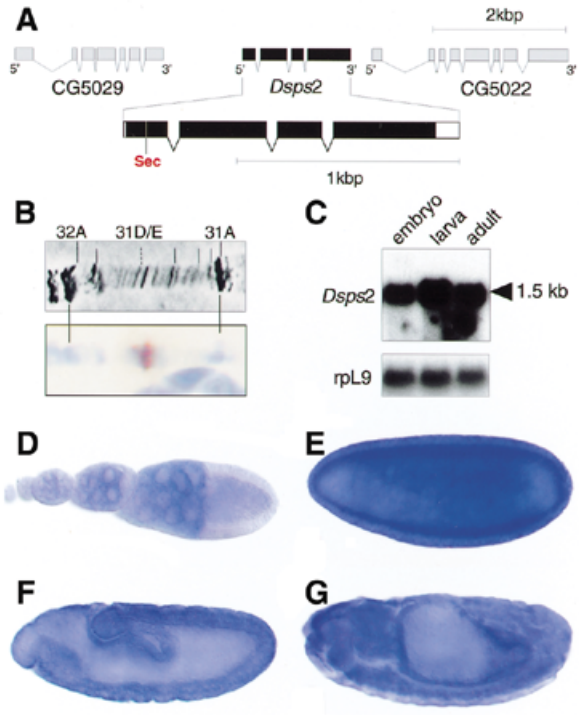

Fig. 1. Structure, location and expression of the *Drosophila sps2* gene. (**A**) Genomic organization of *Dsps2* showing that the gene is composed of four exons; coding sequences (black bars) and untranslated regions (open bars) are indicated. Note the position of the UGA in position 24 of the deduced protein. (**B**) *In situ* hybridization of *Dsps2* cDNA to polytene chromosome showing a signal in section 31D/E (see text). (**C**) Northern blot showing a single transcript in poly(A)$^+$ RNA of embryos, larvae and adults; rpL9 is a control for similar RNA loading. (D–G) *In situ* hybridization to ovaries (**D**), blastoderm (**E**), gastrula (**F**) and a germ band retracted embryo (**G**) showing that maternal *Dsps2* transcripts are expressed in nurse cells (D) and that zygotic expression occurs in a spatially restricted pattern (see text). Orientation: anterior to the left, lateral view, dorsal is top (E–G). For staging see Campos-Ortega and Hartenstein (1985).

('*LacZ/TGA/luc* reporter'). We transfected cultured *Drosophila* Schneider cells with DNA of *LacZ/TGA/luc* reporter genes (see Methods) with or without the *Dsps2* 3′UTR (pDsps2, pNo; Figure 3B). The results indicate that the 3′UTR of *Dsps2* resulted in translational readthrough activity as shown by luciferase activity (Figure 3C) as previously demonstrated in response to the mammalian SECIS-element (Kollmus *et al.*, 1996). Addition of limiting components of the selenoprotein biosynthesis pathway such as selenium and/or overexpressed $tRNA^{Sec}$ caused an increase of the SECIS-dependent readthrough activity (Figure 3D), indicating that the 3′UTR of *Dsps2* acts in a Sec-specific manner.

Next, we asked whether mammalian SECIS elements can also increase readthrough activity in *Drosophila* cells. For this, we used *LacZ/TGA/luc* reporter genes, which contained functionally characterized SECIS-elements of pig heart phospholipid hydroperoxide glutathione peroxidase mRNA (PHGPx) (Methods; Figure 3B). Figure 3C shows that readthrough activity is increased by SECIS-elements of pig PHGPx, indicating that the components of selenoprotein biosynthesis are functionally

