Dominant modifiers of the *polyhomeotic* extra-sex-combs phenotype induced by marked *P* element insertional mutagenesis in *Drosophila*

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Summary

Members of the *Polycomb* group (*Pc*-G) and *trithorax* group (*trx*-G) of genes, as well as the enhancers of *trx*-G and *Pc*-G (*ETP*), function together to maintain segment identity during *Drosophila* development. In order to obtain new marked *P* mutations in these genes, we screened for dominant modifiers of the extra-sex-combs phenotype displayed by males mutant for the *polyhomeotic* (*ph*) gene, a member of the *Pc*-G group. Five *P*{*lacW*} insertions in four different genes were found to stably suppress *ph*: two are allelic to *trithorax*, one is the first allele specific to the *Minute*(*2*)*21C* gene, and the remaining two define new *trx*-G genes, *toutatis* (*tou*) in 48A and *taranis* (*tara*) in 89B10-13. *tou* is predicted to encode a 3109 amino acid sequence protein (TOU), which contains a TAM DNA-binding domain, a WAKZ motif, two PHD zinc fingers and a C-terminal bromodomain, and as such is likely to be involved in regulation of chromatin structure as a subunit of a novel chromatin remodelling complex. In a previous study, we found that insertion of a *P*{*ph*} transposable element containing *ph* regulatory sequences creates a high frequency of mutations modifying *ph* homeotic phenotypes. One such insertion enhanced the *ph* phenotype and we show that it is a new allele of *UbcD1*/*eff*, a gene encoding a ubiquitin-conjugating enzyme that is involved in telomere association and potentially in chromatin remodelling.

1. Introduction

In *Drosophila melanogaster*, segment identity along the anterior–posterior axis is established during early embryonic stages by the differential activity of the homeotic genes of the BX-C and ANT-C complexes (Lewis, 1978; Kaufman *et al.*, 1980; Akam, 1987; Duncan, 1987; McGinnis & Krumlauf, 1992). Thereafter, cell fate must be maintained throughout development. Genetic analysis has revealed three distinct classes of homeotic trans-regulator genes involved in the maintenance of segmental identity: the *Polycomb* group genes (*Pc*-G), the *trithorax* group genes (*trx*-G) and the enhancers of *trithorax* and *Polycomb* (*ETP*) (Gildea *et al.*, 2000).

Mutations of the *Pc*-G cause a variety of homeotic phenotypes including transformation of meso-thoracic and meta-thoracic legs into pro-thoracic legs, partial transformation of the wing to haltere, and posteriorly directed transformations in abdominal segments (Lewis, 1978; Kennison, 1995). When in combination, *Pc*-G mutations act synergistically on homeotic gene regulation, inducing more dramatic homeotic transformations than the mere addition of each mutant effect (Jürgens, 1985). The products encoded by *Pc*-G genes maintain the repression of homeotic genes outside their normal domain of expression previously established by other developmental regulators (Struhl & Akam, 1985; Wedeen *et al.*, 1986; Dura & Ingham, 1988; Jones & Gelbart, 1990; McKeon & Brock,

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1991; Simon *et al.*, 1992). The *Pc*-G is thought to contain as many as 40 different genes (Jürgens, 1985), including *Polycomb* (*Pc*) (Lewis, 1978; Duncan & Lewis, 1982) and *polyhomeotic* (*ph*) (Dura *et al.*, 1985, 1987). Consistent with their proposed repressor function, the products of these two genes, PC and PH, respectively, have been shown to bind to about 120 common discrete sites on the polytene chromosomes, including BX-C and ANT-C (Zink & Paro, 1989), as well as to transgenes carrying DNA sequences from either *Antennapedia* (Zink *et al.*, 1991) or *bithoraxoid* (DeCamillis *et al.*, 1992). Many different findings suggest that *Pc*-G products may act in multimeric complexes which would induce local and inherited changes in chromatin conformation at *Polycomb* responsive elements (PREs), preventing transcriptional activity (Locke *et al.*, 1988; Paro & Hogness, 1991; Franke *et al.*, 1992; Fauvarque & Dura, 1993; Orlando & Paro, 1993; Martin & Adler, 1993; Rastelli *et al.*, 1993; Chan *et al.*, 1994; Lonie *et al.*, 1994; Fauvarque *et al.*, 1995; Zink & Paro, 1995; Shao *et al.*, 1999; van der Vlag *et al.*, 1999; Horard *et al.*, 2000; Tie *et al.*, 2001; for reviews see Cavalli & Paro, 1998; Brock & van Lohuizen, 2001).

The level of expression of homeotic genes must also be maintained within their normal domains. Loss-of-function mutations in *trx*-G genes cause homeotic transformation owing to insufficient expression of homeotic genes (Kennison & Tamkun, 1988; Huang & Dawid, 1990; Mazo *et al.*, 1990; Breen & Harte, 1993; Sedkov *et al.*, 1994; LaJeunesse & Shearn, 1995). A particular feature of *trx*-G genes is that their mutations can suppress those in the *Pc*-G genes (Ingham, 1983). Most members of the *trx*-G were screened according to this criterion (Kennison & Tamkun, 1988; Kennison, 1995). The *trithorax* product (TRX) binds to polytene chromosomes at homeotic complexes and several other sites, suggesting a direct role of *trx*-G products in the regulation of the corresponding genes (Kuzin *et al.*, 1994; Chinwalla *et al.*, 1995). Molecular and genetic data argue in favour of the hypothesis that *trx*-G products function in the opening of chromatin to allow efficient transcriptional activity at their target sites (Tamkun *et al.*, 1992; Farkas *et al.*, 1994; Kuzin *et al.*, 1994; Fauvarque *et al.*, 1995; Chinwalla *et al.*, 1995). The proteins of the newly described *ETP* class are needed for both activation and repression of chromatin transcription (Gildea *et al.*, 2000).

