The 35UZ transposon of *Drosophila melanogaster* reveals differences in maintenance of transcriptional control between embryonic and larval stages

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ABSTRACT   The *D. melanogaster* transposon P[35UZ] contains a *lacZ* reporter gene fused to 35 kb of *Ubx* upstream sequences which drive a *Ubx*-like expression in embryos and in metathoracic imaginal discs. Transposition of P[35UZ] followed by analysis of different lines in wild-type and mutant backgrounds allowed us to analyze the interplay between *Ubx* regulatory elements, including the Polycomb response element (PRE), located inside the transposon and *cis*-acting regulatory elements, located outside. We found that all lines show a *Ubx*-like β-galactosidase expression pattern in the embryo, but proximity to strong imaginal enhancers can change this pattern drastically. These data illustrate how maintenance of gene expression depends on the chromosomal environment and on dynamic interactions between all developmentally regulated enhancers located close to a promoter under *PcG* control.

KEY WORDS: *silencing maintenance, developmental enhancers, PREs, polycomb group genes, Ubx*

Maintenance of the expression domains of many selectors, as the *Hox* genes, depends on the negative *trans*-regulators coded by the *Polycomb* group of genes *(PcG)*. These proteins form multimeric complexes which bind to *cis*-regulatory Polycomb response elements (PRE) in the genes to be silenced (reviews: Paro, 1995; Pirrotta, 1997, 1998). *PcG* mediated repression is thought to induce a chromatin structure whereto proteins are allowed access according to shape and size (McCall and Bender, 1996). This phenomenon has been extensively studied with transposons containing a PRE, a reporter gene and a transcription activator binding element such as the GAL4 binding sequence UAS. Silencing of such a construct can be overcome by an excess of the transcription activator GAL4 (Zink and Paro, 1995). During embryogenesis, reversal is consistently obtained by a single burst of GAL4 production from a hs-GAL4 construct, whereas during the larval stages one burst of the activator apparently cannot permanently revert PRE-mediated gene repression (Cavalli and Paro, 1998), suggesting that it might show increased stability during the longer interphases of larval development.

Here, we have attempted a complementary approach to study factors that affect gene expression patterns in larvae, by analyzing a *lacZ* reporter gene controlled by regulatory sequences of the *Ultrabithorax (Ubx)* gene at different chromosomal locations and in different genetic backgrounds. Endogenous *Ubx* is expressed in the embryonic epidermis from PS5 to PS13, with a strong peak in PS6, and in larvae in the posterior meso- and metathoracic disc compartments (review: Irvine *et al.*, 1991). The two *Ubx* regulatory regions both contain embryonic control elements, imaginal enhancers and PREs (Fig. 1A) (review: Pirrotta *et al.,* 1995). Most upstream embryonic control elements are contained in the 35 kb of *Ubx* sequences in transposon P[35UZ] (Fig. 1B) which drives a *Ubx*-like *lacZ* expression in embryos and in metathoracic discs (Irvine *et al.*, 1991). This pattern due solely to upstream sequences mimics a *bona fide Ubx* in embryos and in larval PS6 (White and Wilcox, 1985; Little *et al.,* 1990).

To assay the influence of different flanking sequences on P[35UZ], we examined lines bearing P[35UZ] at various chromosomal sites. Three transgenic lines, 35UZ-1 to -3, were generated by Irvine *et al.* (1991), and we recovered further 35UZ lines by mobilizing P[35UZ] from 35UZ-3. Southern blot analyses showed that six new lines (called the T lines) had a single insertion. Five

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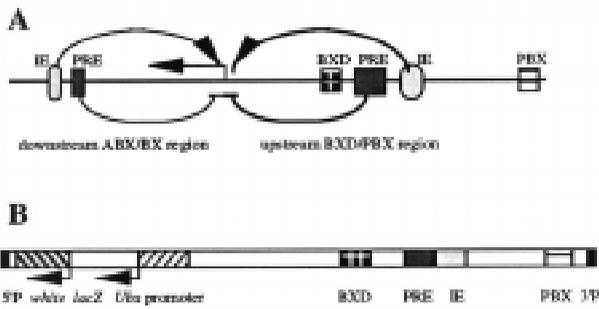
Fig. 1. *Ubx* regulation and map of transposon P[35UZ]. (A) *Ubx regulation by PREs (square boxes) and imaginal enhancers (IE, ovals) from the upstream and downstream regions are schematically outlined; sizes of boxes and ovals indicate the relative strengths of the corresponding regulatory elements.* **(B)** *Regulatory elements included in P[35UZ], represented as patterned boxes: the minimal Ubx promoter, the BXD and PBX elements, and the strong PRE. IE refers to imaginal enhancers (Pirrotta et al., 1995).*

contain an intact P[35UZ] transposon and one shows a small deletion in the 5' P-element sequences.

