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Effects of the su(Hw) insulator protein on the expression of the divergently transcribed *Drosophila yolk protein* genes

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The suppressor of Hairy-wing [su(Hw)] protein mediates the mutagenic effects of the gypsy retrotransposon by blocking enhancer activity. These repressive effects are general, can occur over long distances and require that the su(Hw) protein is bound between the affected enhancer and promoter. The effects of the su(Hw) binding region on *yolk protein* (*yp*) gene expression were determined. These genes are regulated by shared enhancers in the intergenic region, which provided a method to examine whether an enhancer blocked by the su(Hw) protein remained functional. We demonstrate that a blocked enhancer is completely active, supporting the proposal that the su(Hw) protein is an insulator protein that acts by forming a new boundary in a pre-existing chromatin domain, thereby preventing the interaction of regulatory elements located upstream of the insertion site with the promoter. In addition, we found that *yp* promoter function is not diminished by sharing enhancers, suggesting that these enhancers are not rate limiting for transcriptional activation. Lastly, our data indicate that *yp* promoter activity is interdependent, such that transcription from one promoter influences the level of activity of the linked promoter.

Keywords: divergent transcription unit/domain boundaries/ *Drosophila*/gene expression/su(Hw) protein

Introduction

In *Drosophila*, integration of the gypsy retrotransposon causes tissue-specific mutations of a number of loci, including *yellow*, *cut* and *Ultrabithorax* (Modolell *et al.*, 1983). Gypsy is 7.5 kb in length, contains long terminal repeats (LTRs) of 482 bp and encodes proteins that are similar in amino acid sequence to retroviral proteins (Marlor *et al.*, 1986; Song *et al.*, 1994). In many cases, insertion of gypsy within a gene causes mutant phenotypes that require a second unlinked modifier locus, the *suppressor of Hairy-wing* [*su(Hw)*] gene. Mutations in this gene specifically reverse the phenotype of gypsy-induced alleles and do not affect mutations induced by any other transposable element (Rutledge *et al.*, 1988). Gypsy insertions can have long-range effects on gene expression; in some cases causing mutations even when located at distances between 10 and 80 kb from a promoter (Peifer and Bender, 1986; Jack *et al.*, 1991).

The *su(Hw)* gene encodes a 110 kDa protein that is expressed throughout development and is present in most tissues (Parkhurst *et al.*, 1988; Harrison *et al.*, 1993). This protein contains a Zn finger DNA binding domain, a leucine zipper motif and acidic domains that are essential for its mutagenic effects (Parkhurst *et al.*, 1988; Harrison *et al.*, 1993). The su(Hw) protein binds specifically to gypsy sequences located 3′ of the 5′ LTR, called the su(Hw) binding region (Spana *et al.*, 1988; Mazo *et al.*, 1989). The su(Hw) binding region contains 12 copies of a 27 bp sequence whose core is similar to the octamer motif and has tracts of A residues which provide a bend in the DNA that is necessary for the interaction of su(Hw) protein (Spana and Corces, 1990).

In most cases, gypsy causes mutations by inactivation of enhancers present in the target genes (Geyer *et al.*, 1986; Peifer and Bender, 1986; Simon *et al.*, 1990; Jack *et al.*, 1991; Qian *et al.*, 1991; Dorsett, 1993). These repressive effects require the entire su(Hw) binding region, as deletion of binding sites or insertion of additional sequences within this region partially restores enhancer activity (Geyer *et al.*, 1988; Peifer and Bender, 1988; Smith and Corces, 1992). The mutagenic effects of the entire gypsy element can be reproduced by insertion of only the su(Hw) binding region (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Roseman *et al.*, 1993). Thus, the su(Hw) binding region is both necessary and sufficient for gypsy mutagenesis.

Inactivation of enhancer function occurs only when the su(Hw) protein is bound between an enhancer and promoter (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Roseman *et al.*, 1993). This indicates that there is a position-dependent component involved in this process and that it is not simply due to the proximity of the su(Hw) protein to the enhancer. Expression of a large number of functionally unrelated genes can be affected by the binding of the su(Hw) protein to gypsy elements inserted in their regulatory regions. This suggests that repression by the su(Hw) protein is general. This conclusion is supported further by the fact that (i) all of the *yellow* enhancer elements can be repressed by the su(Hw) protein, even those unaffected in the original gypsy-induced mutation (Geyer and Corces, 1992); (ii) enhancer elements present in genes which do not have gypsy-induced mutations can be inactivated by the placement of the su(Hw) binding regions in the appropriate position (Holdridge and Dorsett, 1991; Roseman *et al.*, 1993); and (iii) the presence of the su(Hw) binding region buffers the mini-*white* gene from both inhibitory and stimulatory position effects caused by adjacent DNA sequences when the mini-*white* gene is inserted in various genomic locations (Roseman *et al.*, 1993).

Several paradigms for repressive effects of DNA binding proteins have been elucidated (reviewed in Levine and Manley, 1989; Jackson, 1991). In most cases, repression results from direct inactivation of the enhancer. To test

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whether the su(Hw) protein disrupts the function of enhancers in this way, we tested whether an enhancer that was blocked by the su(Hw) protein was still able to activate transcription. In these studies, we assayed the activity of a blocked enhancer using the divergently transcribed *yolk protein* (*yp*) genes as a model system. These genes, called *yp1* and *yp2*, encode two of the three abundant food proteins and are expressed in an identical pattern only in adult female fat bodies and follicle cells of the ovaries (Barnett and Wensink, 1981; Brennan *et al.*, 1982; Garabedian *et al.*, 1986). Expression of these genes is controlled by shared fat body and ovarian enhancers located in the intergenic region which regulate expression independently in each tissue (Garabedian *et al.*, 1985, 1986; Logan *et al.*, 1989; Logan and Wensink, 1990; Abrahamsen *et al.*, 1993). Using these divergent transcription units, we tested whether inactivation of the shared fat body enhancers caused a loss of transcription from one or both *yp* promoters. Our studies demonstrate that a blocked enhancer is still functional. These findings support the proposal that su(Hw) protein acts as an insulator protein to block communication between enhancer and promoter elements. Furthermore, this system allowed examination of regulatory processes involved in controlling expression of a divergent transcription unit.

Results

Insertion of the su(Hw) binding region into the yp intergenic region does not alter temporal or tissue-specific expression

The effects of the su(Hw) protein on enhancer function were investigated using the divergently transcribed *yp* genes. The 430 bp su(Hw) binding region was inserted into the *yp* intergenic region of the p[CR2] vector (Figure 1). This vector contains a modification of the *yp* genes, such that *yp1* sequences downstream of +37 are fused to the *Drosophila* alcohol dehydrogenase (*Adh*) gene and *yp2* sequences downstream of +105 are fused to the *Escherichia coli LacZ* gene (Logan and Wensink, 1990). These fusion genes carry the entire *yp* intergenic region and are expressed with the correct developmental specificity (Logan and Wensink, 1990). This vector is particularly useful because the activity of the *yp* genes can be determined independently, using either histochemical or spectrophotometric analyses.