In order to obtain new marked *P* mutations in *trx*-G and *Pc*-G genes, we screened for dominant modifiers of the extra-sex-combs phenotype displayed by *ph⁴¹⁰* mutant males. Five *P{lacW}* insertions in four different genes were found to stably suppress *ph*: two of these are allelic to *trx*; one is the first allele specific to the *Minute(2)* in *21C(M(2)21C)* gene; and the remaining two define new *trx*-G genes, *toutatis* (*tou*) in 48A and

taranis (*tara*) in 89B10-13. *tou* is predicted to encode a 3109 amino acid sequence protein (TOU) which is likely to be involved in the regulation of chromatin structure. In a previous study, we found that insertion of a *P{ph}* transposable element containing *ph* regulatory sequences creates a high frequency of mutations modifying *ph* homeotic phenotypes (Fauvarque & Dura, 1993). Here, we show that for one of these insertions, the mutant phenotype is specifically due to the disruption of the *UbcD1/eff* gene in 88D, that we thus define as a *Pc*-G gene. The *UbcD1* gene encodes a ubiquitin-conjugating enzyme required for proper telomere behaviour (Cenci *et al.*, 1997), and which may be a chromatin remodelling factor involved in telomere position effect and position effect variegation (G. Cenci, F. Verni & M. Gatti, personal communication). Strong *UbcD1⁻* homozygous mutants die during embryogenesis and show derepression of the homeotic gene *Scr*; i.e. heterozygous males show a small but significant number of additional sex comb teeth on T2 legs. These results support the hypothesis that the *P{ph}* transposon can be used for preferentially directed mutagenesis into genes which interact with *ph* in homeotic gene regulation.

2. Materials and methods

(i) *Fly strains and culture*

All strains were maintained on standard culture medium at 25 °C. Except where otherwise stated, alleles are described in Lindsley & Zimm (1992) and in Fauvarque & Dura (1993). The X chromosome *y w mlac* contains four *PlacW* elements (Bier *et al.*, 1989). *P{ry⁺ Δ2–3}*(99B) is a stable source of transposase (Robertson *et al.*, 1988) inducing mobility of the *PlacW* element. Before mutagenesis, the strain *w¹¹¹⁸*, the strain *y w mlac*, the strain *w¹¹¹⁸; P{ry⁺ Δ2–3}*(99B) and the strain *ph⁴¹⁰w* were isogenized for autosomes *2* and *3*.

(ii) *Mutagenesis*

The strains *y w mlac*, *w¹¹¹⁸; P{ry⁺ Δ2–3}*(99B) and *w¹¹¹⁸* were controlled to have no effect on the meso- and meta- to pro-thoracic leg transformations observed in *ph⁴¹⁰* males.

(a) *Jump*

y w mlac females were mated to *w¹¹¹⁸; P{ry⁺ Δ2–3}*(99B) males and two different crosses (A and B) were then performed with the male and female progeny. In cross A, *y w mlac/w¹¹¹⁸; P{ry⁺ Δ2–3}*(99B)/+ females were mated to *ph⁴¹⁰w*/Y males. In cross B, *y w mlac*/Y; *P{ry⁺ Δ2–3}*(99B)/+ males were mated to *ph⁴¹⁰w* females.

(b) *Screen*

As the partial transformation of wing to haltere induced by the viable ph^{410} mutation is undetectable in $ph^{410}/+$ females but strongly enhanced in a Pc^- context (Dura *et al.*, 1985), female progeny from crosses A and B presenting this transformation were selected as potentially bearing mutations similar to *Pc*. Screening for *ph* suppressor mutations in F1 males from cross B (males from cross A do not carry the ph^{410} chromosome) was based on total suppression of the extra-sex-comb teeth ectopically present on the meta-thoracic legs of more than 90 % of ph^{410} males. The number of flies screened was estimated from the proportion of male descendants from cross B carrying at least one new autosomal insertion, i.e. having red eyes (20 %). This proportion gives us the ratio of flies carrying a mutagenized chromosome in our experiment. By analogy, we estimated that a similar proportion of females would carry a mutagenized chromosome. According to this ratio, 11400 effectively mutagenized chromosomes were screened for enhancement of ph^{410}, and 2500 mutagenized chromosomes for suppression of ph^{410}.

(c) *Establishment of the new mutant lines*

The selected males and females were individually backcrossed with the ph^{410} strain to control for the penetrance and stability of the suppressor or enhancer effect. Suitable insertions were outcrossed 10 times with the strain w^{1118} isogenic for chromosomes 2 and 3 and tested again for the suppressor/enhancer effect. For multiple insertions (2 of the 5 selected cases), each insertion was studied separately. Finally, in order to verify that the mutation was specifically due to the insertion of the *P* element, we produced new alleles by jumpout: *white* derivative excisions were produced for each insertion which modified ph^{410} phenotypes as described in Dura *et al.* (1993), i.e. such that the same genetic background was retained. *white* excisions were then tested with ph^{410} and classified either as revertants or as new mutant alleles.

(ii) *Interaction with* ph *and* Pc: *scoring of the homeotic transformation of meta-thoracic and meso-thoracic legs into pro-thoracic legs*

The partial transformation of second and third legs to first legs in $Pc^{16}/+$ or in ph^{410}/Y males results in the ectopic appearance of sex comb teeth on the second and third legs of males. These transformations were scored as follows: (1) In ph^{410} males, third legs were ranked into three classes depending upon the number of extra teeth (class 1, 0 to 2; class 2, 3 to 5; class 3, more than 5). (2) In $Pc^{16}/+$ males, second legs were ranked as for (1). The chi-square test was applied to

determine the statistical significance of probability differences.