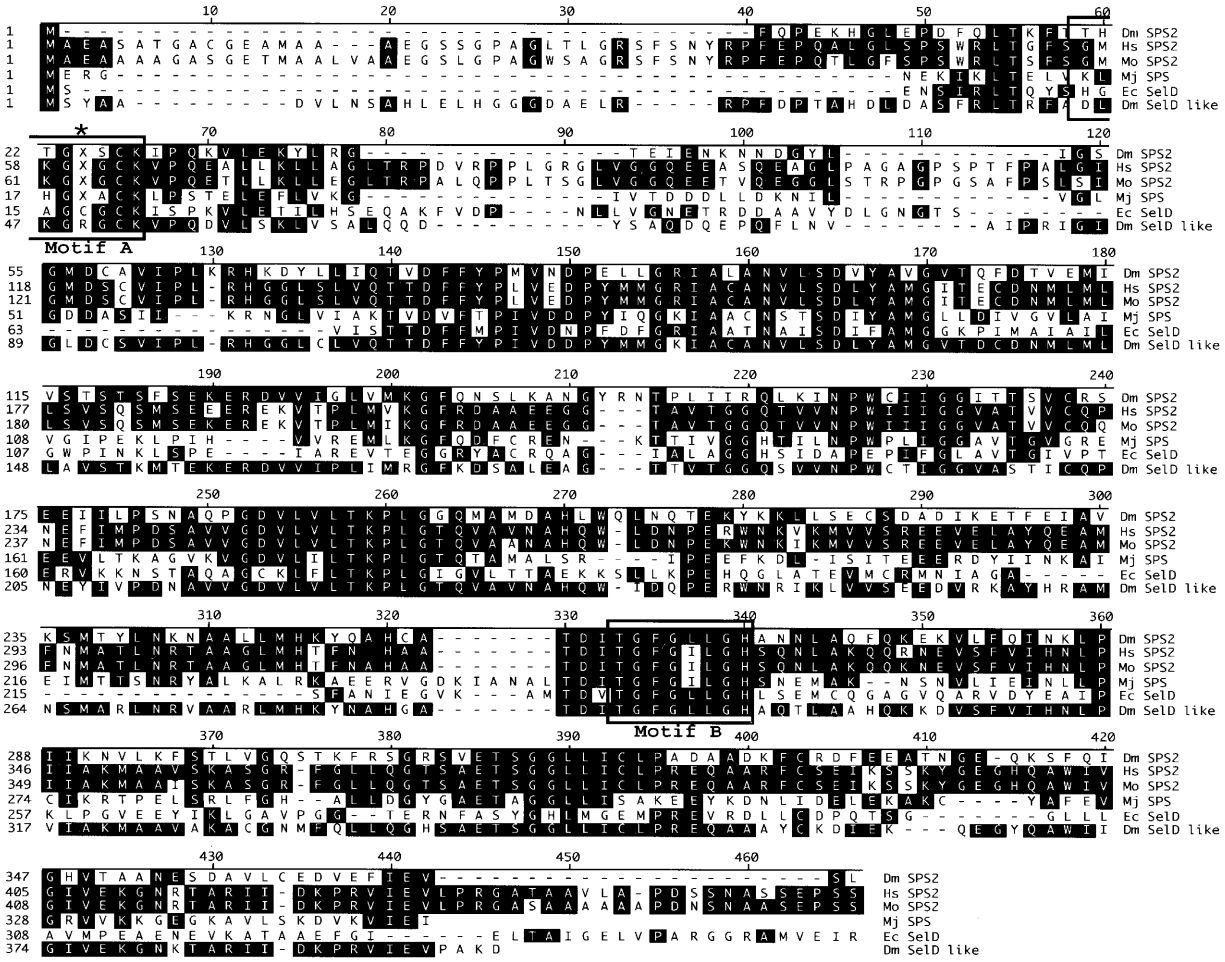

Fig. 2. Sequence comparison of DSPS2 and the known homologs. Alignment showing the *Drosophila* (Dm SPS2), human (Hs SPS2), mouse (Mo SPS2), *M. jannaschii* (Mj SPS), and *E. coli* (Ec SelD) SPS proteins and *Drosophila* SelD like protein (Dm SelD like). Position corresponding to Cys-17 of *E. coli* SelD is indicated by an asterisk, X denotes the Sec residue. Boxes: catalytic center (Motif A) and the ATP/GTP binding site (Motif B).

conserved between mammals and fly. The same result was obtained with the SECIS-containing 3′UTR of rat 5′ deiodinase mRNA (data not shown). The reduced readthrough efficiency mediated by the mammalian SECIS elements might be due to minor incompatibilities between the mammalian and fly selenoprotein biosynthesis systems or reflect a distance effect between the UGA and the SECIS element, an aspect not addressed by our study.