The effects of insertion of the su(Hw) binding region on the temporal and spatial expression pattern of the *yp* genes were determined. Recent studies suggest that, in certain contexts, the su(Hw) protein may activate transcription (Smith and Corces, 1995). For this reason, we wanted to ensure that the su(Hw) protein did not alter *yp* expression in any unexpected way. The su(Hw) binding region was inserted 340 bp upstream of the *yp1* promoter in p[CR2], creating p[ZARG] (Figure 1). Several independent transformed lines were obtained that carried one insertion of p[ZARG]. Histochemical staining was carried out on tissues isolated from third instar larvae and from adult males and females of three independent p[ZARG] lines. As expected, no β-galactosidase or alcohol dehydrogenase (ADH) expression was observed during larval stages or in adult males (data not shown, Figure 1). In adult females, both *yp* genes in p[ZARG] were expressed in a tissue-specific pattern that was indistinguishable from control p[CR2] lines; β-galactosidase staining was detected only in fat bodies and ovarian follicle cells. Expression of the *yp* genes in the ovary occurs in a restricted, cell type-specific manner (Logan and Wensink, 1990). These genes are active in the columnar follicle cells, beginning in stage 8 egg chambers and continuing through to stage 10. Spotty follicle cell staining also is apparent in later staged 12 and 13 egg chambers (Figure 1; Logan and Wensink, 1990). Transgenic lines carrying p[ZARG] showed *yp1* and *yp2* expression in these same cell types, at the appropriate stages of oogenesis. The expression of *yp1* in p[ZARG] ovaries was not expected because the position of insertion of the su(Hw) binding region was between the previously characterized ovarian enhancers and the *yp1* promoter and should have prevented *yp1* expression in this tissue. However, further analysis of the *yp1* upstream region indicates that a third ovary-specific regulatory element is present just upstream of the *yp1* promoter, consistent with the observed expression pattern (K.S.Scott and P.K.Geyer, unpublished result). To verify that p[ZARG] was expressed exclusively in females, extracts isolated from whole male or female flies were assayed using a more sensitive spectrophotometric method. Only females expressed the *yp* genes in p[ZARG] (Table I). We conclude that the su(Hw) binding region does not affect the tissue-, temporal or sex-specific patterns of expression of the *yp* reporter genes.

The su(Hw) binding region represses the yp fat body enhancer elements

We determined the effects of su(Hw) protein on the function of the *yp* fat body enhancers. The *yp* intergenic region contains two enhancers which direct expression of these genes only in the fat body (Figure 1; Garabedian *et al.*, 1985, 1986; Logan *et al.*, 1989; Logan and Wensink, 1990; Abrahamsen *et al.*, 1993). Fat body cells are located throughout the head, thorax and abdomen of the fly. To study *yp* gene expression in this tissue independent of ovarian expression, dissected carcasses were used in all studies. The su(Hw) protein was expected to block the fat body enhancers from activating the *yp* promoters because previous studies suggested that the su(Hw) protein acts as a general repressor of enhancers (Roseman *et al.*, 1993). The su(Hw) binding region was placed at several positions within the *yp1/yp2* intergenic region (Figure 2). These constructs were transformed into flies using P element-mediated germline transformation. Only transgenic lines that carried one, unrearranged P element insertion were analyzed. Whenever possible, at least three transgenic lines were studied.

First, the activity of the *yp2* gene in the various transgenes was analyzed. A quantitative analysis of β-galactosidase activity was used to assess the level of *yp2* promoter function in dissected carcass extracts. Extracts from transgenic flies carrying the p[CR2] transposon were assayed in parallel and assigned a value of 100 activity units. The activities of the various su(Hw) binding region derivatives of p[CR2] were expressed as a percentage of wild-type activity (Figure 2). Insertion of the su(Hw) binding region upstream of both fat body enhancers (p[ZARU]) produced transgenic flies which had a high level of expression from the *yp2* promoter. Interestingly,