(iv) *Quantification of the modification of the antenna-to-leg phenotype in* Dll^{MP} *flies*

Mutations isolated on the basis of modification of the *ph* extra-sex-combs phenotype were assayed to determine whether they also modified the dominant, but not fully expressed, transformation of the arista to leg in Dll^{MP} flies, chosen as being representative of the dominant phenotype induced by *trx*-G mutations. Males from balanced mutants were first crossed with w^{1118}; *TM3*, $Sb/Tp(2:3)ap^{Xa}$ females. Males carrying the mutation over $Tp(2:3)ap^{Xa}$ (*Xa* in Tables) were recovered and crossed with Dll^{MP}/CyO females. Phenotypes of mutant chromosome$/Dll^{MP}$ flies were compared with those of $Tp(2:3)ap^{Xa}/Dll^{MP}$ flies, since $Tp(2:3)ap^{Xa}$ does not modify the Dll^{MP} phenotype. Scores were assigned with respect to the percentage of the arista length transformed to leg as follows: 1, 0–10 %; 2, 10–75 %; 3, 75–100 %.

(v) *Antibody labelling*

Embryos 0–16 h old were stained with antibodies against the *Ultrabithorax* gene product (UBX) (White & Wilcox, 1984) and the *Sex combs reduced* gene product (SCR) (Glicksman & Brower, 1988) as described by Ingham & Martinez-Arias (1986). Detection of UBX or SCR was performed in the progeny of $mer^4/TM3$, *hblacZ* adults.

(vi) *Polytene chromosome* in situ *hybridization*

Cytogenetic localization of transposon insertion sites was performed as described in Fauvarque & Dura (1993).

(vii) *Isolation of genomic DNA flanking* UbcD1^{mer1} P *insertion*

Inverse PCR amplification of genomic DNA flanking the *P* element insertion in the $UbcD1^{mer1}$ allele was carried out as described by E. Jay Rehm in the BDGP web site (http://www.fruitfly.org/about/methods/inverse.pcr.html). 1·6 kb of 3′ *P*-element flanking DNA was recovered by inverse PCR amplification with Pry1 and Pry2 oligos from $UbcD1^{mer1}$ DNA digested with *Msp*I. Sequence analysis of the PCR product shows that the mer^1 *P* element is inserted at the beginning of the *UbcD1* gene (position 27329 of GenBank AE003706).

(viii) *Isolation of genomic DNA flanking the* tou^1 P *insertion and predicted exon–intron structure of* tou

A 6·4 kb *Eco*RI genomic DNA fragment (pT1) 3′ to the tou^1 insertion site was cloned by plasmid rescue

(Bier *et al.*, 1989). The nucleotide sequence adjacent to the *PlacW* element was determined from pT1 using a *P* LTR-specific primer (5′-CGACGGGACCACCTT-ATGTTATTTCATCATG-3′). A BLAST analysis of the recently completed genomic sequence indicates that the *PlacW* transposon inserted at position 154461 of GenBank AE003825. Comparison of the flanking genomic sequence with expressed sequence tags (ESTs) from the BDGP cDNA project (Rubin *et al.*, 2000) revealed that *tou¹* is inserted within a large (∼23 kb) intron of a ∼37 kb gene for which a part has been annotated as CG10897 in the complete genome analysis (Adams *et al.*, 2000). The following eight cDNAs correspond to *tou*: LP12012 (split by the 23 kb intron), HL04413, LP06732, LD19326, GH-22615, GH23205, LD06608 and LD09110. LP12012 is a chimeric cDNA including part of CG9691. The exon–intron structure and open reading frame of *tou* was deduced from sequence comparison between the genomic sequence and these various cDNAs, along with visual inspection of consensus split sites. A putative alternative exon of 153 bp was inferred from the 5′ end of GH22615. Two alternative polyadenyl-ation sites separated by 637 bp are inferred from the 3′ end of GH22615 and LP06732 and may account for the two *tou* mRNAs detected by Northern blot analysis (Drees *et al.*, 1987). Several *P* insertions were detected in the 23 kb intron, including *EP2530* and *EP2532* (Rorth *et al.*, 1998). The latter are inserted at the same position, 904 bp upstream of the *tou¹* insertion.

(ix) *Isolation of genomic DNA clones and identification of* rpA2 *RNA*

A genomic clone (pbel3) flanking a *P* element insertion region was recovered from $M(2)21C^{beo}$ strain by plasmid rescue. A genomic DNA *Drosophila* library (Maniatis *et al.*, 1978) was screened with pbel3. Overlapping clones (four phages) spanning 22 kb were recovered. The fragments surrounding the insertion point were identified on Southern blots of both genomic and phage DNA and subcloned into the Bluescript vector. Within these subclones, pbs102 is a 1·8 kb *Sac*II–*Eco*RI (linkers) fragment and pbs103 is a 1·45 kb *Sac*II–*Sac*II fragment. In order to further characterize the insertion point 332 bp were sequenced from pbs103, and 357 bp were sequenced from pbel3. Once assembled these sequences give 608 bp sur-rounding the insertion point of the *PlacW* element. The *rpA2* fragment was amplified from genomic DNA of the w^{1118} strain using the following degenerate primers: S1: AGTC(A/G)AA(C/A)AGACC(A/G)-AAGCCCATGTC and S2: GAGCT(C/T)GC(G/C)-TCGGTCTACGC(C/G)TCCCTCAT(C/T)CT. A 0·8 kb *rpA2* RNA was identified on developmental Northern blots made with polyA⁺ RNAs isolated from the indicated stages probed with *rpA2* or pbs102. Another 1·4 kb RNA from an unidentified gene was also detected on these Northern blots when probed with pbs103 and pbel3.

