Kiger, John A., Jr., and Christopher I. Ho. 2001. *P* repressor as a tool to analyze *GAL4/UAS* enhancer trap phenotypes in *Drosophila melanogaster*. *Dros. Inf. Serv.* 84: 188-199.

P repressor as a tool to analyze *GAL4/UAS* enhancer trap phenotypes in *Drosophila melanogaster*.

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Abstract

P Repressor suppresses expression of *UAS* transgenes. Using mutant *P* elements that make Repressor but cannot make Transposase, we demonstrate the utility of Repressor for analysis of *GAL4/UAS* phenotypes in three paradigms. 1. Mitotic crossing over was used to remove a mutant *P* element from clones to generate genetic and phenotypic mosaics. We investigated the cellular specificity of wing phenotypes produced by ectopic expression of Protein kinase A catalytic subunit (PKAc) in wing hemocytes and in wing epithelial cells. 2. Repressor permits the survival of adults carrying lethal combinations of *GAL4* and *UAS P* elements. Balanced lethal stocks of mutant *P* element and lethal *GAL4/UAS* combinations have been created that can be used in genetic screens to identify genes that physiologically interact with the *GAL4/UAS* combination to suppress lethality. 3. In the study of *GAL4/UAS* phenotypes that are pleiotropic or are modified by other aspects of the genotype, a mutant *P* element can be used to demonstrate that the observed effects are indeed caused by GAL4-driven expression of the *UAS* transgene rather than by some genetic interaction created by the genetic background.

Introduction

The *P* element-based *GAL4/UAS* system developed by Brand and Perrimon (1993) has become a standard tool to target gene expression to specific cells in *Drosophila melanogaster* in order to observe *in vivo* effects on the phenotype. In the course of studies using this system, we found that wildtype *P* elements suppress *GAL4/UAS* phenotypes. It has been shown for P strains derived from natural populations that *P* Repressor partially blocks transcription of *P-LACZ* fusion genes by binding to the *P* promoter (Lemaitre and Coen, 1991). Repression of the *P* promoter by Repressor is not complete, however, since a significant level of transcription is required to maintain Repressor synthesis (Roche *et al.*, 1995). Like *LAC-Z* transcription, *GAL4* transcription is initiated from the *P* promoter in *GAL4* transgenes (Brand and Perrimon, 1993), suggesting a mechanism for at least a part of the suppression we observe. *P* repressor has also been shown to suppress transcription from heterologous promoters carried in *P* elements by a chromatin-based mechanism involving *Polycomb*-group genes (Roche *et al.*, 1995; Roche and Rio, 1998). Therefore, it is possible that suppression of *GAL4/UAS* transgene phenotypes is due to synergism in the repression of transcription from both *GAL4* and *UAS P* elements.

Here we investigate the potential utility of *P* Repressor as a means of genetically and phenotypically manipulating *GAL4/UAS* combinations. For such studies it would be highly desirable (essential in some cases) that these *P* elements do not change their chromosomal locations due to the action of *P* Transposase. This consideration led us to investigate the properties of mutant *P* elements that make Repressor but cannot make Transposase (Karess and Rubin, 1984; Misra and Rio, 1990; Rio,

1991). We have tested two such mutant *P* elements in three paradigms and found them to perform as useful tools for the analysis of *GAL4/UAS* combinations.

First, we show that *P* Repressor turns over sufficiently rapidly to be used to create genetic mosaics enabling clonal analyses of *GAL4/UAS* phenotypes. Mitotic crossing over has been used to investigate wing phenotypes produced by ectopic expression of Protein Kinase A catalytic subunit (PKAc). We show that wing phenotypes can be caused by ectopic PKAc expression in either wing epithelial cells or in wing hemocytes and relate these phenotypes to previous studies. These results provide novel evidence that wing hemocytes represent a unique population of hemocytes, one that is not recruited from the general circulation. The implications of this hemocyte population for wing morphogenesis are discussed

Second, *P* Repressor permits adults carrying lethal combinations of *GAL4* and *UAS P* elements to survive. Balanced lethal stocks of mutant *P* and *GAL4/UAS* combinations are easily produced. These stocks can be used in genetic screens to identify genes that physiologically interact with the *GAL4/UAS* combination to suppress the lethal effect.

Third, in the study of *GAL4/UAS* phenotypes that are pleiotropic or may be modified by other aspects of the genotype, a mutant *P* element can be used to determine if the observed phenotypic effects are caused by GAL4-driven expression of the *UAS* transgene rather than by some other genetic interaction created in crossing strains with different genetic backgrounds.

Results

Suppression of GAL4/UAS phenotypes by Wildtype P elements.

Expression of *UAS-PKAc* transgenes using the *GAL4-30A* enhancer-detector strain produces severely blistered or collapsed wings and melanotic growths that are frequently found in the head. These effects are caused by PKAc expression in hemocytes around the time of eclosion (Kiger *et al.*, 2001). To study these effects we have used a chromosome carrying both the *GAL4-30A* transgene and the *UAS-PKAc 15.3* transgene. When flies carrying this chromosome are crossed to flies of interest, the *GAL4-30A, UAS-PKAc 15.3* combination is transmitted from the first parent as a unit, allowing effects of genes or transgenes transmitted from the second parent to be assessed from the phenotype of progeny that receive both.