To demonstrate that the *Dsps2* 3′UTR mediates SECIS function in a living organism, we expressed *LacZ/TGA/luc* reporter genes with or without the *Dsps2* 3′UTR in transgenic embryos using the GAL4/UAS system (Brand and Perrimon, 1993) (Figure 4A). Embryos expressing a *Dsps2* 3′UTR-containing reporter gene showed significantly higher luciferase activities than those lacking the *Dsps2* 3′UTR (Figure 4B). In parallel to the enzymatic assays, embryonic homogenates were examined by immunostaining of western blots showing that the UGA stop codon is indeed suppressed in a *Dsps2* 3′UTR-dependent manner and that a β-galactosidase/luciferase fusion protein is produced (Figure 4B; inset). Furthermore, the *Dsps2* 3′UTR-dependent readthrough activity is reduced more than 2-fold in embryos homozygous for a *Dsps2* deficiency, which derived from heterozygous *Dsps2* deficiency females (Figure 4B). Residual DSPS2 in these embryos can be attributed to maternal *Dsps2* activity (see Figure 1D, E). In the absence of distinct *Dsps2*, this activity could not be removed since additional essential genes are uncovered by the smallest available *Dsps2* deficiency. Alternatively, part of the residual activity could also be contributed by *Dsps1*, provided that it carries SPS activity *in vivo* as suggested by Alsina *et al.* (1999).

The results reported here establish that: (i) *Drosophila* possesses the two vertebrate-type isoenzymes of SPSs; (ii) the SECIS element of *Dsps2* mRNA functions both in transfected cells and transgenic embryos; (iii) the translational readthrough can be stimulated by addition of critical components of both Sec synthesis and Sec incorporation; and (iv) transgene-derived DSPS2 of the expected size can be immunostained on western blots (our unpublished result), suggesting that *Drosophila* SPS2 is a selenoprotein. This conclusion is supported by the observation that its homologs in *Haemophilus influenzae*, the archaeon,

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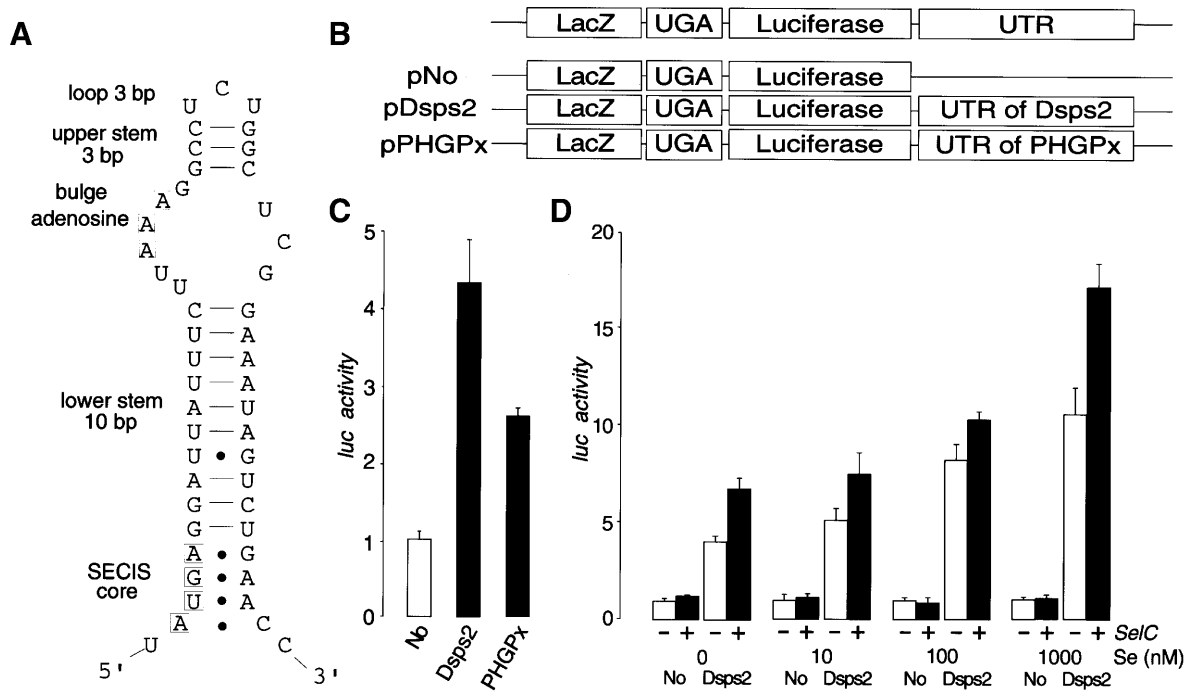