3. Results and discussion

(i) *Five* PlacW *insertions stably suppress the* polyhomeotic *extra-sex-combs phenotype*

We used *PlacW* element mutagenesis (Bier *et al.*, 1989) to search for suppressors and enhancers of *ph*. The screen was performed on males and females carrying one viable mutant allele of *ph* (ph^{410}). The selection criteria were: appearance of a wing-to-haltere transformation in $ph^{410}/+$ females, and sup-pression of the meta- to pro-thoracic leg transform-ation in ph^{410}/Y males (see Section 2). No insertion was found to stably induce a wing-to-haltere trans-formation among 11 400 females. In contrast, for 2500 mutagenized chromosomes, we isolated five independent insertions showing stable suppression of the transformation of meta- into pro-thoracic leg in ph^{410} males. Four of the five suppressor mutations are due to a homozygous lethal insertion. The remain-ing one is subviable with homozygous flies showing a slight wing defect: about 80 % of homozygous adults exhibit an interrupted vein 5, and the wing has a downward-turned appearance (not shown). For all five insertions, the *white* derivative excisions, which are revertant either for the lethality or for the downward-turned wing phenotype, no longer show suppression of the ph^{410} extra–sex-combs phenotype. Thus, in each strain, suppression of *ph* is attributed to the *PlacW* insertion.

Surprisingly, whereas five independent suppressor insertions were obtained from among the relatively few mutagenized chromosomes screened, no enhancer insertion was isolated from among 11 500 observed mutagenized chromosomes. One reason could be linked to the subviability of the ph^{410} mutation: a suppressor mutation may induce better or normal viability, and therefore the 2500 chromosomes ob-served may represent an underestimate of the real number of mutagenized chromosomes. In contrast, enhancer mutations might diminish the chances of survival of the $ph^{410}/+$ females.

(a) trx

Our screen was designed to isolate *trx*-G genes and we indeed found two lethal insertions which do not complement the lethality of either the deficiency *Df(3R)red-P*93 covering the *trx* locus, or the *trx^E2* allele, and are therefore defined as new alleles of *trx*, namely *trx^lac1* and *trx^lac2*. Both alleles are larval lethal and strongly suppress *ph* (Table 1). The three other mutations complement all the known *trx*-G genes

Table 1. *Effect on the* ph^{410} *extra-sex-combs phenotype*

Allele	Class 1	Class 2	Class 3	χ^2	P	Effect	*In situ* localization
Df(3R)sbd^{45}/ +	108	17	1	86	< 0·001	Suppressor	
Control^a	25	26	39				
M(2)21C/ +	44	16	10	36	< 0·001	Suppressor	
Control^a	16	10	44				
M(2)21C^{beo}/ +	83	26	3	75	< 0·001	Suppressor	21C
Control^b	18	45	37				
mer^1/ +	1	15	36	30	< 0·001	Enhancer	88D
Control^b	17	21	10				
mer^4/ +	0	10	41	59	< 0·001	Enhancer	
Control^c	9	45	10				
mer^4/ +	13	46	147	10,5	< 0·01	Enhancer	
Control^a	8	9	19				
tara^1/ +	37	23	4	23	< 0·001	Suppressor	89B
Control^b	6	24	12				
tou^1/ +	96	37	5	57	< 0·001	Suppressor	48A
Control^b	22	38	30				
tou^2/ +	55	29	4	59	< 0·001	Suppressor	
Control^d	5	31	29				
trx^{lac1}/ +	49	3	0	90	< 0·001	Suppressor	*trx* allele
Control^b	3	29	28				
trx^{lac2}/ +	76	23	1	124	< 0·001	Suppressor	*trx* allele
Control^b	1	35	52				

Controls are: *a*, + ; *b*, Xa; *c*, TM3; *d*, CyO.

Table 2. *Effect of* Minute *alleles on* ph^{410} *extra-sex-combs phenotype*

Minute	Class 1	Class 2	Class 3	χ^2	P	Effect
M(2)56F/ +	97	68	68	26	< 0·001	Suppressor
Control	20	24	59			
M(2)58F/ +	132	133	70	83	< 0·001	Suppressor
Control	18	14	71			
M(2)60E/ +	121	0	0	160	< 0·001	Suppressor
Control	2	13	34			

Control is *Xa.*

listed in Kennison (1993). This high proportion of *trx* alleles (2 of 5) might be explained by the fact that *trx* is a large locus of about 25 kb (Mozer & David, 1989; Breen & Harte, 1991) which is also reportedly a hotspot for *P* element insertion (J. Kennison, personal communication). Furthermore, *trx* mutants are particularly strong suppressors of the *ph* extra-sex-combs phenotype compared with other known mutations of the *trx*-G (this study and M.O.F & J.-M.D. unpublished results).