When *GAL4-30A, UAS-PKAc 15.3 / CyO* flies are crossed to flies of two different P strains carrying multiple *P* elements (Rio, 1991) the non-*CyO* progeny have a completely normal phenotype, i.e. the *GAL4-30A, UAS-PKAc 15.3* phenotype is completely suppressed (data not shown). To study the effects of a single *P* element, we employed a chromosome carrying the $cact^{BQ}$ mutant, which was induced by *P* mobilization using the P strain Harwich (Daniel St Johnston, personal communication). This mutation is caused by insertion of a full length *P* element into *cact* exon 2(Bergmann *et al.*, 1996). The $cact^{BQ}$ chromosome produces complete suppression of the *GAL4-30A, UAS-PKAc 15.3* phenotype (Figure 1A). Attempts to prove identity of the $cact^{BQ}$ mutation and the suppressor by mobilization of the *P* element and selection for reversion of $cact^{BQ}$ were not successful, necessitating mapping of the suppressor locus. The suppressor maps to the location of $cact^{BQ}$ as discussed in MATERIALS AND METHODS. This *P* element is evidently transcriptionally active because we have found that the $cact^{BQ}$ chromosome can mobilize a *UAS* transgene (data not shown).

To test the phenotypic specificity of suppression, we examined the effects of the $cact^{BQ}$ chromosome on other combinations of *GAL4* enhancer-detector strains and *UAS-transgenes*. In addition to expression in hemocytes, *GAL4-30A* is strongly expressed in the salivary glands of 3rd instar larvae as shown using a *UAS-GFP* transgene. The $cact^{BQ}$ chromosome strongly reduces the level of GFP

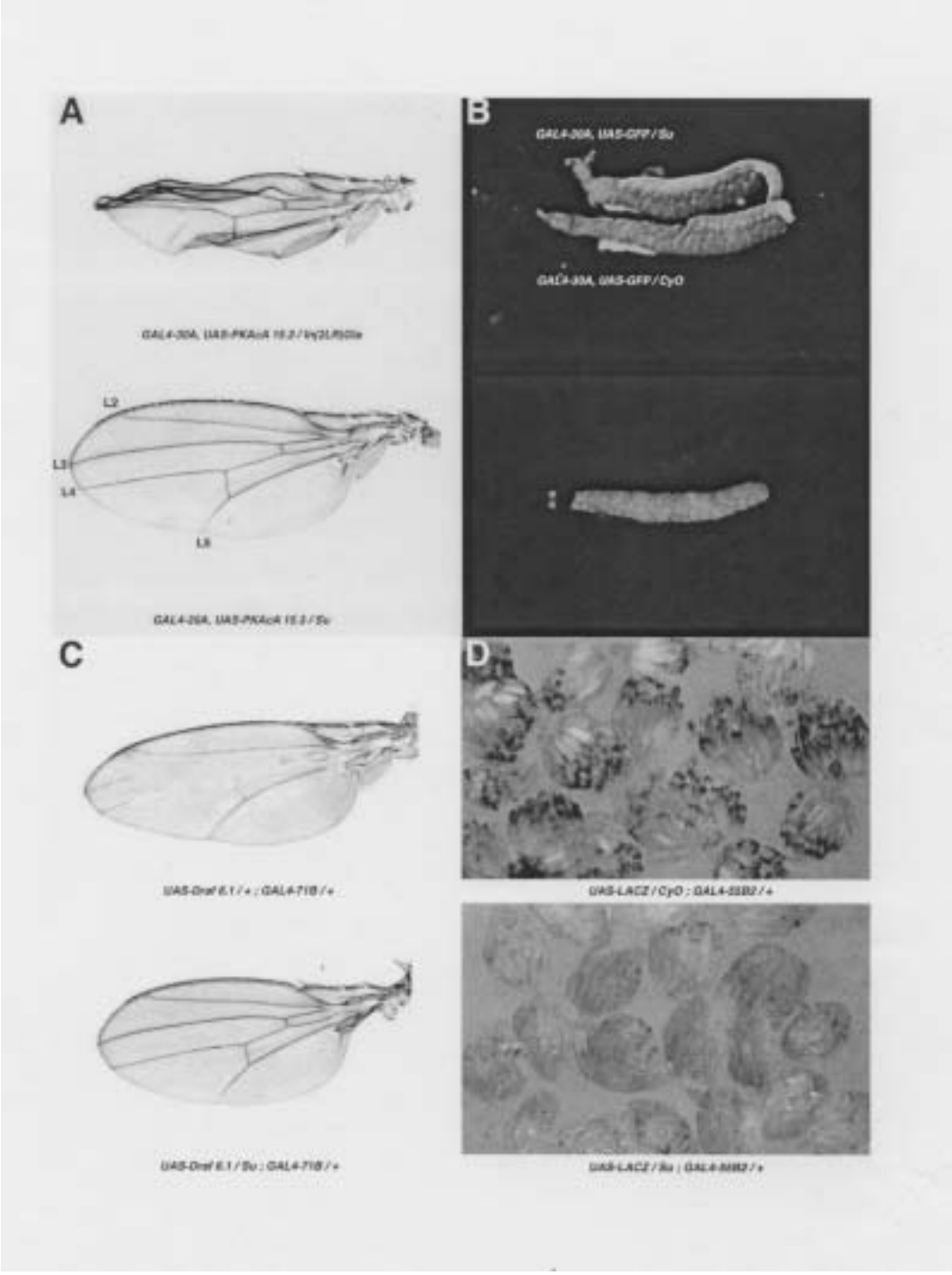

Figure 1. The effects of the $cact^{BQ}$ chromosome, designated *Su*, on the phenotypes of different *GAL4/UAS* combinations. (A) The collapsed wing phenotype produced by *GAL4-30A* and *UAS-PKAc 15.3*. Indicated in the bottom panel are the longitudinal vein designations of the normal wing. (B) Fluorescence in salivary glands produced by *GAL4-30A*, and *UAS-GFP*. The top panel shows salivary glands illuminated from the side using tungsten light. The bottom panel shows the same salivary glands using epifluorescence. (C) The wing phenotype produced by *UAS-Draf 6.1* and *GAL4-71B*. (D) X-gal staining of LACZ activity in ovarian follicle cells produced by *UAS-LACZ* and *GAL4-55B2*.