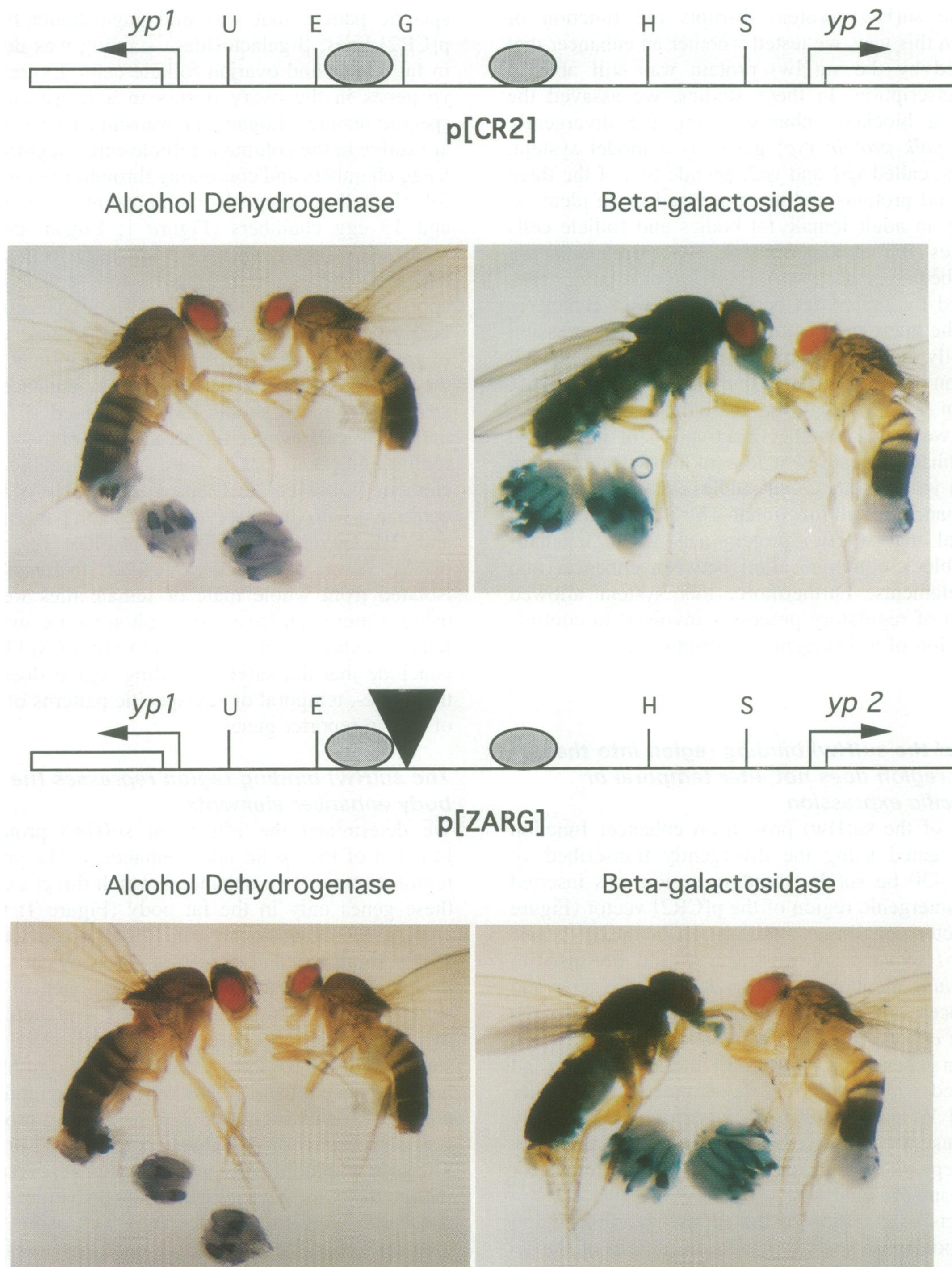

Fig. 1. Tissue- and sex-specific expression of the *yp* transgenes. Shown are two constructs which contain the *yp* fusion genes. The *yp1* and *yp2* promoters are shown as arrows with the direction of transcription indicated. The *yp1* promoter is fused to the ADH-coding sequences, whereas the *yp2* promoter is fused to sequences encoding the *E.coli* β-galactosidase. The intergenic region contains two fat body enhancers which are represented as ovals. Within the intergenic region are a number of unique restriction sites that were used to prepare the su(Hw) binding region derivatives of p[CR2]. The positions of these sites are indicated by a capital letter: U, *Stu*I; E, *Eco*0109; G, *Bgl*II; H, *Hin*dIII; and S, *Sac*I. The black triangle in p[ZARG] represents the su(Hw) binding region. Below the diagram of each construct are the photographs of the corresponding transgenic flies (females, left; males, right) that were stained for ADH or β-galactosidase activity. The staining patterns of flies carrying either transgene are female-specific and restricted to fat bodies and ovarian follicle cells.

the level of *yp2* activity was ~3.5 times higher than that seen for the control p[CR2] transposon. To determine whether hypertranscription of the *yp2* promoter was dependent upon the su(Hw) protein, one line of flies carrying the p[ZARU] transposon was crossed into a *su(Hw)*$^-$ background. We found that *yp2* activity remained elevated relative to the wild-type control (Figure 3), suggesting that this effect was not caused by the su(Hw) protein.

Placing the su(Hw) binding region between the fat body enhancers (p[ZARG]) reduced *yp2* expression to ~24% of the p[CR2] level, suggesting that the upstream enhancer

was blocked by the su(Hw) protein, while the downstream enhancer remained active. The 75% decrease in *yp2* activity suggests that the fat body enhancer located between the *Eco*0109 (E, Figure 2) and the *Bgl*II (G, Figure 2) restriction sites is the stronger of the two fat body enhancers. This observation is consistent with previous results concerning the fat body control elements (Garabedian *et al.*, 1986; P.Wensink, personal communication). The fat body enhancer located between *Eco*0109 and *Bgl*II corresponds to the well characterized FBE1 element which contains multiple binding sites for transcriptional activators (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; An and Wensink, 1995).

Table I. Sex-specific expression of a *yolk protein* transgene carrying the su(Hw) binding region

Strain	ADH activity	
	Units/1 female	Units/10 males
Adh^{fn6} cn;ry^{506}	0.002 ± 0.005	0.004 ± 0.003
p[CR2];Adh^{fn6} cn; ry^{506}	0.627 ± 0.020	0.002 ± 0.002
p[ZARG]; Adh^{fn6} cn; ry^{506} line 14	0.593 ± 0.009	0.001 ± 0.001
Adh^{fn6} cn; p[ZARG] ry^{506} line 18	0.536 ± 0.021	0.002 ± 0.003
Adh^{fn6} cn; p[ZARG] ry^{506} line 39-2	0.547 ± 0.014	0.002 ± 0.001
Strain	**β-Galactosidase activity**	
	Units/1 female	Units/10 males
y ac; ry^{506}	0.002 ± 0.003	0.004 ± 0.005
p[CR2]; ry^{506}	1.233 ± 0.020	0.003 ± 0.002
y ac p[ZARG]; ry^{506} line 14	0.279 ± 0.021	0.000 ± 0.001
y ac;p[ZARG] ry^{506} line 18	0.303 ± 0.037	0.003 ± 0.001
y ac; p[ZARG] ry^{506} line 39-2	0.322 ± 0.030	0.002 ± 0.001

Reported are the averages of three enzymatic assays of different preparations of whole fly extracts. Each individual assay was performed in triplicate and gave <15% error. For ADH, a unit corresponds to 1×10^{-3} A_{340}/5 min. For β-galactosidase activity, one unit corresponds to 1×10^{-3} A_{595}/1 h.

Insertion of the su(Hw) binding region downstream of both enhancers (p[ZARH], –340 relative to the *yp2* cap site) caused an additional decrease in *yp2* promoter activity to ~8% of the p[CR2] level, with an unchanged pattern of sex- and tissue-specific expression (data not shown). This indicates that the second fat body enhancer was blocked. The remaining *yp2* expression may result from either an incomplete block of enhancer function or residual, non-enhancer-activated transcription from the *yp2* promoter. In the latter case, transcription may remain sex- and tissue-specific because of additional control elements which reside outside of the fat body enhancers, perhaps directly associated with the promoter.

These data suggest that the su(Hw) protein can disrupt the activities of both fat body enhancers. To control for distance effects resulting from the insertion of the su(Hw) binding region, a 429 bp region of pBR322 was placed at the same position as the binding region in p[ZARH] to create p[ZAPH]. The activity of the *yp2* promoter in three independent transgenic lines carrying p[ZAPH] was ~95% of that in p[CR2], demonstrating that the increased distance did not cause the decreased *yp2* activity of p[ZARH] (Figure 2).

