A leucine zipper domain of the *suppressor of Hairy-wing* protein mediates its repressive effect on enhancer function

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The *suppressor of Hairy-wing* [*su(Hw)*] protein mediates the mutagenic effect of the gypsy retrotransposon by repressing the function of transcriptional enhancers controlling the expression of the mutant gene. A structural and functional analysis of *su(Hw)* was carried out to identify domains of the protein responsible for its negative effect on enhancer action. Sequence comparison among the *su(Hw)* proteins from three different species allows the identification of evolutionarily conserved domains with possible functional significance. An acidic domain located in the carboxy-terminal end of the *Drosophila melanogaster* protein is not present in *su(Hw)* from other species, suggesting a nonessential role for this part of the protein. A second acidic domain located in the amino-terminal region of *su(Hw)* is present in all species analyzed. This domain is dispensable in the *D. melanogaster* protein when the carboxy-terminal acidic domain is present, but the protein is nonfunctional when both regions are simultaneously deleted. Mutations in the zinc fingers result in *su(Hw)* protein unable to interact with DNA in vivo, indicating a functional role for this region of the protein in DNA binding. Finally, a region of *su(Hw)* homologous to the leucine zipper motif is necessary for the negative effect of this protein on enhancer function, suggesting that *su(Hw)* might exert this effect by interacting, directly or indirectly, with transcription factors bound to these enhancers.

[Key Words: Leucine zipper domain; *su(Hw)*; transcriptional enhancer function]

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Insertion of the gypsy retrotransposon into various *Drosophila* genes results in mutations with phenotypes that can be reversed by second site mutations in the *suppressor of Hairy-wing* [*su(Hw)*] gene (Modolell et al. 1983). This finding suggests a direct involvement of the *su(Hw)* protein in the generation of mutant phenotypes by gypsy, because the lack of a functional *su(Hw)* protein results in a reversion of the gypsy-induced phenotype. *su(Hw)* is a zinc finger protein that binds to a specific sequence, similar to the octamer motif, located in the 5'-transcribed untranslated region of gypsy (Spana et al. 1988; Dorsett 1990; Spana and Corces 1990). The necessary and sufficient requirement of *su(Hw)* protein for gypsy mutagenesis has been demonstrated in the case of *hsp70*, *yellow*, and *cut* alleles induced by this retrotransposon (Holdridge and Dorsett 1991; Jack et al. 1991; Geyer and Corces 1992; Smith and Corces 1992). The temporal and spatial expression of the two latter genes is

controlled by tissue-specific transcriptional enhancers located in the intron and/or the 5' region of the respective locus (Geyer and Corces 1987; Liu et al. 1991). In both cases, insertion of the gypsy element interferes with the expression of the gene in those tissues regulated by enhancers located distally from the gypsy insertion site with respect to the promoter (Jack et al. 1991; Geyer and Corces 1992). In the case of *yellow*, the phenotypic effect of gypsy can be reproduced when the *su(Hw)*-binding sequences are present in the original gypsy insertion site, suggesting that the *su(Hw)* protein alone is responsible for the induction of the mutant phenotype (Spana and Corces 1990; Geyer and Corces 1992). This negative effect of *su(Hw)* on transcription is not enhancer specific, because insertion of the *su(Hw)*-binding site in different regions of the *yellow* gene is able to inhibit the function of any enhancer located distally from the *su(Hw)*-binding region with respect to the *yellow* promoter (Geyer and Corces 1992). Furthermore, the presence of the *su(Hw)*-binding region flanking the *white* gene can buffer *white* expression from position effects of adjacent sequences independent of the location of the *white* gene in the genome (Roseman et al. 1993). The

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su(Hw) protein can also interfere with regulatory sequences located in genes for which gypsy-induced mutations have not been found. For example, the insertion of the *su(Hw)*-binding region in the 5' end of the *hsp70* gene, between the heat shock element and the promoter, interferes with proper heat shock induction of transcription (Holdridge and Dorsett 1991).