(b) M(2)21C

An insertion which we named *belenos* (*beo*) was localized to position 21C on the second chromosome by *in situ* hybridization to polytene chromosomes. *beo* is homozygous lethal, and lethal over the deficiency *Df(2L)al* (21B8-C1; 21C8-D1) also known, by virtue of its extreme *Minute* phenotype, as *M(2)21C*. The *P*

element insertion, which we now call *M(2)21C^{beo}*, is situated in the 5′ transcribed region of the acidic ribosomal protein gene *rpA2* previously localized to 21C (Wigboldus, 1987; Olson *et al.*, 1993) and disrupts its coding region (data not shown, see Section 2). When compared with heterozygous *Df(2L)al*/ + flies, *M(2)21C^{beo}*/ + flies show a weaker characteristic *Minute* bristle phenotype, and a shorter developmental delay, i.e. 5–10 h versus 24–25 h, suggesting that *M(2)21C^{beo}* is not a null mutation. We tested whether the suppression of the *ph* extra-sex-combs phenotype was specific to this particular *Minute* mutation or rather was simply a common side-effect of *Minute*-induced developmental delays, as has been reported, although in most cases the data were not shown (see Kennison & Russell, 1987, and references therein for a discussion of this problem). *M(2)21C^{beo}*, as well as the corresponding deficiency *Df(2L)al* (= *M(2)21C*), and three other *Minute* strains *M(2)56F*, *M(2)58F* and

Table 3. *Effect on* $Dll^{MP}/+$ *antenna-to-leg phenotype*

Allele	Class 1	Class 2	Class 3	χ^2	P	effect
$Df(3R)sbd^{45}/+$	18	46	46	55	< 0·001	Enhancer
Control	69	32	10			
$M(2C)21C^{beo}/+$	31	42	42	35	< 0·001	Enhancer
Control	62	27	8			
$M(2)21C/+$	84	47	25	9	≈ 0·01	Enhancer
Control	98	23	21			
$M(2)56F/+$	35	44	11	16	< 0·001	Enhancer
Control	57	19	6			
$M(2)58F/+$	32	48	26	20	< 0·001	Enhancer
Control	55	30	7			
$M(2)60E/+$	13	33	60	50	< 0·001	Enhancer
Control	41	21	9			
$mer^4/+$	105	4	0	28	< 0·001	Suppressor
Control	86	23	14			
$tou^2/+$	61	12	11	1·3	> 0·5	No effect
Control	46	15	9			

Control is *Xa*.

Table 4. *Effect on the* $Pc^{16}/+$ *extra-sex-combs phenotype*

Allele	Class 1	Class 2	Class 3	χ^2	P	Effect
$mer^4/+$	4	16	42	38·7	< 0·001	Enhancer
Control[a]	16	16	2			
$tara^1/+$	98	27	0	12·6	< 0·001	Suppressor
Control[b]	15	17	16			
$tou^1/+$	33	11	0	13·4	< 0·001	Suppressor
Control[c]	11	22	0			

Controls are: *a*, *Xa*; *b*, *TM3*; *c*, *CyO*.

$Df(2R)M60E (= M(2)60E)$ have a similar suppressing effect on the *ph* extra-sex-combs phenotype, as well as an enhancer effect on a *trx*-G mutation, $Dll^{MP}/+$, as assayed by the antenna-to-leg transformation (Tables 2, 3). Therefore, no specificity of action on homeotic transformations can be attributed to $M(2)21C^{beo}$. These results indicate that slowing of developmental rate has a general effect on homeosis, resulting in the suppression of the *ph* extra-sex-combs phenotype.

(c) toutatis

The insertion *toutatis*[1] (tou^1) maps at 48A on chromosome 2, where the genes $E(Pc)$ and *en* are located. tou^1 is not allelic with these genes as both $E(Pc)$ and en^{59} complement the wing defect present in homozygous tou^1 flies; however, the deficiency $Df(2R)en^{SFX31}$ does not, indicating that the gene *tou* is present in the region covered by this deficiency (48A1; 48B5-7). Among w^- excisions there were no lethal *tou* alleles, but one allele, tou^2, exhibits the same wing defect as tou^1 and has a strong suppressor effect on the transformation of meta- into pro-thoracic leg

(Table 1). $Df(2R)en^{SFX31}$ does not complement the wing phenotype of tou^2 but owing to the presence of *Enhancer of Polycomb* ($E(Pc)$) in this same deficiency, it was not possible to test $Df(2R)en^{SFX31}$ for modification of the *ph* extra-sex-combs phenotype. tou^1 also induces suppression of Pc^{16}, another *Pc*-G mutation (Table 4) we cloned by plasmid rescue 6·4 kb of genomic DNA flanking the tou^1 P element. Sequencing indicated that the insertion is located at 48A6 next to *en* (Fig. 1*A*). Analysis of the neighbouring genomic sequence identified two other P elements (*EP2530* and *EP2532*) located 904 bp upstream of the tou^1 insertion. Like tou^1 the *EP2530/EP2532* insertions are homozygous subviable and both exhibit the same wing phenotype in combination with each other, or with the $Df(2R)en^{SFX31}$ deficiency, indicating that they represent distinct alleles of the same gene. The tou^1 and $tou^{EP2530/EP2532}$ insertions are located within the first intron of a ~ 37 kb gene, annotated as CG10897 by the complete genome database, that expresses two abundant large mRNAs (transcript number VI of Drees *et al.*, 1987). *tou* is predicted to encode a 3109 amino acid sequence protein (TOU) which contains a TAM DNA-binding domain, a