fluorescence in the salivary glands of *GAL4-30A, UAS-GFP* larvae (Figure 1B). The $cact^{BQ}$chromosome suppresses the wing phenotype caused by ectopic expression of a wildtype *Draf* cDNA driven by *GAL4-71B* (Figure 1C) and significantly reduces *UAS-LACZ* expression driven by *GAL4-55B2* in follicle cells of the ovary (Figure 1D). Thus the $cact^{BQ}$chromosome acts as a general suppressor of *GAL4/UAS* phenotypes. Although we have not independently mapped suppressor activity for each of these phenotypes, it is reasonable to believe (given the results presented below) that in all cases suppression is due to the *P* element in *cact.*

Mosaic analyses of wing phenotypes caused by ectopic PKAc expression using the SalI mutant Repressor.

The wildtype *P* element carries a single transcription unit whose primary transcript can be spliced to produce 87kD Transposase or a C-terminally truncated, 66kD Repressor (Rio, 1991). The mutant *P[ry$^+$; Sal I]* element contains a frameshift mutation near the middle of exon 4 of the transcription unit, causing polypeptide termination 10 amino acids downstream of that site, inactivating Transposase but not Repressor (Karess, and Rubin, 1984; Robertson, and Engels, 1989). *P[ry$^+$; Sal I] 89D* suppresses the *GAL4-30A, UAS-PKAc 15.3* phenotype as effectively as the $cact^{BQ}P$ element (data not shown). We tested the ability of the *SalI* Repressor to generate mosaics following mitotic crossing over as follows. Females of genotype *y, w, P[ry$^+$; hs-FLP] ; FRT82B / TM3, Sb* were crossed to males of genotype *y, w /* Y*; GAL4-30A, UAS-PKAc 15.3 / +; FRT82B, SalI 89D, y$^+$/ +*. Larvae were, or were not, heat shocked as described in Materials and Methods. The incidence of y^+ progeny adults, homozygous or heterozygous for *FRT82B*, exhibiting one or more wing blisters is recorded in Table 1. The data show that the presence of a wing blister requires both a heat shock during larval development and homozygosity for *FRT82B*, demonstrating that the blisters must be the result of mitotic crossing over. The blisters in general are quite large and tend to be round, whereas mitotic clones induced in wing epithelial cells are usually smaller and elongated along the proximal-distal axis (Garcia-Bellido and Merriam, 1971). One of the blistered wings is shown in Figure 2A. The blister encompasses a large portion of the anterior wing margin where the large bristles can be seen to be y^+. The presence of a nearby *y* bristle is evidence of mitotic recombination having occurred in epithelial cells that produced this wing. The *y* phenotype cannot be scored reliably in the wing blade cuticle or the wing hairs, however.

Table 1. *hsFLP*-Induced mitotic recombination in *P[ry$^+$; SalI] /* + flies.

Gal4/UAS Combination	Incidence of Flies with Abnormal Wings		
	Heat Shock *FRT/FRT*	No Heat Shock *FRT/FRT*	Heat Shock *FRT/ +*
GAL4-30A, UAS-PKAc 15.3	47/147	0/132	0/133
UAS-PKAcF 1.1; Gal4-71B	48/108	0/93	

Having established (1) that the *SalI* Repressor turns over rapidly enough to permit mosaic analysis and (2) that the *GAL4-30A, UAS-PKAc* phenotype is amenable to such analysis, we constructed strains that permit mosaic analysis of the wing blade using the f^{36a} marker. The f^{36a} mutant curls or bends the wing hairs. Because each cell in the wing differentiates a hair (unless it differentiates a larger structure), the extent of a mitotic clone in the wing blade can be determined at the cellular level. Figure

2B shows the wing of a fly of genotype *w, P[ry$^+$; hs-FLP] 9F, f^{36a} / y, w, f^{36a}; GAL4-30A, UAS-PKAc 15.3 / + ; 82B, SalI 89D, y$^+$, f$^+$/ 82B* heat shocked during development. A round blister encompasses a portion of vein L2. Anterior to L2 a large clone of f^{36a} hairs runs through the blister while on the other side of L2 the hairs are f^+. Such observations indicate that the genotype of the wing epithelial cells does not control blister formation, consistent with previous conclusions that blisters are caused by PKAc expression in hemocytes within the wing (Kiger *et al.*, 2001).

We then turned to investigate the effect of PKAc expression in wing epithelial cells using *GAL4-71B* known to be expressed in the wing pouch of the wing imaginal disc (Brand and Perrimon, 1993). *GAL4-71B*-driven expression of strong *UAS-PKAc* transgenes is lethal while expression of weak *UAS-PKAc* transgenes allows some adult survivors to eclose. These survivors generally have wings too shrunken and deformed to permit analysis. We have used *UAS-PKAcF 1.1*, a transgene that is lethal with *GAL4-71B*, for mosaic analysis using *SalI 89D* to suppress lethality and permit development of normal adults. When larvae of genotype *y, w, P[ry$^+$; hs-FLP] / y, w ; UAS-PKAcF 1.1 / +; GAL4-71B, 82B, SalI 89D, y$^+$/ 82B* are heat shocked during development (Table 1) almost half the adult flies had ectopic wing veins, located between veins L3 and L4, in otherwise normal wings.