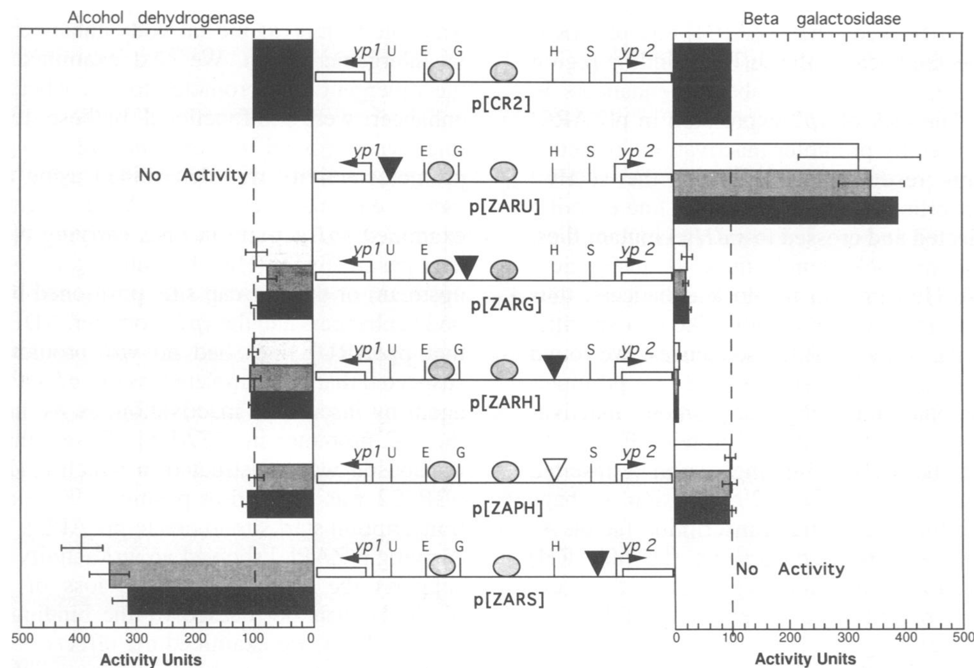

Fig. 2. Structures and activities of p[ZAR*X*] constructs. Shown are the constructs (center) carried by transgenic flies that were assayed for either ADH activity (left) or β-galactosidase activity (right). For ADH, an activity unit corresponds to the rate of change in OD_{340}/s/fly. For β-galactosidase, an activity unit corresponds to the rate of change in OD_{595}/min/fly. The average level of activity for the p[CR2] transformant was set to 100% (solid black bar), and experimental values for the individual transformed lines are expressed relative to this value. No activity means that values were <0.2% of the control. Results obtained are from multiple independent transformed lines, each represented as a stippled bar. Each independent transformed line was assayed in triplicate. The symbols are described in the legend to Figure 1, except that an open triangle represents the insertion of spacer pBR322 DNA.

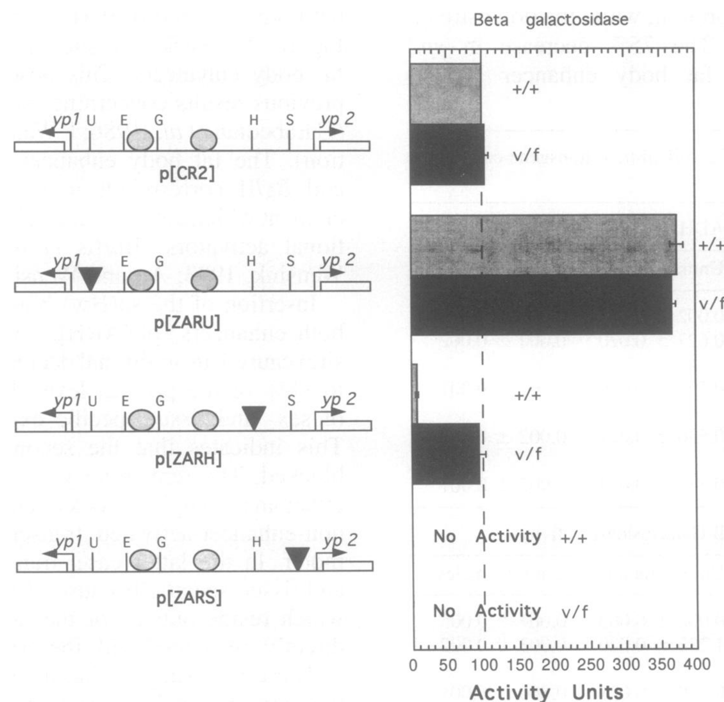

Fig. 3. Effects of a *su(Hw)* mutant background on *yp* gene expression in p[ZAR*X*] transgenes. To determine whether changes in *yp* expression were direct effects of the su(Hw) protein, several transgenes were crossed into a $su(Hw)^{v}/su(Hw)^{f}$ mutant background and then β-galactosidase activity was determined as described in Figure 2. Enzymatic activity in a wild-type *su(Hw)* background is shown as (+/+), while β-galactosidase activity in a mutant *su(Hw)* background is shown as (v/f). Each independent transformed line was assayed in triplicate. The symbols are described in the legend to Figure 1.

Addition of the su(Hw) binding region at a second site downstream of both fat body enhancers (159 bp upstream of the *yp2* promoter, p[ZARS]) produced transgenic lines which had no *yp2* promoter activity. The observed difference in *yp2* activity between the p[ZARH] and p[ZARS] lines was not expected because the su(Hw) binding region should have blocked the same number of enhancers in each transposon. The lack of *yp2* expression in p[ZARS] appeared to be caused by promoter inactivation. To determine whether this resulted directly from the su(Hw) protein or from insertional mutagenesis, one line carrying p[ZARS] was selected and crossed to *su(Hw)* mutant flies. In a *su(Hw)* mutant background, there is insufficient function of the su(Hw) protein to block enhancers, thus the su(Hw) binding region is essentially like non-specific, spacer DNA, similar to the pBR322 sequences. We found that in *su(Hw)* mutant p[ZARS] flies, the *yp2* promoter was still not functional, indicating that promoter inactivation was independent of the su(Hw) protein (Figure 3). We conclude that the su(Hw) binding region is inserted into DNA sequences critical for *yp2* expression, perhaps into a binding site for an essential transcription factor. As a control, we crossed one line carrying the p[ZARH] transposon into a *su(Hw)* mutant background. In this case, *yp2* expression increased to a level comparable with that seen in p[CR2] (Figure 3). Taken together, these experiments indicate that the difference in *yp2* expression between p[ZARH] and p[ZARS] results from insertional inactivation of the *yp2* promoter in p[ZARS]. These studies suggest that the su(Hw) protein disrupts enhancer but not promoter function, consistent with what is observed at other loci (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Roseman *et al.*, 1993).