A
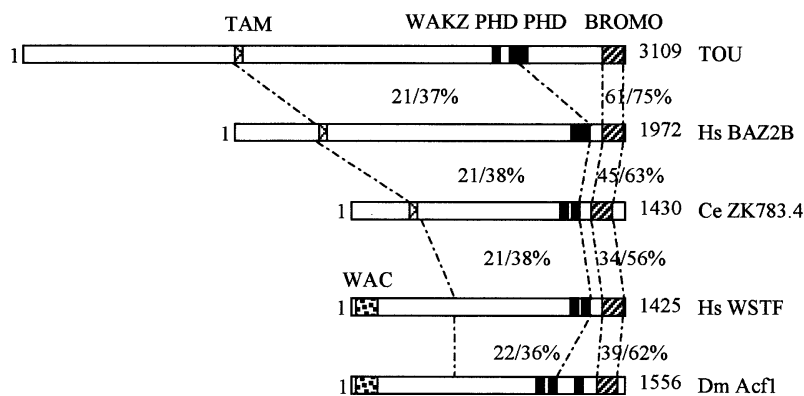
tou locus at 48A3-6
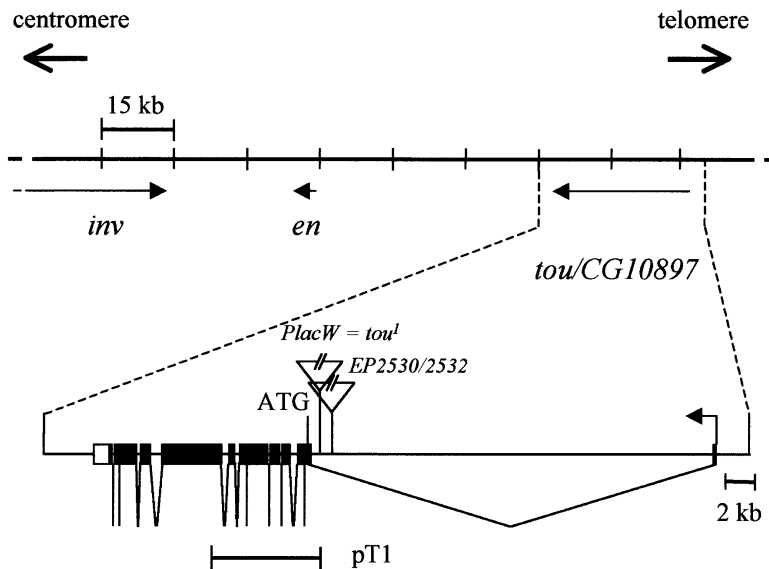
B

Fig. 1. Molecular analysis of the *tou* gene and comparison of the predicted TOU protein with related WAL family proteins. (*A*) A physical map of the *tou* region on chromosome 2R, cytological interval 48A3-6 (centromere, left; telomere, right) including the *invected* (*inv*), *engrailed* (*en*) and *tou* loci, is shown in the upper part. The 5′ to 3′ orientations of the three corresponding transcription units are shown below the DNA line. An enlarged view of the predicted exon–intron structure of *tou* is shown beneath and has been entered into GenBank under accession no. AF314193. Note that only a part of this structure has been annotated by the BDGP as CG10897. The neighbouring *P* element insertion alleles *tou¹* and *tou^{EP2530/2532}* are indicated above the DNA line by inverted triangles. The position of the 5′ end of the LP12012 cDNA (nucleotide 131650 of GenBank AE003825) is indicated by an arrow (the precise transcription initiation site has not been determined). Only the major transcription unit is shown with boxes representing exons; ATG is the presumptive initiator codon, filled boxes indicate protein coding regions, and broken lines indicate introns. For simplicity a putative alternative polyadenylation site is not represented. The position of the *Eco*RI genomic fragment recovered by plasmid rescue is shown below the DNA line. (*B*) The motif structure of the conceptually translated TOU protein along with related WAL family proteins from human (Hs), *Caenorhabditis elegans* (Ce) and *Drosophila* (Dm). Overall regions of homology (% identity/similarity), as determined by BLAST 2 sequence analysis (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html), are indicated by dashed lines. The GenBank accession numbers are: BAZ2B (BAA89212), ZK783·4 (T34516), WSTF (AAC97879) and Acf1 (AF148962). TAM, TTF-IIP5/ARBP/MeCP1 DNA binding domain; WAKZ, WSTF/Acf1/KIAA0314/ZK783·4 motif; PHD, PHD zinc finger; BROMO, bromodomain.

WAKZ motif, two PHD zinc fingers and a C-terminal bromodomain (Fig. 1*B*). Proteins with extensive homologies with the TOU protein are also detected in the worm and human genomes (GenBank accession numbers AAC24421 and BAA89212, respectively). TOU belongs to the recently defined WAL family of

chromosomal proteins (Poot *et al.*, 2000), including *Drosophila* ACF1, the large subunit of ACF/CHRAC (ATP-utilizing chromatin assembly and remodelling factor/chromatin accessibility complex; Ito *et al.*, 1999) and human WSTF, the Williams syndrome transcription factor (Lu *et al.*, 1998). ACF/CHRAC also contains the ISWI protein, a DNA-dependent ATPase related to the BRM *trx*-G protein. Recently, ISWI has been shown to be required for gene expression and the maintenance of higher-order chromatin structure *in vivo* (Deuring *et al.*, 2000). Together, these data indicate that TOU is likely to be involved in the regulation of chromatin structure as a subunit of a chromatin remodelling complex. Interestingly, the *tou* gene corresponds to the gene VI in Strutt & Paro (1997) shown by immunoprecipitation experiments with *in vivo* cross-linked chromatin to be a physical target for PH and PSC proteins. This suggests that PH and PSC may be involved in regulating the expression of *tou*.