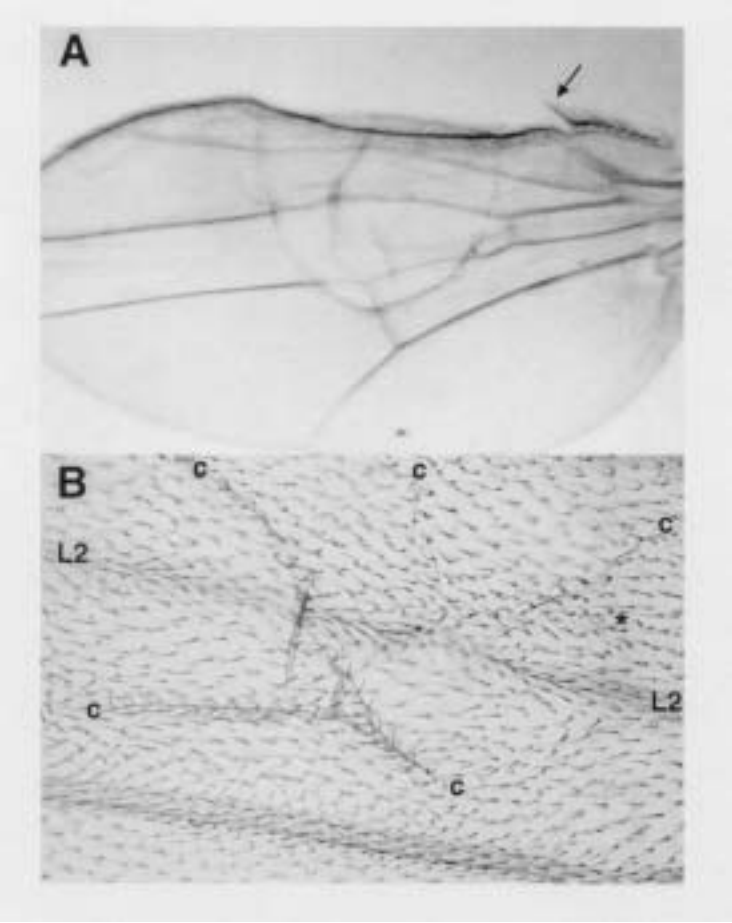

Figure 2. Mosaic blistered wing phenotypes produced by heat shock induced mitotic recombination. (A) Wing of a fly of genotype *y, w, P[ry$^+$; hs-FLP]/ y, w ; GAL4-30A, UAS-PKAc 15.3 / + ; 82B, SalI, y$^+$/ 82B.* *y* is easily scored only in the large bristles along the anterior margin of the wing. The very large bristle (arrow) is *y*. Those bristles encompassed by the large blister are y^+. (B) Wing of a fly of genotype *w, P[ry$^+$; hs-FLP] 9F, f^{36a} / y, w, f^{36a}; GAL4-30A, UAS-PKAc 15.3 / + ; 82B, SalI, y$^+$, f$^+$/ 82B.* A blister (area bounded by creases marked "c") encompasses a portion of L2. A large clone of f^{36a} hairs (identified to the right by "*") runs through the blister anterior (above) to L2 without crossing the vein. Most of the hairs posterior (below) to L2 are f^+.

We then constructed stocks that allow clones in the wing blade to be marked with f^{36a}. A wing showing such clones is shown in Figure 3A. Interestingly, only clones falling between L3 and L4 have phenotypic effects. Clones located elsewhere do not disturb the normal wing pattern. Figure 3B shows the distal portion of the wing in Figure 3A at higher magnification. In the region of the normal L4 vein which is missing there is a large clone marked with f^{36a} bounded on each side by ectopic veins that appear to be a mirror-image duplication. Most of these veins have f^{36a} hairs on them except near the distal ends. These veins extend to the wing margin where socketed f^+ bristles can be seen that are

characteristic of L2 or L3 but not of L4. Figure 3C shows an ectopic vein in another wing joining L3 and L4. The ectopic vein is clearly of the L3 type because it has sensilla that are characteristic only of L3. Figure 3D shows a clone of f^{36a} hairs along the anterior edge of L4. Within the clone is an ectopic sensillum characteristic of L3 and curiously nearby there is a small gap in L4.