These results indicate that the *su(Hw)*-mediated mutagenic effect of gypsy is attributable to an inhibitory effect of the *su(Hw)* protein on enhancer elements that control the expression of the mutated gene. The lack of specificity in the nature of the affected enhancers and the directionality of the inhibitory effect suggest a few alternatives to explain the mechanism underlying this phenomenon. One possibility is that binding of the *su(Hw)* protein causes changes in the conformation of the adjacent chromatin that spread distally with respect to the promoter and interfere with the binding of transcription factors to distal enhancers. The requirement for a directional (away from the promoter) spreading of chromatin changes can be explained if binding of *su(Hw)* to DNA creates boundaries between higher order domains of gene activity (Roseman et al. 1993). A second possibility is that the inhibitory effect of *su(Hw)* on enhancer function is attributable to the interaction of this protein with enhancer-bound transcription factors as they try to reach the transcription complex by tracking along the DNA or looping the intervening sequences (Geyer and Corces 1992). This model explains the directionality of the *su(Hw)* effect better than that based on chromatin changes, but the direct contact of *su(Hw)* with transcription factors might require a degree of specificity in the interaction that is at odds with the apparent universal effect of *su(Hw)* on all enhancers tested.

To further the understanding of the mechanism by which *su(Hw)* affects enhancer function, we have sought to determine whether specific domains of *su(Hw)* are involved in the negative effect of this protein on tissue-specific transcription. Here, we report a functional study of the *su(Hw)* protein carried out by analysis of extant *su(Hw)* mutants, as well as new mutations induced in vitro and introduced into the fly by P element-mediated transformation. These studies suggest that an extended amphipathic α -helix of the *su(Hw)* protein with structural characteristics similar to the leucine zipper motif might be responsible for the inhibitory effect of *su(Hw)* on enhancer-promoter interactions, whereas two acidic domains located in the amino- and carboxy-terminal ends of the *su(Hw)* protein might play auxiliary functions in this process.

Results

Mutations in su(Hw) only affect oogenesis

To gain insights into the normal role of the *su(Hw)* protein and the mechanism by which *su(Hw)* mediates the mutant effect of gypsy, we have analyzed in detail the phenotypic consequences of the lack of the *su(Hw)* gene

product. The only phenotypic effect of null mutations in the *su(Hw)* gene is female sterility. Females carrying the *su(Hw)^V* null allele (Harrison et al. 1992) are unable to lay eggs because the egg chambers degenerate before completion of oogenesis. Egg chambers from *su(Hw)^V* females appear less regular in shape and spaced more closely on the ovarioles than wild-type chambers (data not shown). In stages 7, 8, and 9, nurse cells of *su(Hw)* mutants can often be seen to shrink away from one another, leaving gaps in the anterior portions of the egg chambers. In addition, wild-type oocytes begin accumulating yolk at stage 8. The stage 8 and 9 egg chambers of *su(Hw)* mutant females show a considerable reduction in deposition of yolk in the oocyte. Egg chambers of the mutant cease to grow and eventually degenerate before stage 10. DAPI staining shows that wild-type ovaries contain nurse cells with homogeneously staining chromosomes within the nucleus after very early stages of oogenesis (King et al. 1956). The DNA appears to be decondensed and spread throughout the nucleus (Fig. 1A). In *su(Hw)* mutant nurse cells, the chromosomes look normal until stage 3 or 4, but afterward become aggregated and condensed (Fig. 1B). The bulbous nature of the chromosomes from *su(Hw)* mutants is reminiscent of that seen fleetingly in normal egg chambers only at stage 4. It seems that *su(Hw)^V* nurse cell chromosomes generally retain the bulbous morphology throughout later stages of oogenesis, suggesting that the *su(Hw)* gene product might be required for decondensation of the nurse cell chromosomes. As *su(Hw)* is a DNA-binding protein capable of interacting with many sites throughout the genome (Spana et al. 1988), it could be hypothesized that the binding of *su(Hw)* directly to these bulbous chromosomes is responsible for decondensation. The chromosomes of follicle cells appear to be unaffected in the mutant.

An additional defect seen in some of the *su(Hw)* alleles is that many egg chambers contain more than the normal 15 nurse cells. This defect is likely to be the result of the fusion of egg chambers rather than the overproliferation of nurse cells, because most of the aberrant chambers contain ~30 nurse cells, twice the normal number. In addition, the anterior nurse cells are often smaller than the posterior, as might be expected because the younger chambers are located more anteriorly in the ovariole. Perhaps the strongest evidence is the presence of some chambers that appear to be in the process of fusing. These chambers are pushed together, and material from degenerating follicle cells can be seen between the cysts (Fig. 1C). These fused chambers continue to develop until stage 9 of oogenesis, arrest, and eventually degenerate similar to nonfused *su(Hw)* egg chambers. These results suggest that the *su(Hw)* protein is not essential in males and is only required in females during oogenesis.

su(Hw) is a ubiquitous nuclear protein

To understand the apparent discrepancy between *su(Hw)* RNA expression in all tissues of the fly and the restric-