An enhancer blocked by the su(Hw) protein still retains full activity

Analysis of the effects of the su(Hw) binding region on *yp2* promoter activity demonstrated that the su(Hw) protein was able to repress the fat body enhancers present in the *yp* intergenic region. We next examined the activity of the divergent *yp1* promoter to see whether the blocked enhancers were still functional. In these studies, transgenic lines were crossed into an *Adh* null background and *yp1* promoter activity was determined using a spectrophotometric enzymatic assay for ADH (Figure 2). We first examined *yp1* activity in lines carrying p[ZARU]. In this transposon, the su(Hw) binding region is located 90 bp upstream of the *yp1* cap site, positioned between both fat body enhancers and the *yp1* promoter. ADH assays showed that p[ZARU] lines had no *yp1* promoter activity. We suspected that the complete loss of *yp1* activity was caused again by insertional inactivation, as we had observed for the *yp2* promoter in p[ZARS]. To test this hypothesis, a transposon was constructed in which a 429 bp region of pBR322 was inserted at position –90 relative to the *yp1* transcription start site to create p[ZAPU]. Transgenic flies carrying p[ZAPU] showed no *yp1* activity (Figure 4). This supports the proposal that the loss of *yp1* activity in p[ZARU] was not caused by the binding of the su(Hw) protein. Next, we examined the effects of insertion of the su(Hw) binding region between the two fat body enhancers (p[ZARG]). In these lines, we observed a slightly reduced *yp1* activity (~88% of the *yp1* promoter in p[CR2]). This lowered activity is consistent with the proposal that, in p[ZARG], the major fat body enhancer (FBE1) can activate *yp1* transcription, while the minor fat body enhancer is blocked by the su(Hw) protein. Lastly, we examined the

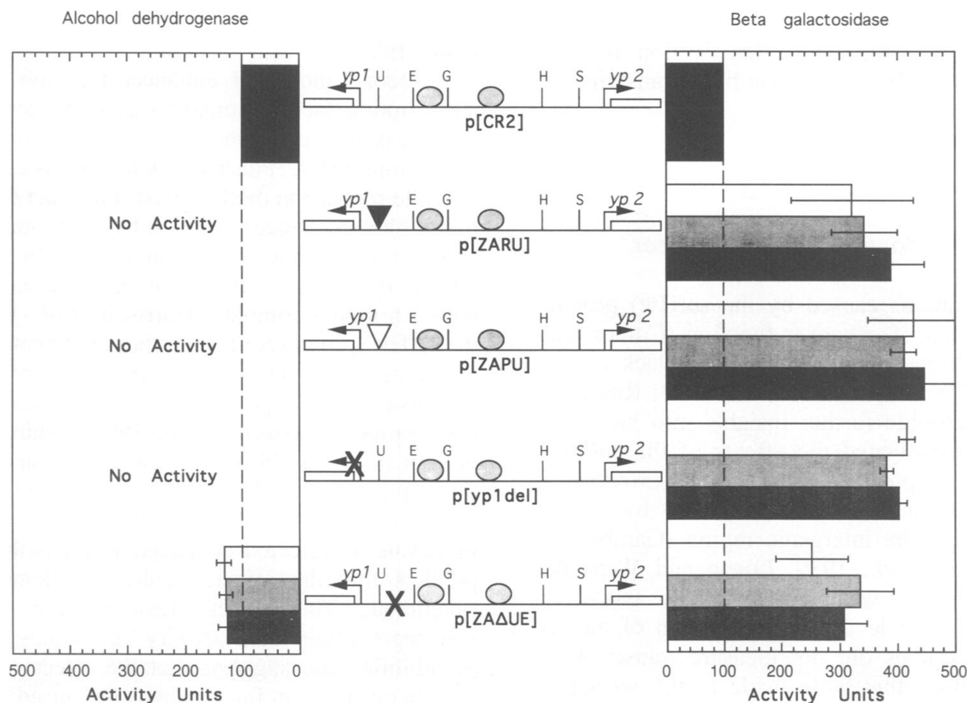

Fig. 4. Hyperactivity of the *yp2* promoter arises independently of the su(Hw) binding region. In several constructs, the expression of the *yp2* fusion gene was at a level higher than the control p[CR2]. p[ZARU] carries a su(Hw) binding region inserted 90 bp upstream from the *yp1* transcription start site, p[ZAPU] carries a 429 bp piece of pBR322 inserted at the same site. The construct p[*yp1*del] carries a 107 bp deletion which includes the *yp1* promoter, indicated by the X. The construct p[ZAΔUE] carries a 110 bp deletion indicated as an X between the two restriction sites. Enzymatic activities were determined as described in Figure 2. Each independent transformed line was assayed in triplicate. The symbols are described in Figures 1 and 2.

level of *yp1* activity in transgenic lines carrying the p[ZARH] and p[ZARS] transposons. In these transgenes, the interaction between the *yp*2 promoter and the fat body enhancers was blocked. In both cases, the level of activity of the divergent *yp1* promoter was high. Flies carrying p[ZARH] showed almost the same level of expression as the *yp1* promoter in p[CR2], whereas flies carrying p[ZARS] showed ~3.5 times as much activity as the *yp1* promoter in p[CR2]. These results directly demonstrate that blocked enhancers still retain wild-type activity. Therefore, the action of the su(Hw) protein is not directed towards either an enhancer or a promoter, but interferes with the communication between these regulatory elements.

Enhanced promoter activity is not dependent upon the su(Hw) protein

We were interested in understanding the basis for hyperactivation of the *yp1* or *yp2* promoter observed in some *yp* transgenes containing a su(Hw) binding region. One possible explanation was that when an enhancer is shared by two promoters, the activity of both promoters is decreased because the number of interactions between the enhancer and promoter pre-initiation complex is cut in half. Using this reasoning, we predicted that any transposon that contained an insertion of the su(Hw) binding region between the fat body enhancers and one promoter, would have 'unshared' enhancers and increased transcription from the opposite promoter. Thus, lines carrying the transposons p[ZARH] or p[ZARS] should show hyperactivity of the *yp1* fusion gene. However, this is not what was observed. The activity of the *yp*1 promoter in p[ZARH] was essentially the same as that observed in the control p[CR2]. This result suggests that hypertranscription of the *yp1* and *yp2* promoters does not result because of exclusive use of the fat body enhancers by one promoter.

Several lines that carried transposons which showed hyperactivation of one promoter (p[ZARU], p[ZAPU] and p[ZARS]) had a complete loss of activity of the divergent promoter due to insertional inactivation. For this reason, we tested the possibility that hypertranscription could result from a loss of transcription from one divergent gene. We created a deletion of the *yp1* promoter that eliminates the TATA box and the start site of transcription producing p[*yp1*del]. Several lines carrying the p[*yp1*del] transposon were obtained. We tested whether this deletion removed the *yp1* promoter by crossing these transposons into an *Adh* null background and carrying out ADH spectrophotometric assays. All of the p[*yp1*del] lines had no detectable ADH activity, indicating that the *yp1* promoter was completely inactive (Figure 4). We next checked the activity of the divergent *yp*2 promoter. As predicted, the *yp*2 activity was hyperactive, showing a 4-fold greater activity than in p[CR2] transgenic flies (Figure 4). This demonstrates that hyperactivation of a promoter can occur in the absence of the su(Hw) binding region.