(d) taranis

Cytogenetic analysis localized the larval lethal *P* element insertion *taranis¹* (*tara¹*) to position 89B on chromosome 3. Deficiency mapping by complementation analysis locates *tara¹* more precisely to 89B10-13 since $Df(3R)sbd^{105}$ (88F9-89A1; 89B9-10) is *tara⁺*, but $Df(3R)sbd^{45}$ (89B4; 89B13) and $Df(3R)sbd^{26}$ (89B9-10; 89C7-D1) are *tara⁻*. $Df(3R)sbd^{45}$, the smallest deficiency deleting *tara* function, proved to be a stronger suppressor of the ph^{410} allele than *tara¹* (Table 1). *tara¹* also induces suppression of the *Pc*-G mutation Pc^{16} (Table 4), and $Df(3R)sbd^{45}$ has a clear enhancer effect on the $Dll^{MP}/+$ antenna-to-leg transformation, Dll^{MP} being a *trx*-G mutation (Table 3). The *tara¹* insertion is located within a broadly expressed essential gene, for which the major part has been annotated as CG6889 in the complete genome database, whose genetic and molecular properties meet all the criteria for classification as a novel *trx*-G member (S. Calgaro, M. Boube, D.L. Cribbs & H.-M. B., submitted). Molecular characterization of *tara* predicts two novel ~ 96 kDa protein isoforms produced by alternative promoters and splicing. The TARA proteins share two evolutionarily conserved motifs with several mammalian proteins, including the recently characterized human nuclear protein cyclin-dependent kinase regulator p34[SEI-1](Sugimoto *et al.*, 1999) and RBT1, a novel transcriptional co-activator interacting with the second subunit of replication protein A (Cho *et al.*, 2000). This observation raises the interesting possibility of a link between *trx*-G function in the epigenetic maintenance of active chromosomal states and cell division control pathways.

(ii) *One* Pc-*like mutation induced by a* P *element carrying* ph *regulatory sequences is inserted into* UbcD1/eff

No *ph* enhancer mutations were isolated by the random mutagenesis approach. Thus, we took advantage of the *P{ph}* transposon – containing *ph* 5′ regulatory sequences driving the *lacZ* gene – which was shown to insert preferentially at loci corresponding to PH/PC binding sites creating a high proportion of insertions interacting with *ph* (Fauvarque & Dura, 1993). In particular, three *P{ph}* insertions were shown to enhance the homeotic transformation of meta-thoracic and meso-thoracic legs into pro-thoracic legs in a ph^{410} and a $Pc^{16}/+$ context, or of antennae to legs in the $ph^{401}/+$; $Pc^3/+$ context. It was not clear, however, whether the phenotype was specifically associated with the *P{ph}* insertion, or was due to the disruption of a gene in its immediate vicinity. Indeed, the presence of *ph* regulatory sequences in transposon *P{418}* induced a partial inactivation of the neighbouring genes *mini-white* and *lacZ* leading to a variegated expression of both genes. This phenomenon – the Developmental Regulator Effect Variegation or DREV – has been interpreted as a consequence of the spreading of a heterochromatin-like structure from *ph* regulatory sequences silencing adjacent reporter genes. Theoretically, this spreading could span many kilobases and therefore cause silencing of genes even far away from the insertion; it might also provoke a titration of the *Pc*-G products involved in the silencing, creating a *Pc*-G-like loss of function phenotype. To exclude these possibilities, we produced white excisions of the *P{ph}* mutant insertion *T11·1* (at 88D). As verified by Southern analysis (not shown), imprecise excisions no longer containing *ph* sequences still showed the mutant phenotype. Therefore, the enhancer effect on ph^{410} extra-sex-combs phenotype is due to the inactivation of the gene in which the transposon is inserted. The *T11·1* insertion is located in the 5′ region of the *UbcD1/eff* gene at position 464–465 (from figure 2 in Cenci *et al.*, 1997) of the ubiquitously expressed 1·7 kb transcript. The *UbcD1* gene encodes a class I ubiquitin-conjugating (E2) enzyme (Cenci *et al.*, 1997) involved in ubiquitin-proteasome-mediated protein degradation (Treier *et al.*, 1992). The *UbcD1* gene is also involved in telomere association (Cenci *et al.*, 1997), and *UbcD1* mutants act as suppressors of both telomeric position effect (TPE) and of position effect variegation (PEV) (G. Cenci, F. Verni & M. Gatti, personal communication).