In embryos, all nine 35UZ lines revealed an epidermal pattern of β-galactosidase (β-gal) activity similar to wild-type (wt) *Ubx* expression (Fig. 2A) (Irvine *et al.,* 1991), with occasional faint ectopic *lacZ* expression anterior to PS6 in late embryos. Strong ectopic β-gal activity was detected only in T17 embryos. In P[35UZ]/+ embryos mutant for an amorphic allele of the *PcG* gene *polyhomeotic (ph), lacZ* expression was found in parasegments anterior to PS5 in all nine 35UZ lines (Fig. 2B), showing that P[35UZ] *lacZ* expression in embryos is basically controlled by *PcG* regulation.

β-gal profiles were next determined in third instar larvae. Six lines showed the '35UZ pattern', a metathoracic disc pattern similar to *Ubx* expression (Fig. 3A), sometimes associated with a few ectopic spots on notum primordia (Table 1). Two lines showed different and reproducible disc staining patterns. Irvine and Wieschaus (1994) previously reported that P[35UZ] at 78A in line 35UZ-1 has induced an allele of the *fringe (fng)* gene, which encodes a signaling molecule involved in cell interactions during development, and that 35UZ-1 larvae exhibit staining in the wing, eye and leg discs, in addition to the 35UZ pattern. In line T5

TABLE 1

PcG DOSAGE AND 35UZ LACZ EXPRESSION PATTERNS

Line	wt larvae[a]	PcG larvae[b]
35UZ-1	35UZ, *fng*-like, eye*	no effect
35UZ-2	35UZ	ectopic
35UZ-3	35UZ, eye*	ectopic
T1	35UZ, eye* (wing)	ectopic
T5	*dac*-like	no effect
T12	35UZ, (wing)	ectopic
T14	35UZ, eye* (wing)	ectopic
T16	35UZ, eye*	ectopic
T17	all discs, brain	n.d.

[a] 35UZ: *lacZ* expression in posterior metathoracic discs (see text). eye*: eye disc with lacZ expression in the region corresponding to mini-white expression in the adult eye. (wing): rare wing discs show weak staining.

[b] ectopic: *lacZ* expression in non-PS6 derived imaginal structures (wing and leg discs); n.d.: not determined.

(Fig. 3C-F), staining was found in the wing disc, eye-antennal disc, and in the femur, tibia and proximal tarsus primordia of the leg discs. Little to no β-gal activity was detected in the haltere discs, in total divergence from the 35UZ pattern. T5 flies exhibit dominant leg bristle modifications on tarsal segment 2, suggesting that the transposon had disrupted a leg-patterning gene. *In situ* hybridization showed the T5 location of P[35UZ] to be at 35F-36A. The *dachshund (dac)* gene at 36A1 is involved in *Drosophila* eye and leg specification. *dac* is strongly expressed in larvae in a pattern that resembles that of T5 (Mardon *et al.,* 1994), and genetic analysis with *dac* deficiencies and loss of function alleles confirmed that T5 is an allele of *dac*.