In the course of our experiments, we created a transposon, p[ZAΔUE], which carries a deletion of sequences just upstream of the *yp1* promoter between –90 and –200 from the transcription start site. This deletion does not remove either fat body enhancer. Three independent transformed lines were obtained and ADH and β-galactosidase assays were carried out (Figure 4). We found that *yp2* activity in these lines was hyperactive (299%) relative to

the control p[CR2], whereas *yp1* activity in this line was close to wild-type (127%). Thus, inactivation of one promoter is not absolutely required for hypertranscription of the linked promoter.

Discussion

The su(Hw) protein does not alter enhancer function

Tissue-specific mutations caused by the su(Hw) protein result from repression of enhancer function (Corces and Geyer, 1991; Holdridge and Dorsett, 1991; Jack *et al.*, 1991; Geyer and Corces, 1992; Dorsett, 1993; Roseman *et al.*, 1993). To provide further insights into how this might occur, we investigated the effects of the su(Hw) protein on *yp* gene expression. These genes provided a useful test system because they are regulated by shared enhancers residing in the intergenic region (Garabedian *et al.*, 1985; Logan *et al.*, 1989; Logan and Wensink, 1990). In these studies, we used enzymatic assays to assess quantitatively the level of transcription of the *yp* genes. While our studies did not measure transcription directly, all alterations that were made to the *yp* genes were in the intergenic control region. Thus, changes in enzymatic activity should reflect a change in the amount of transcription, as the processing and stability of the transcribed RNAs should not be altered.

Using this system, we directly examined the question of whether a blocked enhancer remained functional. Our results show that insertion of the su(Hw) binding region into the *yp* intergenic control region altered gene expression in the same position-dependent manner as was previously observed (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Roseman *et al.*, 1993). Furthermore, these studies add the *yp* fat body enhancers to the large number of enhancers which can be repressed directly by the su(Hw) protein, consistent with its properties of affecting enhancer function in a general way. Most importantly, our experiments demonstrate that an enhancer which is blocked by the su(Hw) protein can still activate transcription at a wild-type level from a divergent promoter (for example, see Figure 2 p[ZARH]).

Models for su(Hw) protein function

Prior to these studies, several models were proposed to explain the negative effects of the su(Hw) protein on enhancer function. These models are dependent upon the mechanism by which enhancers activate transcription. One model of enhancer function proposes that they are entry points for transcription factors that track along the DNA until they reach a promoter. In this context, the su(Hw) protein may block the progression of these factors (Geyer and Corces, 1992). Although results obtained in this study are consistent with the su(Hw) protein interfering with tracking of transcription factors, it is unlikely that this protein acts simply as a barrier to tracking because some su(Hw) mutations which disrupt enhancer blocking activity do not interfere with DNA binding (Harrison *et al.*, 1993). Alternatively, the su(Hw) protein could impede transcription factors from scanning the DNA by direct protein–protein interactions. However, this hypothesis is difficult to reconcile with the fact that the su(Hw) protein is a general repressor of enhancer function (Roseman *et al.*, 1993).

A second model of enhancer function proposes that transcription factors bound to an enhancer interact with those assembled at the promoter by looping out the intervening DNA (Ptashne, 1988). In this context, several possible repression models exist. First, the su(Hw) protein might block enhancer function by capturing the enhancer complex during the looping process or by disrupting the interaction of the enhancer-bound transcription factors with a bridging complex (Harrison *et al.*, 1993). Second, the su(Hw) protein could alter the conformation of adjacent chromatin such that a repressive type of chromatin is established which spreads over the enhancers causing their inactivation (Harrison *et al.*, 1993). Third, the su(Hw) protein might establish a domain boundary which limits the activity of the enhancers (Roseman *et al.*, 1993). These first two models predict that the su(Hw) protein would inactivate an affected enhancer, whereas the third model predicts that the affected enhancer element would be functional. The direct demonstration that blocked enhancers retain full activity eliminates the first two possibilities and suggests that the effects of the su(Hw) protein on enhancer function may be caused by the creation of a domain boundary. This proposal is consistent with the general nature of the repressive effects of the su(Hw) protein. Domain boundaries may disrupt interactions between enhancers and promoters by folding or assembling in such a way as to increase the likelihood of interactions between regulatory elements within a domain, while decreasing these interactions between domains (Vazquez and Schedl, 1994).

Shared enhancers do not limit promoter activity

The sharing of fat body enhancers between *yp* promoters did not lower the amount of transcription from either promoter. This conclusion is based on transgenic lines carrying p[ZARH]. The insertion site of the su(Hw) binding region in this construct caused a disruption of the interaction between both fat body enhancers and the *yp2* promoter, creating a situation where the fat body enhancers were only able to interact with one promoter, *yp1*. In this situation, *yp1* activity did not increase above the control p[CR2]. This suggests that promoter activation by the fat body enhancers is not rate limiting and that enhancers can be used efficiently by the two divergent promoters. These results contrast with those obtained for an upstream promoter element (UPE). When two divergent promoters shared a single UPE, mutual inhibition of both promoters was observed, indicating that transcription factors bound to the UPE can only interact with one transcription complex (Adami and Babiss, 1992).

The yp promoters show interdependence

These experiments demonstrated that the transcriptional activity of one gene can be significantly increased over that observed in the control p[CR2] transposon. This effect did not involve the su(Hw) protein, as it could be recreated completely in modified transposons which lacked the su(Hw) binding region. In three cases, hypertranscription of one promoter resulted from inactivation of the divergent promoter. However, these effects are not caused directly by transcription, as a deletion of part of the intergenic

region upstream of the *yp1* promoter, which retained promoter activity, caused hypertranscription of the *yp2* reporter gene. These studies suggest that transcription of each *yp* promoter is not completely independent. The insertion of the su(Hw) binding region does not alter this interdependence, because hypertranscription does not always occur when the su(Hw) binding region is placed in the intergenic region.

One possible explanation for the observed hypertranscription is that assembly of a pre-initiation complex at these promoters requires a factor which is rate limiting for transcription. The pre-initiation complexes assembled at the *yp* promoters are likely to be extremely similar, if not identical, because these promoters are expressed in the same temporal and spatial patterns. If sequences required for assembly of the pre-initiation complex are altered, as may have occurred in p[*yp1*del], p[ZARU], p[ZAPU] and p[ZAΔUE], then the limiting factor may be freed, thereby facilitating transcription of the divergent promoter. This proposal requires that in p[ZAΔUE], assembly of the *yp1* pre-initiation complex occurs independently of the limiting factor. This may arise because the enhancers are placed in closer proximity to the *yp1* promoter. Furthermore, our results suggest that the limiting factor acts through the region deleted in p[ZAΔUE]. This factor cannot simply be a repressor of transcription, as the sequences between –90 and –200 are present in the p[*yp1*del] transposon which shows hypertranscription of the *yp2* fusion gene. The linkage between the two promoters may affect the overall rate of transcription because, in this configuration, competition may be due to the local concentration of binding sites. Thus, the introduction of an additional *yp1* promoter elsewhere in the genome would not necessarily result in a detectable decrease in *yp* gene expression.