The *T11·1* insertion, renamed *UbcD1^{mer1}*, is larval lethal. New transposase-induced mutant *UbcD1^{mer1}* alleles were examined and one embryonic lethal allele *UbcD1^{mer4}* was further studied. Both *UbcD1^{mer1}* and *UbcD1^{mer4}* are lethal with $Df(3R)ry^{506–85C}$ (87D1-2;
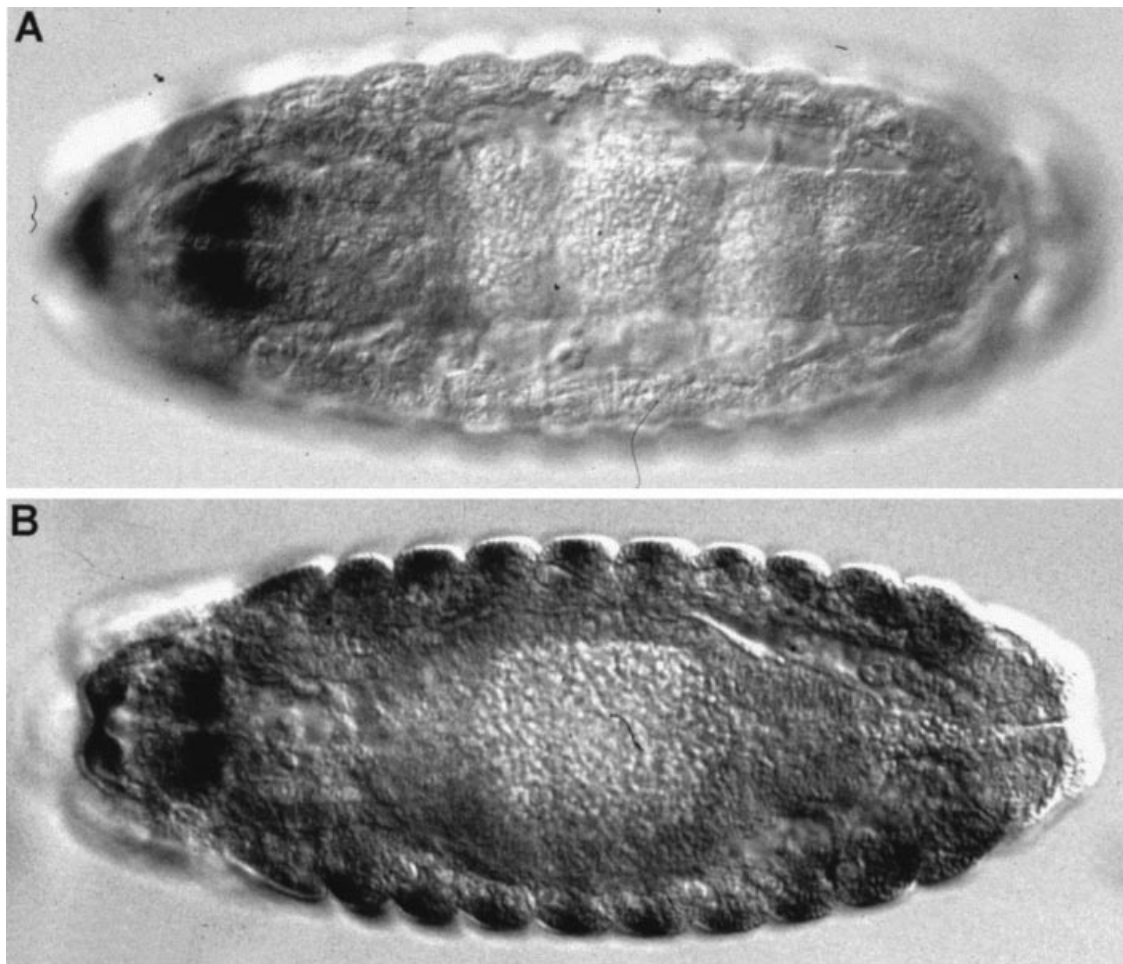
Fig. 2. Detection of SCR protein in germ band-retracted embryos. (*A*) SCR is expressed in the head of wild-type embryos. (*B*) A clear ectopic expression of SCR in posterior segments of $UbcD1^{mer4}$ mutant embryos is observed.

88E5-6) and neither complements the male sterility of $UbcD1^{eff-8}$ or the recessive lethality of $UbcD1^{eff-s1782}$. Like $UbcD1^{mer1}$, $UbcD1^{mer4}$ enhances the phenotype of the *Pc*-G mutation Pc^{16} (Table 4), and also has a clear suppressor effect on the antenna-to-leg transformation induced by $Dll^{MP}/+$, a *trx*-G mutation (Table 3). Unfortunately, we were not able to generate deficiency-bearing males without the rescuing duplication associated with their Y chromosome, and were thus unable to assay for modification of the *ph* extra-sex-combs phenotype; however, deficiency-bearing females showed a characteristic wing phenotype interpretable as a partial wing-to-haltere transformation. Moreover, $UbcD1^{mer4}/+$ heterozygous males showed a small but significant number of additional sex-comb teeth on T2 legs compared with the wild-type; i.e. 11/1406 versus 0/3178, respectively. We assayed for modifications in the expression of both SCR and UBX in $UbcD1^{mer4}$ mutant embryos. SCR is normally expressed in the labial lobes and the first thoracic segment (Mahaffey & Kaufman, 1987; Riley *et al.*, 1987). While SCR continued to be normally expressed in the head, we observed a clear derepression in the more posterior segments; i.e. staining with antibody against SCR was detected in posterior thoracic and abdominal segments (Fig. 2). No obvious changes of UBX pattern were observed in $UbcD1^{mer4}$ embryos.

Thus, we have demonstrated that modification of *ph* homeotic phenotypes by the *P{ph}* insertion *T11·1* defines *UbcD1* as a new *Pc*-G gene. A recent study shows that mono-ubiquitination of histone H1 mediated by the coactivator $TAF_{II}250$, a central component of TFIID, is involved in activation of eukaryotic transcription (Pham & Sauer, 2000). Interestingly, $TAF_{II}250$ may have an intrinsic ubiquitin-conjugating E2 activity. Therefore, it is likely that the *Pc*-G gene *UbcD1* acts as a general chromatin remodelling factor via histone ubiquitination.

4. Conclusion

The identification of new *Pc*-G and *trx*-G genes indicates that the genome is not saturated for these kind of mutations, and at least twice as many *Pc*-G genes probably remain to be discovered, although

their isolation by random mutagenesis has proved difficult (Landecker *et al.*, 1994). Moreover, the phenotypic modifications induced by the putative new *Pc*-G genes tend to be rather weak, at least in the heterozygous state, the condition in which they were screened (Kennison & Tamkun, 1988; Harding *et al.*, 1995; this study). One reason for the difficulty in obtaining *Pc*-G mutations may be the potential property of some previously described *Pc*-G proteins, now referred to as *ETP* in Gildea *et al.* (2000), to both activate and repress transcription (for review: see Brock & van Lohuizen, 2001). In contrast to the random mutagenesis approach, enhancer mutations identifying new *Pc*-G genes might be better isolated with the *P{ph}* transposon described in Fauvarque & Dura (1993). As we have shown in an alternative approach, this *P{ph}* transgene increased the probability of obtaining insertions in genes interacting with *ph* more than 100-fold. It may also allow the recovery of new kinds of mutations not obtained in other screens, as evidenced by the unambigous identification of the *T11·1* insertion as a new *Pc*-G mutation affecting the *UbcD1* gene.

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