To check whether the transposon inserted in line T5 possessed all *cis*-acting sequences necessary to drive a *bona fide Ubx*-like pattern in larvae, we remobilized P[35UZ] at 36A. New lines were examined for *lacZ* patterns in imaginal discs (Table 2), and their molecular analysis included hybridization to a genomic 4.1 kb fragment located 5' of P[35UZ] at 36A. In four lines, a second
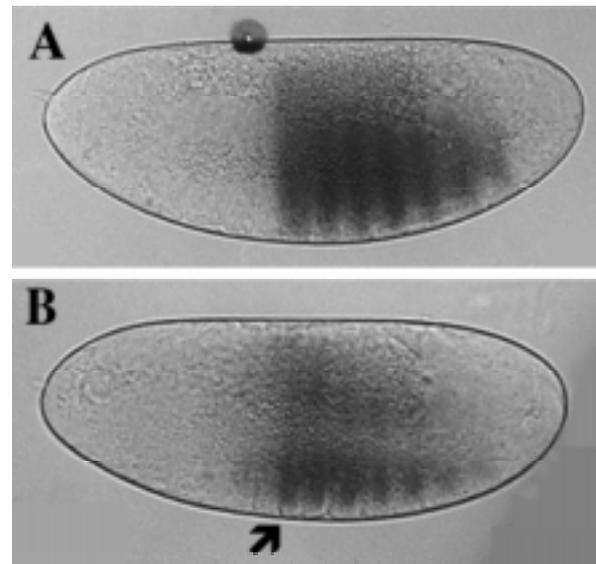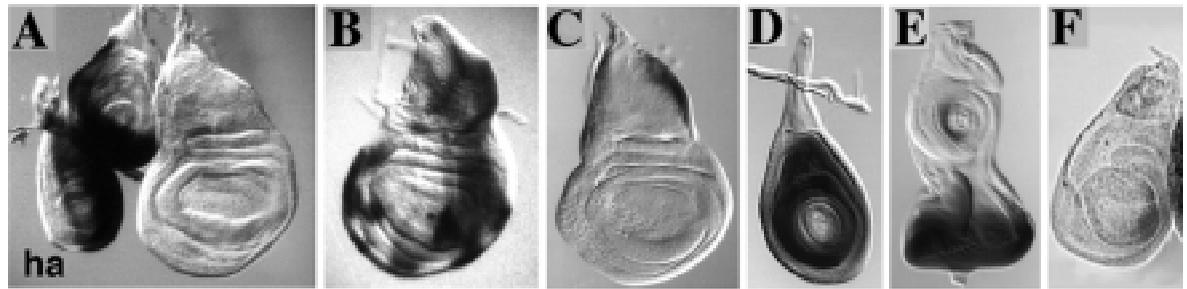

Fig. 2. P[35UZ] *lacZ* expression in embryos. *β-gal activity in T5/+* **(A)** *and ph505/Y;T5/+ embryos* **(B)** *at germ band retraction reveals lacZ derepression anterior to PS6 (arrow) in ph505;T5/+ individuals; anterior is to the left and dorsal up.*

transposon was inserted in addition to P[35UZ] at 36A. All four exhibited a complex pattern consisting of both the 35UZ and *dac*-like patterns. Two further lines were completely deleted of *lacZ* sequences at 36A, but possessed a P[35UZ] (36A) derivative inserted at a new site, and both showed the 35UZ pattern. We concluded that the P[35UZ] transposon at 36A of line T5 is identical to the original transposon at 34D in 35UZ-3.

Imaginal *lacZ* patterns were tested for dependence on *PcG* regulation in *mxc16a-1*/Y or *phbr*/Y larvae (Table 2). In all lines that exhibited the 35UZ pattern, such *PcG* mutant P[35UZ]/+ larvae showed strong ectopic *lacZ* expression in the wing discs (Fig. 3B), and often in other discs as well. *PcG* dosage had no effect on the *dac*-like expression pattern of line T5. Similarly, the *fng-lacZ* pattern of line 35UZ-1, examined in clones in *ph505*/+;35UZ-1/+ individuals, showed no modification in *ph* null clones (not shown). As the modified pattern of *lacZ* expression in imaginal discs, and the lack of response to *PcG* gene dosage of lines 35UZ-1 and T5

Fig. 3. P[35UZ] *lacZ* expression in imaginal discs. *lacZ expression follows a 35UZ pattern in third instar discs, which is de-regulated in ph^{br}/Y larvae.* **(A)** *Wing, haltere and L3 discs from a T1 larva.* **(B)** *Wing disc from a ph^{br}/Y;T1/+ larva. T5/CyO larvae exhibit staining in wing* **(C)**, *leg* **(D)** *and eye-antennal discs* **(E)**, *but little to no staining in haltere discs* **(F)**.

cannot be due to alterations of the transposon, they must be attributed to new *cis*-regulatory sequences. We propose that in lines T5 and 35UZ-1 the P[35UZ] transposon is located close to developmental enhancer elements (of *dac* and *fng*) that drive *lacZ* expression in novel imaginal disc domains, and thus escape *PcG* regulation.