Interdependence of transcription from the divergent *yp* promoters has been reported previously (Logan *et al.*, 1989). In this case, fat body expression of the *yp1* gene was severely inhibited in transgenes that contained the *yp1* promoter linked to an impaired *yp2* promoter. The *yp2* promoter was damaged by deletion of sequences downstream of –2 bp from the *yp2* transcription start site. When additional *yp2* DNA sequences (to +105 bp) were replaced, *yp1* expression was restored. These results were interpreted to indicate that a damaged promoter region causes a non-productive interaction between *yp2* promoter and the fat body enhancers, interfering with their ability to interact with the *yp1* promoter. While the underlying mechanism for this effect may be different from that responsible for hypertranscription, these two observations imply that transcription from one promoter has a substantial influence on the level of activity from the divergent promoter.

Multiple insulator regions exist in the Drosophila genome

Several regions of the *Drosophila* genome are proposed to function by establishing domain boundaries. The specialized chromatin structures, scs/scs′, surrounding the 87A7 *hsp70* heat shock loci, insulate transgenes from chromosomal position effects and block enhancer activity when inserted between a promoter and enhancer, both properties which are associated with boundary elements (Kellum and Schedl, 1991, 1992). In addition, two regions within the *bithorax* complex, Mcp and Fab-7, appear to act as domain boundaries (Karch *et al.*, 1994). Deletion of these regions from within the *bithorax* complex causes a gain-of-function phenotype due to inappropriate activation of the *Abd-B* gene by posterior control elements. Thus, the Mcp and Fab-7 regions appear to prevent regulatory elements from functioning inappropriately on the *Abd-B* promoter. None of these putative domain boundaries shares DNA sequence homology, suggesting that the genome contains a number of boundary regions which bind distinct proteins and have unique characteristics. Consistent with this idea is the observation that boundary sequences possess different properties. For example, the Mcp and Fab-7 regions cause pairing-dependent repression of *white* expression in transgenic flies (Vazquez *et al.*, 1993), whereas the scs/scs′ sequences and the su(Hw) binding region do not (Kellum and Schedl, 1991; Roseman *et al.*, 1993). The presence of many insulators with different functional capacities may impart flexibility for controlling the type of interactions that can occur between regulatory elements in a given region of the genome during the differentiation and development of an organism.

Materials and methods

DNA constructions

The su(Hw) binding region was inserted into the plasmid vector *yp* cassette (a gift of P.Wensink) to construct the various p[ZAR*X*] and p[ZAP*X*] transgenes. The *yp* cassette contains *yp* DNA starting from 37 bp downstream of the *yp1* promoter, through the 1.2 kb *yp* intergenic region, and terminating 105 bp downstream of the *yp2* start site of transcription. All of the enhancers required for wild-type expression of both *yp1* and *yp2* are present in this fragment. The su(Hw) binding region (R), containing 12 potential binding sites for su(Hw) protein, was isolated from the Rep 1 plasmid which contains gypsy sequences between nucleotides 647 and 1077 as numbered in Marlor *et al.* (1986). The su(Hw) binding region was blunt end-ligated into the *yp* cassette, which previously had been digested and blunt end-repaired at several unique restriction sites within the intergenic region, to create the *yp*–R–*X* cassettes. With respect to the *yp1* promoter, the unique restriction sites were located either downstream of both fat body enhancers (*Stu*I, –90) to form the ZARU cassette, between the two enhancers (*Bgl*II, –340) to form the ZARG cassette, or upstream of both enhancers (*Hin*dIII, –884; *Sac*I, –1046) to form the ZARH and ZARS cassettes, respectively. To control for effects of insertion of DNA sequences at particular sites in the *yp* intergenic region, a similarly sized fragment of pBR322 (3961 to 29; Watson, 1988) was inserted at either the *Stu*I or the *Hin*dIII sites to form the ZAPU and ZAPH cassettes, respectively. The p[ZAΔUE] cassette was made by digesting the *yp* cassette with *Stu*I and *Eco*0109, purification of the fragment containing the *yp* genes and religation. This plasmid carries a deletion of a portion of the *yp* intergenic region between –90 and –200 relative to the *yp1* transcription start site.

To test for effects of *yp1* transcription on the opposing promoter, a construct was made in which a portion of the *yp1* promoter was deleted. This construct, *yp1* deletion cassette, carries a 107 bp deletion which is centered around the promoter, and removes the TATA box and the start site of transcription. The *yp1* deletion cassette was made by replacing sequences of the *yp* cassette between *Xba*I (+40) and *Eco*0109 (–200) with a 149 bp PCR fragment made by amplification using as a 5′ primer sequences around –67 (–67 *Xba*I primer: 5′-CATCTAGACTTGCT TTTCGCTGCCTC-3′) and as a 3′ primer sequences around –216 (–216 *Eco*0109 primer: 5′-GCACCTGCTAAGTCATCAGTGGGGT-3′). The PCR-amplified DNA was sequenced, which verified that no base substitutions occurred during the PCR process. This DNA was digested with *Xba*I–*Eco*0109, and the fragment was inserted into the *Xba*I–*Eco*0109 *yp* cassette.

The *yp* intergenic region from each of the modified *yp* cassette plasmids was subcloned into the P element transformation vector pZAV1

(a gift of P.Wensink) to make the plasmids p[ZAR*X*]. In this way, the promoter region of *yp1* was placed upstream of the coding region for the *Drosophila Adh* gene. Similarly, the *yp2* promoter was placed upstream of the coding region for bacterial β-galactosidase. pZAV1 contains the xanthine dehydrogenase gene (*rosy*) as a transformation marker. Plasmid DNA isolation and DNA enzymology were carried out by standard procedures (Ausubel *et al.*, 1994).

Genetic manipulations

Flies were raised at 25°C, 70% humidity on standard corn meal/agar medium. The mutations and chromosomes used in this study are described in Lindsley and Zimm (1992). Germline transformation was carried out as described by Rubin and Spradling (1982). The host strain used in these experiments was *y ac; ry^{506}* which carries a deletion of a portion of the X chromosome containing the *yellow* and *achaete* loci and the ry^{506} mutation. DNA concentrations used in these experiments were 400 μg/ml of the ZAR*X* construct and 100 μg/ml of the 'wings clipped' helper plasmid pπ25.7 (Karess and Rubin, 1984). Transformants were recognized by the ry^+ phenotype and used to establish stocks. Additional independent insertion lines were obtained by mobilizing the transposon in one line with the *Sb* ry^{506} P(ry^+Δ2-3) (99B) chromosome (Robertson *et al.*, 1988). For each independent line, the number of insertions and the integrity of the transposon were determined by DNA Southern analysis. Only lines with single insertions were analyzed, and at least three lines per construct were obtained.