Fig. 3. 3′UTR-dependent readthrough of UGA in *Drosophila* tissue culture cells. (**A**) Predicted secondary structure of the 3′UTR of *Dsps2* showing a form II SECIS element [see text and details in Grundner-Culemann *et al*. (1999)]. (**B**) Diagrams of transfection constructs for experiment. (**C**) Readthrough activity as determined for different reporter genes [for nomenclature see (B)] containing different 3′UTRs in transiently transfected Schneider cells. Luciferase activities were normalized versus β-galactosidase activity and 'Luc activity' is represented as a fold-increase over the baseline value which is given by No-UTR. ('Luc activity' mean values of six independent transfection experiments and standard deviation are indicated). Note higher *Dsps2* 3′UTR-dependent readthrough activity as compared to SECIS elements of mammalian glutathione peroxidase (pPHGPx). (**D**) Effect of selenium (adding sodium selenite to the medium; see Methods) or *SelC* (by overexpressing co-transfected *DselC* as indicated by a +; see Methods) on *Dsps2* 3′UTR-dependent readthrough activity.

M. jannaschii, mouse and human carry a UGA-encoded Sec corresponding to Cys-17 in the activity center of SPS (Guimaraes *et al*., 1996).

The genomes of *C. elegans* and *Drosophila melanogaster* are sequenced (The *C. elegans* Sequencing Consortium, 1998; Adams *et al*., 2000). Whole genome database searches for *selD* homologs in the two species (www.sanger.ac.uk/Projects/C_elegans/ and www.fruitfly.org/), which represent model systems for unraveling molecular pathways by functional genetics, revealed only one *selD* homolog in *C. elegans* which contains a Cys in the critical position of the enzyme. The conservation of two isoforms of a key enzyme of selenoprotein biosynthesis, DSPS1 (Persson *et al*., 1997; Alsina *et al*., 1999) and DSPS2, in *Drosophila* and the readthrough activity obtained in response to the mammalian SECIS elements in *Drosophila* cell culture make it possible to use fly genetics to elucidate the precise function of each of the isoforms and to identify yet unknown components of the selenoprotein biosynthesis pathway more easily than in the vertebrate system.

METHODS

Cloning, sequencing and molecular procedures. Cloning of the *Dsps2* cDNA was initiated with an expressed sequence tag (EST) obtained from the Berkeley *Drosophila* Genome Project (BDGP). Two of the longest cDNA clones obtained from a poly(A)$^+$ RNA-derived library of 2–18 h old *Drosophila* embryos (Stratagene) were sequenced. Sequence comparison and secondary structure prediction were performed using the MegAlign program (DNASTAR Inc.) and the RNA-fold program of the GCG package (Genetic Computer Group), respectively. Database searches were done with both the *Drosophila* and *C. elegans* genomic sequences (www.fruitfly.org/ and www.sanger.ac.uk/Projects/C_elegans/).

Vectors pBPLUGA, and pBPHGPx3U (Kollmus *et al*., 1996) were obtained from Dr McCarthy. The *Dsps2*-3′UTR fragment was PCR-amplified (primers: 5′-AGA TCT AAT TAA TCA TTC AAC TTA TGA G-3′ and 5′- GGT ACC ATC GAC AAA CGA TTT TAT TAT-3′) and subcloned into *Bgl*II–*Kpn*I sites of pBPLUGA (pBselD). Reporter gene fragments (*LacZ/luciferase*; with or without 3′UTRs) of pBPLUGA, pBPHGPx3U, and pBselD were subcloned into the *Eco*RV sites of pAc5.1 C (Invitrogen; pNo, pPHGPx and pDsps2, see Figure 3) and into the *Not*I–*Kpn*I sites of pUAST (Brand and Perrimon, 1993; pUAS-No-UTR and pUAS-Dsps2, see Figure 4). *DselC* DNA was PCR-amplified (primers: 5′-GGT AAC CTT ACT CTT AAA ATT CCC AAG TGC TTA-3′ and 5′-GCG GCC GCC AGT TGC CTA GAT CGG GAG ACT CTG–3′) and subcloned into *Kpn*I–*Not*I sites of the pAc5.1 C (pSelC). Northern blots and whole-mount *in situ* hybridization of preparations and chromosomes are described (Persson *et al*., 1997; Vorbrüggen *et al*., 2000).