mini-white in the 35UZ lines behaves like numerous other transposons carrying both a *mini-white* transposition marker and a PRE: they depend on regulatory elements located both in the P[35UZ] transposon and in the flanking genomic sequences (Fauvarque and Dura, 1993; Pirrotta and Rastelli, 1994; Gindhart and Kaufman, 1995). Indeed, in adult flies, eye pigmentation patterns of six among the nine 35UZ lines are graded and uneven, and sensitive to *PcG* gene dosage. Several *PcG* gene mutations strongly increased 35UZ eye-color, whereas loss of the *Ubx*-activating *zeste* product diminished 35UZ-3 *mini-white* expression. In four lines where *mini-white* is expressed in a posterior eye crescent, we observed a stronger β-gal activity in the posterior eye disc when *PcG* gene dosage was reduced. Interestingly, whereas the eyes of adult 35UZ-1 and 35UZ-3 flies are entirely colored in a *w ph^{410}* mutant background, *ph^{br}/Y;35UZ-1/+* and *ph^{br}/Y;35UZ-3/+* larvae never showed *lacZ* expression in the whole eye disc. Therefore, the regulation of the *mini-white* and *lacZ* genes can be uncoupled at certain insertion sites.

Our data illustrate the many possible levels of interaction between regulator elements contained in P[35UZ] and in its flanking sequences. In most cases, the 35 kb of *Ubx* upstream sequences suffice to serve as a buffer against any influence of flanking sequences, in embryos and in larvae. But the *fng*-like and *dac*-like *lacZ* expression patterns in lines 35UZ-1 and T5 indicate that *PcG* mediated silencing depends on all *cis*-regulatory elements located in the vicinity of the promoter, and can be reversed

TABLE 2

CHARACTERISTICS OF T5 VARIANTS

Line	lacZ[a]	Molecular[b]
T5	*dac*-like	P at 36A
1, 3, 4, 5, 6, 7	none	ΔP at 36A
2	*dac*-like	ΔP at 36A
101	none	ΔP at 36A
13, 26, 29, 30	*dac*-like + 35UZ	P at 36A; T
40	35UZ	Df at 36A; T
17	35UZ	ΔP at 36A; T

[a] imaginal disc *lacZ* pattern; for explanations see Table 1 and text.

[b] ΔP: partial deletion of P[35UZ] at 36A; Df: deletion of flanking genomic sequences at 36A; T: transposition of P[35UZ] on chromosome 2.

when P[35UZ] is inserted near strong imaginal enhancers. The *Contrabithorax^2* mutation of *Ubx*, that also lifts *PcG*-mediated gene regulation, corresponds to a similar change in flanking sequences in the endogenous locus with a breakpoint less than 10 kb upstream from the P[35UZ] fragment (Bender *et al.*, 1985).

The sample of nine 35UZ lines does not show how often such situations occur on the chromosomes because PRE-carrying P elements are not inserted randomly in the genome (Fauvarque and Dura, 1993), and because we may have selected insertion sites where P[35UZ] expression was particularly affected during the larval stages, and hence underestimated the number of sites where *PcG* regulation of P[35UZ] remained intact. Indeed, such flanking sequences may silence the *mini-white* gene entirely. Control of endogenous *Ubx* expression in embryos and larvae depends on distinct enhancers (Christen and Bienz, 1994; Pirrotta *et al.,* 1995). *Ubx* is activated in embryos by the segmentation genes and repressed, first by *hunchback* and *tailless* and later on by the *PcG* (review: Pirrota *et al.,* 1995). The imaginal enhancers are activated after germband retraction and drive expression in many imaginal tissues. The spatial restrictions imposed on them during the larval stages were entirely attributed to regulation by the *PcG*. Poux *et al.* (1996) suggested that a correct initial *Ubx* expression pattern in embryos is necessary for normal expression in imaginal tissues. Our data support this conclusion since the only line (T17) which exhibits a partially abnormal β-gal profile during embryogenesis did not show a wt pattern in larvae either. But this condition is not sufficient, as demonstrated by imaginal *lacZ* expression in lines 35UZ-1 and T5.