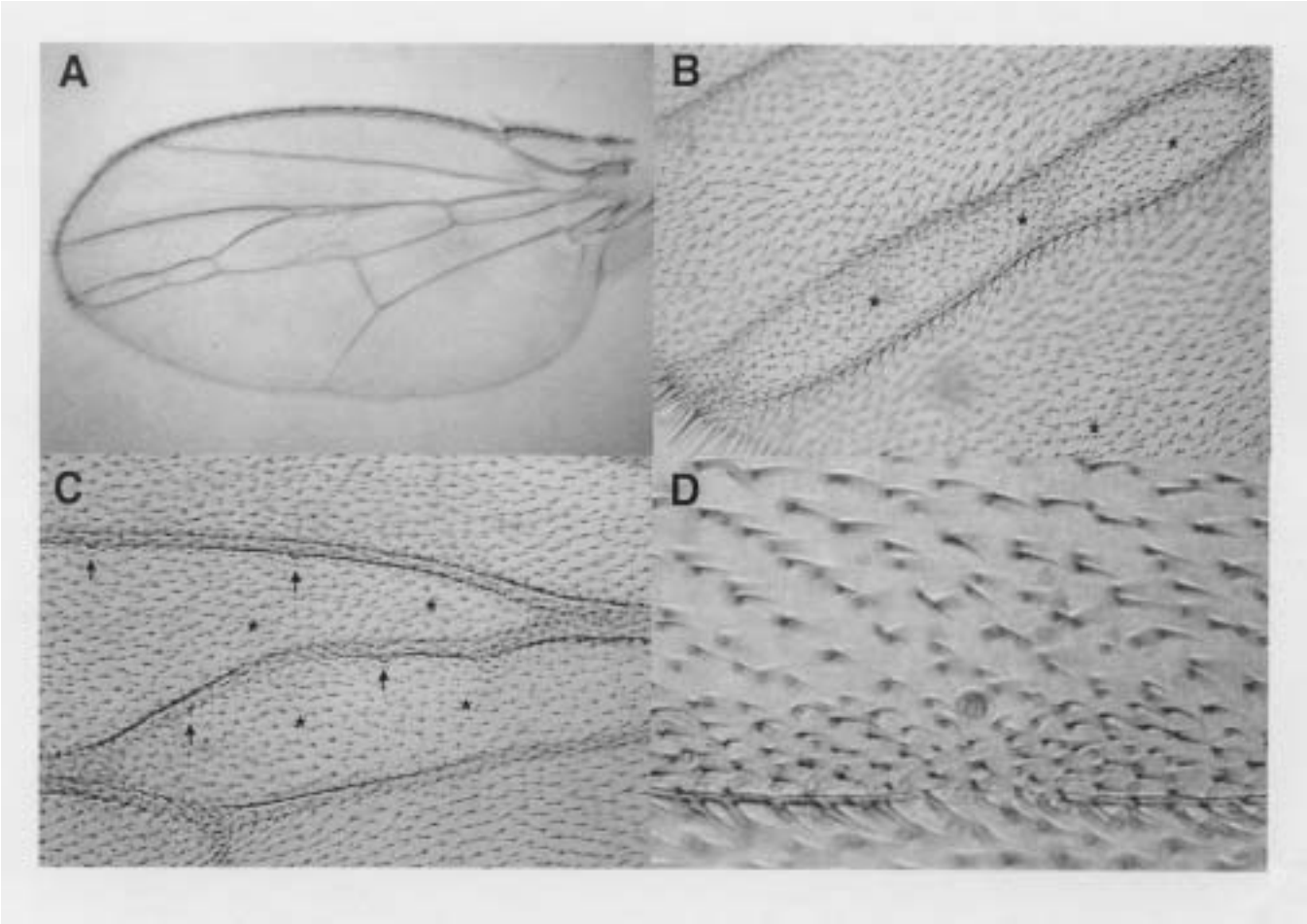

Figure 3. Mosaic wing veination phenotypes produce by heat shock induced mitotic recombination in flies of genotype *w, P[ry$^+$; hs-FLP]9F, f^{36a} / y, w, f^{36a}; UAS-PKAcF 1.1 / +; GAL4-71B, 82B, SalI, y$^+$, f$^+$ / 82B*. (A) Ectopic vein formation in the intervein region between L3 and L4. (B) Distal portion of the wing in A at higher magnification centered on the L4 region. Note the large clone of f^{36a} hairs (marked by "*") filling the region between the doubled veins and the f^+ socketed large bristles where the doubled veins meet the margin of the wing. Socketed bristles are characteristic of the ends of L2 and L3 but not of L4. Note also a region of f^{36a} hairs in the lower right corner (marked by "*") that has no effect on the pattern of the wing. (C) In another wing, an ectopic vein joins L3 and L4. Note the f^{36a} hairs on both sides of the ectopic vein (marked by "*") and the sensilla (arrows) on the normal L3 and on the ectopic vein. Normally only L3 has such sensilla. (D) Another wing at higher magnification showing a clone of f^{36a} hairs along the anterior edge of L4. Note that an ectopic sensillum characteristic of L3 has formed within the clone and that posterior to the sensillum there is a gap in L4.

Genetic manipulation of lethal GAL4/UAS combinations using the 66k mutant Repressor.

We have shown in the above section how *P[ry$^+$; Sal I] 89D* can be used to suppress the lethality of the *GAL4-71B* and *UAS-PKAcF 1.1* combination in creating a genotype that permits mosaic analysis of this *GAL4/UAS* phenotype. We have tested the utility of the mutant *P[ry$^+$; 66k]* inserted in the chromosome II balancer *CyO* for suppressing lethality caused by the PKAc inhibitor PKIF in the combination *GAL4-PP3, UAS-PKIF 5-1.* These *GAL4* and *UAS* transgenes are linked to chromosome III (Kiger *et al*., 1999). *P[ry$^+$; 66k]* is a deletion construct that removes the downstream half of intron 3, including the 3' splice site, and most of exon 4 of the *P* transcription unit so that only the normal 66kD Repressor protein is produced (Misra and Rio, 1990). Males of genotype *w* / Y *; CyO, P[ry$^+$, 66k] / + ; GAL4-PP3* / + were crossed to females of genotype *y, w ; UAS-PKIF 5-1.* The expected progeny classes and (numbers recovered) were: *Cy* / + *; UAS* / + (118); + / + *; UAS* / + (114); *Cy* / + *; UAS / GAL4* (97); + / + *; UAS / GAL4* (0). Thus, suppression of lethality is quite effective.