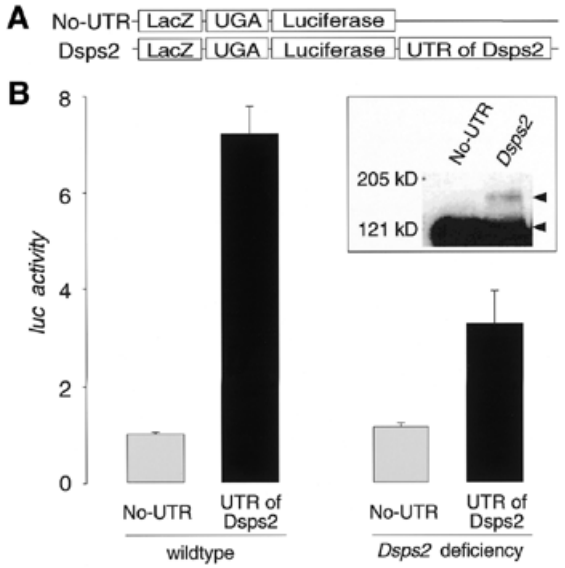

Fig. 4. SECIS element-dependent readthrough of the UGA stop codon in *Drosophila* embryos. (**A**) Schematic representation of the reporter genes used to construct transgenic embryos (see Methods). (**B**) Readthrough activity as determined from extracts of wildtype (left side) or *Dsps2* deficiency mutant embryos (right side; see text; readthrough activity was determined as described in the legend of Figure 3). Relative activities represent mean values from six independent collections of embryos; standard deviations are indicated. Inset: western blot indicating that embryonic extracts contain a 3′UTR-dependent β-galactosidase/luciferase fusion protein as revealed by anti-β-galactosidase-antibody staining. The fusion protein and UGA-terminated β-galactosidase are indicated by arrowheads.

Transgenic flies and cell transfections. Transgenic flies were generated by P-element-mediated germline transformation using pUAS-No-UTR and pUAS-Dsps2 DNA (Rubin and Spradling, 1983). Independent transformants were crossed with the actin-Gal4 driver (Bloomington Stock Center) to examine *luciferase* and *LacZ* activities in 15–24 h old wildtype and *Df(2L)J27* mutant embryos which lack the *Dsps2* gene (www.fruitfly.org).

Schneider Cells (Schneider, 1972) were transfected with 10 µg of plasmids (pNo, pPHGPx or pDsps2) and 5 µg of pBluescript (Niessing *et al.*, 1999). For overexpression of *DselC*, 5 µg of pSelC instead of pBluescript were co-transfected with pNo or pDsps2. Twenty-four hours after transfection, fresh medium or medium with various concentrations of sodium selenite (Sigma) were added. Cells were harvested for enzymatic assay 48 h after transfection.

Enzymatic assay and western blot analysis. Cell lysates and homogenates of embryos were prepared in 'Reporter Lysis Buffer' (Promega). LacZ activities (and the LacZ reference; Sigma) were determined from 100 µl samples diluted with 500 µl assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 12.5 mM β-mercaptoethanol) and 100 µl substrate (4 mg/ml *o*-nitrophenyl β-D-galactopyranoside in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4). Reactions were stopped with 500 µl 1M $NaCO_3$. LacZ activity was determined at 405 nm. Luciferase activity was measured in a PharMingen luminometer after injection of 100 µl Luciferase Assay Reagent (Promega).

Proteins were separated by SDS–PAGE. Western blots were stained with anti-β-galactosidase rabbit serum (Cappel; final dilution of 1:1000) and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) (secondary antibodies, dilution: 1:10000) assayed by SuperSignal west Dura Extended Duration substrate (PIERCE).

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