Our data further confirm that the chromatin structure imposed by *PcG* regulation is not irreversibly closed (Zink and Paro, 1995; McCall and Bender, 1996; Cavalli and Paro, 1998). Indeed, *PcG* mediated repression seems to allow a regular reassessment during *Drosophila* development of the activity state of all genetic elements in the vicinity of the inactivated promotor. *Drosophila* HOM genes include very large regulatory domains compared to many other loci, and such a reassessment mechanism could provide a possible explanation for the evolutionary origin of this particular gene structure. Indeed, one way to keep HOM gene promoters 'out of reach' from other enhancers activated during development could have been to surround each promoter by long stretches of DNA which helped to maintain it efficiently silenced by the *PcG*.

Experimental Procedures

Fly strains and culture

Flies were raised at 25°C. *multi sex combs* mutants were *mxc^{G43}* and *mxc^{16a-1}* (Santamaría and Randsholt, 1995; Docquier *et al.,* 1996). For

description of other mutants and balancer chromosomes see Lindsley and Zimm (1992).

35UZ-3 and T5 remobilization

To mobilize P[35UZ] at 34D from line 35UZ-3 w^{1118}/w^{1118}; 35UZ-3/ 35UZ-3 females were mated to $w^{1118}/Y;CyO/Sp;Dr$ P[Δ2-3]/*TM6* males providing the P(Δ2-3;99D) source of transposase (G0). Thirty-eight individual w^{1118}/Y;35UZ-3/*CyO*;*Dr* P[Δ2-3]/+, G1 progeny were crossed to a w^{1118} or CDX:*y w f/Y* tester strain. In G2, a single fly with modified eye-color was selected per cross, which gave rise to the T lines. For remobilization of P[35UZ] at 36A from line T5, w^{1118}/w^{1118};T5/*CyO* females were crossed to $w^{1118}/Y;CyO/Sp;Dr$P[Δ2-3]/*TM3* males. In G1, $w^{1118}/Y;CyO/$T5;*Dr*P[Δ2-3]/+ or $w^{1118}/Y;Sp/$T5;*Dr* P[Δ2-3]/+ males were individually crossed to $w^{1118}/w^{1118};Xa/CyO;TM3$ females. Putative new P[35UZ] lines were screened among $w^{1118}/Y;CyO/$T5*;*TM3*/+ or $w^{1118}/Y;Xa/$T5*, G2 males by a modification of the T5/+ eye-color or of the dominant T5 leg bristle phenotype. G2 males were then mated individually to $w^{1118}/w^{1118};Xa/$*Cyo;TM3* females.

X-gal staining

Embryos were treated as described (Docquier *et al.,* 1996). Imaginal discs from third instar larvae were fixed in 1xPBS, 3.7% formaldehyde, washed in 1xPBS, and stained like embryos. Tissues were mounted in glycerol. ph embryos represent 25% of progeny from $ph^{505}/FM7$ females crossed to P[35UZ] line males. Effects of *PcG* gene mutations on *lacZ* expression patterns were evaluated in imaginal discs of yellow larvae from y^1 $mxc^{16a-1}/FM7$ or y^1 $ph^{br}/FM7$ females crossed to P[35UZ] males. ph^{505} clones were induced by irradiating first instar ph^{505}/+;35UZ-1/+ larvae with 1000 rad, and examined in X-gal stained third larval instar discs.

Hybridizations

Hybridization on polytene chromosomes was according to Ashburner (1989), using a biotinylated *mini-white* probe. Southern hybridizations were carried out by standard procedures (Sambrook *et al.,* 1989). Blots were revealed by autoradiography or by Phosphorimager^{TM} (Molecular Dynamics).

P[35UZ] sequence and location analysis

Probes for characterization of P[35UZ] sequences and insertions were: 5' and 3' specific P-element sequences; a 1.9 kb *Xba*l-*Cla*l *mini-white* fragment and a *lacZ* probe**;** the 4.1 kb *Ubx* promoter fragment and plasmids BXD 3105bs and PBX 3101bs (Saari and Bienz, 1987; Müller and Bienz, 1991); plasmids 2212B6 and 2212H6.5 (covering a PRE and adjacent imaginal enhancers) (Pirrotta *et al.,* 1995); pEMBL19St*bxd*D6.1,30 and cPw*b*bxd6.2 that together cover cPw*b*bxd35 (Irvine *et al.,* 1991). A 4.1 kb fragment flanking P[35UZ] at 36A was isolated from a partial genomic T5/ *CyO* library in pBluescript, enriched for fragments containing both *mini-white* and adjacent 2nd chromosome genomic DNA. Positive clones were screened with a *mini-white* probe.

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