Females of genotype *y, w / w ; CyO, P[ry$^+$, 66k] / + ; UAS-PKIF 5-1 / GAL4-PP3* were then crossed to males of genotype *w* / Y and progeny males of genotype *w* / Y*; CyO, P[ry$^+$, 66k] / + ; GAL4-PP3 , UAS-PKIF 5-1/* + selected on the basis of eye color. Single males were then used to produce balanced stocks of the genotype *w ; CyO, P[ry$^+$, 66k] / + ; GAL4-PP3 , UAS-PKIF 5-1/ TM3.* We also have maintained this *GAL4/UAS* combination in a stock in which the females are *y, w ; SalI 89D, y$^+$* and the males are *y, w ; GAL4-PP3, UAS-PKIF 5-1/ SalI 89D, y$^+$*. The fact that crossing over does not occur in males preserves the combination. Such a stock of course needs selection every generation so that only *y, w ; SalI 89D, y$^+$* females breed the next generation. Stocks such as these can be used to screen flies for dominant phenotype-specific suppressors of the *GAL4/UAS* combination by a single cross.

Analysis of GAL4/UAS phenotypes as a function of the genetic background.

GAL4/UAS combinations often exhibit quite pleiotropic effects. For example, in addition to wing blisters and melanotic growths in the head, described above, flies carrying the *GAL4-30A, UAS-PKAc 15.3* chromosome exhibit a developmental delay of about 2 days compared to sibs that do not carry this combination. One or another of these pleiotropic phenotypes might be caused by mutations created by the insertion of one or both of these *P* elements into the chromosome or by some other aspect of the genetic background. Introducing *P[ry$^+$; SalI]* into the genetic background causes suppression of all three phenotypes, and its subsequent removal from the genetic background restores all three phenotypes. Thus, the pleitropy of *GAL4-30A, UAS-PKAc 15.3* must be caused by GAL4-driven expression of the *UAS* transgene. Quantitative data on suppression of the wing blister and melanotic growth phenotypes by two different *P[ry$^+$; SalI]* insertions are given in Table 2.

Table 2. Suppression of *GAL4-30A, UAS-PKAc 15.3* pleiotropic phenotypes.

P element	Collapsed Wings	Melanotic Growths
	Fraction of flies	
P[ry$^+$; SalI]3D		
Present	0/152	0/152
Absent	118/118	22/118
P[ry$^+$; SalI]89D		
Present	2/229	1/229
Absent	233/233	89/223

Genetic suppression or enhancement of a *GAL4/UAS* phenotype can be a means to identify interacting gene products and to establish epistatic relationships. When an interaction has been detected, the specificity of the interaction can be tested by introducing a mutant *P* element. We previously reported a dominant lethal interaction between the X-linked *batone* (*bae*) mutant and the *GAL4-30A, UAS-PKAC 15.3* chromosome (Kiger *et al.*, 2001). After eclosion, *batone* mutant flies are unable to

expand their wings, a phenotype that resembles the more extreme expression seen in some individuals carrying the *GAL4-30A, UAS-PKAc 15.3* chromosome. We used *SalI 89D, y^+*, which assorts independently of both *GAL4-30A, UAS-PKAc 15.3* and *bae*, to repress expression of the *GAL4/UAS* combination in one class of sibs and not the other, to determine whether this interaction is due to PKAc expression. To wit, males of genotype *y, w /* Y*; GAL4-30A, UAS-PKAc 15.3 / CyO, Roi ; FRT82B, SalI 89D, y^+/ TM3, Sb* were crossed to *y, w, bae* females. Of the *y, w, bae / +* or Y ; *GAL4-30A, UAS-PKAc 15.3 / +* progeny, 89 carried *SalI 89D, y^+* and none carried *TM3, Sb*. This result shows that PKAc must be expressed for the lethal interaction to take place.

Discussion

P Repressor as a suppressor of UAS transgenes.

The multiple *P* elements in P strains and *P[ry^+; Sal I] 89D* repress transcription of *P-LACZ* fusion genes (Lemaitre and Coen, 1991). Quantitative measurements of LACZ activity in larvae show a 40% reduction by *P[ry^+; Sal I] 89D* and 65% reduction by Harwich P strain: relative values reported are 1.5 [Harwich P strain], 2.6 [*P[ry^+; Sal I] 89D]* and 4.2 [*P[ry^+; RI] 86A*, inactive control]. While we have not made quantitative measurements, based on the effect of *$cact^{BQ}$* on expression of the *UAS-LACZ* or *UAS-GFP* transgenes (Figure 1B and 1D), it appears that we are seeing more than a 65% reduction in expression of these *GAL4/UAS* combinations. However, suppression is obviously not complete. Table 2 showing the frequency of flies exhibiting complete suppression of wing blistering and melanotic growths demonstrates greater than 99% suppression. The difference between these examples is certainly due to the different natures of the assays. The level of LACZ activity or of GFP fluorescence is related more or less directly to the level of transcription and translation of mRNA. On the other hand, morphological phenotypes are more likely to be subject to threshold effects.

We have found *GAL4/UAS* combinations that cannot be suppressed with the two mutant *P* elements we have tested. For example, we were unable to suppress lethality and create a balanced stock of the combination *GAL4-JW1* and *UAS-PKIF 1-1* using *P[ry^+; Sal I] 89D.* The usefulness of mutant *P* elements must be tested on a case-by-case basis. Suppression of the phenotypic effects of *UAS* transgenes expressing highly toxic products may be difficult to achieve due to a low threshold of tolerance. The use of a stronger promoter to express a Repressor cDNA could be a solution to this problem. Nevertheless, we have demonstrated the usefulness of mutant *P* elements in particular cases in three different experimental paradigms. Lee and Luo (1999) have used Gal 80 in a similar manner to our use of Repressor to carry out mosaic analyses. The two systems should complement each other in the Drosophila toolbox.