Figure 1. Phenotype of *su(Hw)* mutations and expression of the *su(Hw)* protein. The three top panels show ovarioles teased apart from formaldehyde-fixed ovaries and stained with DAPI. In the wild-type sample (A), the third egg chamber from the left contains nurse cell nuclei with a bulbous chromosome morphology, thus identifying it as a stage 4 egg chamber. Later during oogenesis, this bulbous chromosome morphology disappears and nurse cell nuclei show homogeneous staining. Ovarioles of *su(Hw)*^V females (B) show the irregular bulbous structure of nurse cell chromatin visible at all stages of oogenesis beyond stage 3. The *su(Hw)*^V chromosome used in these studies carries a P-element construct containing the RNA polymerase II 15-kD subunit (*RpII15*) gene to rescue the lethality associated with this strain (Harrison et al. 1992). (C) DAPI staining of fusing egg chambers from *su(Hw)*^V females. The three bottom panels show 6- μ m frozen sections of several tissues from larvae, pupae, and adults fixed in formaldehyde, incubated with affinity-purified anti-*su(Hw)* antibodies followed by rhodamine-conjugated secondary antibody, and viewed by epifluorescence. (D) A section through an adult female abdomen with staining in the nurse cell and follicle cell nuclei of the ovaries; (E) a section through an adult thorax showing nuclear localization of *su(Hw)* in the proventriculus, a gut structure; (F) an adult thorax also showing localization of *su(Hw)* protein in the nuclei of muscle cells.

tive mutant phenotype, and to test whether the enhancer-specific effects of *su(Hw)* on the expression of the *yellow* gene are attributable to a restriction in the spatial expression of this protein, we have determined the tissue distribution of *su(Hw)* protein during *Drosophila* development. Frozen sections through adults, pupae, and larvae were immunoreacted with anti-*su(Hw)* antibodies (Spana et al. 1988), incubated with a rhodamine-conjugated secondary antibody, and viewed under epifluorescence (Fig. 1). *su(Hw)* protein was detected in the nuclei of cells from all stages of development; therefore, *su(Hw)* is a ubiquitous nuclear protein. Also, staining was detected in males as well as females, contrary to what

might be expected from the ovary-specific phenotype of *su(Hw)* mutations. Sections of flies from the *su(Hw)*^V null mutation immunoreacted with these antibodies failed to stain above background levels (data not shown), indicating that the antibodies recognize only the *su(Hw)* protein in situ.

These results indicate that *su(Hw)* protein is present in the nuclei of all *Drosophila* cells. The role of this protein in tissues other than the nurse cells is dispensable or the function of *su(Hw)* can be replaced by other proteins with similar properties. These results also clarify the role of the *su(Hw)* protein in mediating the mutagenic effect of gypsy on the *yellow* gene. The specificity of the inhibitory effect of *su(Hw)* on enhancers located distal to the *su(Hw)*-binding site with respect to the promoter could not be attributable to the presence of the protein in the tissues where *yellow* expression is controlled by these enhancers, whereas unaffected tissues lack *su(Hw)* protein. Rather, the specificity must depend on the particular mechanism underlying the inhibitory effect. To further understand the molecular basis of this effect we have determined the nature of putative conserved structural domains in the *su(Hw)* protein that could have functional significance in its interaction with enhancers in the *yellow* gene.

Structural conservation of the *su(Hw)* protein in different *Drosophila* species

As a first step in determining the nature of structural domains of functional importance for the role of *su(Hw)*, both during oogenesis and in the generation of gypsy-induced phenotypes, we have determined the amino acid sequence of this protein in several *Drosophila* species. The *su(Hw)* gene was cloned from *Drosophila ananassae* and *D. virilis* by cross-hybridization with the gene from *D. melanogaster*. Figure 2 shows the deduced amino acid sequence for all three proteins.

The amino-terminal region of the protein, including the first 218 amino acids, is poorly conserved, with only 20% identity and 46% similarity. This region of the protein contains a highly acidic domain located between residues 154 and 202 in *D. melanogaster*. In spite of the low overall sequence conservation in this domain, the acidic characteristic is well preserved: 23 of 48 amino acid residues are aspartic acid or glutamic acid in *D. melanogaster*, as well as 23 of 49 in *D. ananassae* and 25 of 49 in *D. virilis*. This high level of conservation in the acidic character of this domain suggests an important role for this region in the function of *su(Hw)*. The central region of *su(Hw)*, located between residues 219 and 623, has been more conserved during evolution than the amino-terminal region. Comparison of this region among all three species indicates 80% amino acid identity and 95% similarity when chemically similar amino acids are taken into account (Fig. 2). This region contains 12 copies of the zinc finger motif, which are presumably involved in the interaction of *su(Hw)* with gypsy DNA. The high degree of conservation not only extends to the zinc fingers but also to some of the interfinger regions