To test the effects of su(Hw) protein on *yp* gene expression, lines containing the p[ZAR*X*] transposons were crossed into a $su(Hw)^v$/$su(Hw)^f$ mutant background. This combination of *su(Hw)* alleles reverses the phenotypes associated with gypsy insertions and is female fertile. $su(Hw)^v$ is a deletion of the *su(Hw)* gene (Harrison *et al.*, 1993), whereas $su(Hw)^f$ is a point mutation in one of the Zn fingers which retains some ability to bind DNA (Harrison *et al.*, 1993). Males that were homozygous for one of the p[ZAR*X*] transposons were crossed to females of the stock $y^-ac^-\ w^{67}\ ct^6\ v^1\ f^1$; *2/CyO;*$bx^{34e}$ $su(Hw)^v$/*TM6*, $su(Hw)^f$, *Ubx*. The resulting male progeny that were $y^-ac^-\ w^{67}\ ct^6\ v^1\ f^1$; *p[ZARX]/CyO* and either $su(Hw)^v$ or $su(Hw)^f$/3 were backcrossed to females of the *su(Hw)* mutant stock. Straight-winged (CyO^+) females that were $su(Hw)^v$/$su(Hw)^f$ (based on the suppressed phenotypes of the X-linked $ct^6\ f^1$ gypsy-induced mutations) were selected. These females carried one copy of the p[ZAR*X*] transposon and were assayed directly for *yp2* activity.

ADH histochemical staining and spectrophotometric assay

Flies were grown in uncrowded conditions at 22°C, collected shortly after eclosion and aged for 2 days on food supplemented with yeast paste. Transgene expression was characterized in two ways. The tissue-specific expression was determined by histochemical staining of adult flies. More quantitative studies were done using spectrophotometric assays of either whole flies (Table I) or dissected carcasses (Figures 2, 3 and 4). Expression from the *yp1* promoter was determined using ADH spectrophotometric assays. For these studies, each transposon was crossed into an *Adh* null background. Transformants carrying the ZAR*X* P element on either the X or third chromosome were crossed to flies from an Adh^{fn6} *cn*; ry^{506} (FCR) strain (a gift from P.Wensink). Flies carrying the transgene (ry^+) and homozygous for the Adh^{fn6} *cn* chromosome were distinguished by their bright orange eye color.

For ADH histochemical analysis, flies were dissected in chilled TB1 buffer [15 mM KPO_4 (pH 7.0), 80 mM KCl, 16 mM NaCl, 5 mM $MgCl_2$, 1% polyethylene glycol], and fixed in 1% glutaraldehyde in Ringer's solution for exactly 5 min. Tissues were washed three times for 10 min in pre-chilled Ringer's solution. ADH staining was performed at room temperature in a container protected from the light. The reaction buffer [1.4 μl potassium phosphate (pH 7.5), 250 μl NBT (nitroblue tetrazolium, 5 mg/ml stock), 25 μl PMS (phenazine methosulfate, 2 mg/ml stock), 50 μl NAD (Sigma grade V, 50 mg/ml stock), and 50 μl 2-butanol] (Logan and Wensink, 1990) was added to the fixed tissue, and the samples were agitated slowly on a rotary shaker. Specific ovarian staining occurred in 10–15 min. Tissues were destained overnight in 70% ethanol, 5% acetic acid.

The spectrophotometric ADH assay was performed essentially as described by Logan and Wensink (1990). Flies were grown and aged as above, and carcasses were isolated by dissection in 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES (pH 6.9). The carcasses were frozen on dry ice in an Eppendorf tube and kept at –80°C for up to 4 weeks before use, without affecting enzymatic activity. Twenty carcasses were homogenized in 200 μl of TB1 to which 2 μl of PMSF (0.1 M PMSF in dimethylsulfoxide) had been added. The homogenates were centrifuged for 10 min in a microfuge tube at 4°C and the resulting supernatants were transferred to a new microfuge tube. The volume was adjusted to 250 μl with additional TB1. Depending on the activity of each construct, aliquots of the extract were added to a 96-well tissue culture dish, and the total volume adjusted to 120 μl with TB1. Less extract was used in assays of lines with high activity. The reaction was started by adding 180 μl of ADH activity buffer [11 μl NAD (50 mg/ml stock), 9 μl 2-butanol, in 160 μl TB1] to each well. Optical density was measured every 30 s for 5 min at 340 nm in a Titretek Multiscan MCC/340 microtiter plate reader. Activity was expressed as a change in OD/s/fly and normalized to the activity of control flies (p[CR2], provided by P.Wensink) which contained a single insertion of a transposon which carries the *yp1/2* reporter genes. Each extract was assayed in triplicate. Errors among these triplicate assays were <15%. In most cases, at least three independent lines corresponding to each transposon were assayed at least three times each.

β-Galactosidase histochemical staining and spectrophotometric assay

The tissue-specific expression pattern from *yp2* was determined using β-galactosidase histochemical staining. Flies were grown under conditions outlined above. Flies were dissected in 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES (pH 6.9). The tissues were stained overnight at room temperature in 10 mM $NaHPO_4$, pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_4Fe(CN)_4$, 0.2% (w/v) X-gal. Tissues were washed in 1× PBS (340 mM NaCl, 10 mM KCl, 3.6 mM KH_2PO_4, 5.5 mM Na_2HPO_4).

The spectrophotometric assay of β-galactosidase activity was performed essentially as described by Simon and Lis (1987). Flies dissected in 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES (pH 6.9) were frozen on dry ice in an Eppendorf tube and kept at –80°C for up to 4 weeks. Twenty carcasses were homogenized in 200 μl of assay buffer (50 mM KPO_4, pH 7.5, 1 mM $MgCl_2$ containing 2 μl 100 mM PMSF). Homogenates were centrifuged for 10 min in a microfuge at 4°C and the resulting supernatants were transferred to a new microfuge tube. The volume was brought to 250 μl with additional assay buffer. Depending on the activity of each construct, aliquots of this 250 μl extract were added to a 96-well tissue culture dish, and the final volume adjusted to 120 μl with additional assay buffer. The reaction was started by adding 180 μl of a 1 mg/ml solution of the substrate, chlorophenol red-β-D-galactopyranoside (CPRG), in assay buffer. Optical density was taken every 15 min for 1 h at 595 nm in a Titretek Multiscan MCC/340 microtiter plate reader. Activity was expressed as the change in OD/min/fly and normalized to the control, p[CR2]. Each homogenate was assayed in triplicate. Errors among these triplicate assays were <15%. With the exception of the single lines mutant for the su(Hw) protein, three independent lines of each construct were assayed. These assays were done at least three separate times.

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