Mosaic analysis of the GAL4-30A, UAS-PKAc 15.3 *wing phenotype.*

We previously presented evidence that the wing blister phenotype of *GAL4-30A, UAS-PKAc 15.3* is due to expression of PKAc in hemocytes in the wing at the time of, or just prior to, wing expansion, where their role may be to synthesize extracellular matrix that binds the wing surfaces together (Kiger *et al.*, 2001). Because a large population of hemocytes in the wing had not been previously described, we have sought ways to confirm that wing blisters are not caused by expression in the wing epithelium which secretes the cuticle that forms the wing surfaces. To do so, we have induced mitotic recombination in 1st instar larval cells soon after hatching. One would think that at the time of wing morphogenesis, hemocytes derived from a mitotically recombined 1st instar larval hemocyte precursor might have become diluted in the larger population of hemocytes derived from non-recombined hemocyte precursors. However, we reasoned that wing hemocytes might not be part of the

general circulation. Milner and Muir (1987) noted that cultured wing discs have adhering hemocytes that become internalized when the disc evaginates so that the hemocytes are then found in the lumen of the wing. Division of adhering hemocytes prior to evagination and/or division of hemocytes in the lumen during the period of wing growth after evagination (Murray, *et al.*, 1995) could produce a clone of hemocytes, over a substantial area of the wing blade, that fails to synthesize extracellular matrix. The observations of wing blisters we present here are evidence that hemocytes are resident in the wing and not part of the circulating hemocyte population. Studies of DNA replication in the developing wing during the first 24 hr following pupariation have not identified any large population of cells other than epithelial cells (Schubiger and Palka, 1987). Expansion of the hemocyte population must occur after 24 hr following pupariation. Understanding of wing morphogenesis will require consideration of when and how these two tissues interact.

Mosaic analysis of the UAS-PKAcF 1.1; GAL4-71B wing phenotype.

Expression of PKAc in the wing epithelium does not cause wing blisters, in contrast to the effects of PKAc discussed above. Expression of PKAc in the wing epithelium produces an effect only in clones located in the wing blade between L3 and L4, where ectopic development of L3 vein material occurs. These effects can be explained by inactivation of the transcription factor Ci-155 (Aza-Blanc and Kornberg, 1999) due to PKAc phosphorylation (Wang *et al.*, 1999; Kiger and O'Shea, 2001) and consequent failure to induce Patched, Knot and Vein activity.

The region between L3 and L4 is the central organizer of the wing specified by the Hedgehog signal emanating from the posterior compartment (Bier, 2000; Mohler *et al.*, 2000). Hedgehog regulates Ci-155 to activate transcription in the organizer region of genes that include *patched*, *knot* and *vein*. Knot is a transcription factor that represses vein development in the blade region between L3 and L4. Vein is a ligand that diffuses beyond the organizer region and induces vein development in cells just outside of the region of Knot expression. Thus, the vein that forms in the anterior compartment is L3 and that that forms in the posterior compartment is L4. Clones of *knot* mutant cells in the wing blade form ectopic vein material but only when the clone is between L3 and L4, just as we see for ectopic PKAc expression (Nestoras *et al.*, 1997).

In Figure 3B, failure of L4 to form could be due to failure of the cells in the clone to produce the Vein ligand that induces L4. The anterior of the mirror-image pair of veins forms because Knot is not expressed in the cells that initiate vein formation. They receive Vein from the cells outside the clone. Being in the anterior compartment, it is L3 vein that forms. The ability of vein material to induce vein formation in nearby cells could explain why the cells at the wing margin forming socketed L3-type bristles are outside the clone (Garcia-Bellido, 1977; Mohler *et al.*, 2000). It is less clear why the posterior of the two mirror-image veins form. However, similar effects are produced by loss of either Patched or Knot activity in clones (Phillips *et al.*, 1990; Nestoras *et al.*, 1997). In Figure 3D, the clone anterior to L4 is smaller than that in Figure 3B and causes only a small gap in L4, suggesting that Vein may be able to diffuse through most of the clone to reach the posterior compartment.

Materials and Methods

Strains

The *UAS* strains employed have been described previously (Kiger *et al.*, 1999) with the exception of the *UAS-Draf* strain, that was created by standard means using a wild type cDNA provided by Klaus Dncker. *GAL4-30A* and *GAL4-71B* are described in Brand and Perrimon (1993) with the corrigendum that their identities are reversed in that paper. *GAL4-55B2* is described in Brand and

Perrimon (1994). All *GAL4* and *UAS* transgenes carry the *mini-w*$^+$ gene and are in a *w* background so that the presence of each transgene can be identified by an eye color phenotype that is more dilute than that caused by *w*$^+$. Frequently, the presence of more than one *mini-w*$^+$ transgene can be detected by the additivity of the eye colors produced, making it possible to carry out crosses between heterozygous transgenic strains and to identify all progeny classes unequivocally.

The P strains employed, derived from natural populations of *D. melanogaster* located in Northern California at Auburn and Wolfskill, were provided by M. M. Green. The *cact*^{BQ} stock was donated by Ruth Steward. The *P[ry*$^+$*; Sal I] 3D* and*P[ry*$^+$*; Sal I] 89D* strains were obtained from the Bloomington Stock Center and William Engels, respectively. *P[ry*$^+$*; SalI] 3D* is inserted into the *diminutive* gene and is also known as *dm*^{P0}. Strains carrying *P[ry*$^+$*; 66k]* were obtained from Donald Rio.

The FRT strains employed were created by meiotic crossing over using chromosomes originally created by Xu and Rubin (1993) and were obtained from the Bloomington Stock Center. The *y, w, P[ry*$^+$*; hs-FLP]* strain was obtained from the Bloomington Stock Center. *P[ry*$^+$*; f*$^+$*] 98B* and *w, P[ry*$^+$*; hs-FLP] 9F, f*^{36a} ; *P[ry*$^+$*; hs-neo; FRT] 82B* strains were obtained from Antonio Garcia-Bellido. All other genetic markers can be found in Lindsley and Zimm (1992). The chromosomes constructed for use in these studies are listed in Table 3.

Table 3. Genotypes of chromosomes constructed for these studies.

1. *y, w, P[ry*$^+$*; SalI]3D, P[ry*$^+$*; hs-neo; FRT]19A*
2. *P[ry*$^+$*; SalI]89D, P[ry*$^+$*; y*$^+$*]96E = SalI89D, y*$^+$.
3. *P[ry*$^+$*; hs-neo; FRT]82B, P[mini-w*$^+$*; hs-πM]87E, P[ry*$^+$*; SalI]89D, P[ry*$^+$*; y*$^+$*]96E = 82B, SalI89D, y*$^+$.
4. *Gal4-71B, P[ry*$^+$*; hs-neo; FRT]82B, P[mini-w*$^+$*; hs-πM]87E, P[ry*$^+$*; SalI]89D, P[ry*$^+$*; y*$^+$*]96E = 71B, 82B, SalI89D, y*$^+$.
5. *P[ry*$^+$*; hs-neo; FRT]82B, P[mini-w*$^+$*; hs-πM]87E, P[ry*$^+$*; SalI]89D, P[ry*$^+$*; y*$^+$*]96E , P[ry*$^+$*; f*$^+$*]98B = 82B, SalI89D, y*$^+$*, f*$^+$.
6. *Gal4-71B, P[ry*$^+$*; hs-neo; FRT]82B, P[mini-w*$^+$*; hs-πM]87E, P[ry*$^+$*; SalI]89D, P[ry*$^+$*; y*$^+$*]96E, P[ry*$^+$*; f*$^+$*]98B = 71B, 82B, SalI89D, y*$^+$*, f*$^+$.

Culture conditions and heat shock.

All crosses were carried out at 25ºC. For heat shock experiments flies were allowed to deposit eggs in food vials for 24 hours. After 24 hours the vial was submerged in a water bath at 38ºC for either one or two hours and then returned to 25ºC.

Microscopy and staining.

Ovaries were stained for LACZ activity using Xgal as described by Brand and Perrimon (1994). Ovaries and salivary glands were photographed using a Leica MZ FLIII fluorescence stereomicroscope. Wings were mounted as described in Kiger *et al.* (1999) and photographed (Figure 1) with a dissecting microscope (Zeiss, Jena, Germany) or (Figures 2 and 3) a Zeiss Axioplan microscope equipped with a Kodak Photomicrography System MDS290.

Mapping the suppressor locus in the cact^{BQ} *chromosome.*

The suppressor locus (*Su*), present on a chromosome carrying the known mutants *b cact*^{BQ} *pr cn*, was mapped initially using a chromosome carrying six dominant markers, *Sp Bl L*^{rm} *Bc Pu*^{2} *Pin*^{B}. Found to be located on the left arm of chromosome II, *Su* was recovered in a recombinant chromosome carrying *Sp b cact*^{BQ} *pr cn*. Next females of genotype *(Su) Sp b cact*^{BQ} *pr cn / +* were crossed to males

of genotype *GAL4-30A, UAS-PKAc 15.3 / CyO, Roi cn.* Preliminary analysis of the progeny indicated that *Su* was located between *Sp* and *cn*. With this knowledge, progeny with crossovers between *Sp* and *cn* were selected over *CyO, Roi cn* and crossed individually to *In(2L) dl^T, b dl^T pr cn sca / CyO.* From this cross, balanced stocks containing individual crossover chromosomes were established, and progeny carrying each crossover chromosome and *In(2L) dl^T* were scored for *b* and *pr* ; female progeny were tested for fertility to determine the *cact* genotype. Females of genotype *$cact^{BQ}$/ In(2L) dl^T* are sterile (Roth *et al.*, 1991). Males carrying each crossover chromosome then were crossed to females of genotype *GAL4-30A, UAS-PKAc / CyO, Roi cn*, and *GAL4-30A, UAS-PKAc 15.3* progeny were scored for *Su*.

Analysis of 331 crossover chromosomes showed that *Su* is located, as is the *cact* gene, between *b* and *pr* (a distance of 6.0 centimorgans). Of these crossovers, 52 single crossovers fell between *b* and *pr* and failed to separate *Su* from *$cact^{BQ}$*. However, 4 triple crossovers separating the two were recovered, 3 moved *Su* to the *b^+ pr^+* chromosome (*Sp Su*) and 1 moved *$cact^{BQ}$* to the *b^+ pr^+* chromosome (*Sp $cact^{BQ}$*). These unusual events may be causally related to the presence of the *P* element. Thus, *$cact^{BQ}$* and *Su* are very closely linked.

Acknowledgments: We thank M. M. Green for critically reading this manuscript. This work was supported by funds of the Agricultural Experiment Station at UC Davis.

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