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Dm MSASKEG-KEKKG-----KLLGVENISPPKDKRPATRMKLLNDVGAGEDSEASTTTTTSRLPPTSRCKRG 63
Da MSAAKEG-KEKKPVATVVKIVEVESVSPSKDKRP-TRMKLLNNLSAS-----SPQTYPPKTD-KRS 58
Dv MSAQEDALPATPPASSSIKISDGGK---PKEKRTGTRMKLLNDVAAKAAVASKGASVSPRLKPE---KRT 64
Dm SVAGSRRIKILNEEILGTPKTEKRGATKSTAPAA---STVKILNEKKTPSATVTVAVETTKIKTSPSKRKKM 131
Da GIKGSRRIKILNEDVLVAPKVEKRRSTKPTSPGATATSTVKILNEKRIVPAEETALETTIKIKISPPKRNKM 128
Dv S-----IKILNNNNDEAQTSTKGGDSVPRPKPPAPASR---YTRSSAPASSAVETAKIKTSPSKKKKM 126
Dm EHYVLQAVKSENTKA--DTTVTVVTEEDDTIDFILADDEEVVPGRIENNNNGQEIIVVTE--DDEDLGEDG-D 197
Da DHYVLQPVVKNNSASQATRGATGEDEDTIAFILGDDDEVVPASTSRNGQEIIVTE--EEEEEEEDLD 197
Dv DHYVLQAKSENNAE--NTTSVVVVEDEDTIDFILADDE--VELGAGAKENGEEFVVSVDDEDDDDDDDE 194
Dm EDGEDSSGKGNSSQTK-IKEIVEHVCCKCYKTFRRVQSLKKHLEFCRYDSGYRLKADMLKNLEKIEKDA 266
Da EGDSPGGSNKNKSGHKAKEIVEHVCCKCYKTFRRVQSLKKHLEFCRYDSGYHLKAECKWNLEKIEKDG 267
Dv DEGVVVEGAKRRSGNNELKEMVEHVCCKCYKTFRRVQSLKKHLEFCRYDSGYHLKADMLKNLEKIEKDA 264
Dm VVMEKKDIFCCSESYDTFHLGHINCPDCPKSFKTQTSYERHIFITHS-EFSDPFCISICNANLRSEALLA 335
Da DVLEKKDIFCCSESYDTFHLGHINCPDCPKSFKTQTSYERHIFITHS-EFSDPFCISICNANLRSEALLK 336
Dv VVMEKKDISFCCSESYDTFHLGHINCPDCPKSFKTQTSYERHIFITHSWSCNDYPCISICNANLRSGALLK 334
Dm LHEEQHKSRSKSYACKICGKDFTRSYHLKRHQKYSSCSNNTDMSCKVCDRVFYRLDNLRLSHLQHLGT 405
Da LHEEQHKSRSKPYACKICGKDFTRSYHLKRHQKYSSCSANENDTMSCKVCDRVFYRLDNLRLSHLKHHLGT 406
Dv LHEQOHLRLGKPFACKICGKDFMCSYHLKCHQKYSSCSANENDTMSCKVCDRVFYRLDNLCAHLQHLGT 404
Dm QVVKKPEYMCHTCKNCFYSLPTLNIHIRTHTGKPFDCDLCKKFSALVALKKHRRYHTGKPYSTVCN 475
Da QVVKKPEYMCCHVCNCFYSLPTLNIHIRTHTGKPFDCDLCKKLSALVALKKHRRYHTGKPYSTVCN 476
Dv QVVKKPEYMCCHVCNCFYSLPTLNIHIRTHTGKPFDCDLCKKFSALVALKKHRRYHTGKPYTCTVCS 474
Dm QAFVAVKVLNRHMKRHTGERPHKCECGKSFIRATQLRTHSKTHIRFPFCEQCEKFKTEKQLERHVKTH 545
Da QAFVAVKVLNRHMKRHTGERPHKCECGKSFIRATQLRTHSKTHIRFPFACDMCEKFKTEKQLERHVKEH 546
Dv QSFVAVKVLNRHMKRHTGERPHKCECGKSFIRATQLRTHSKTHIRFPFACDMCEKFKTEKQLERHVVDH 544
Dm SRTKRPVFSACECKRNFRTPALLKEHMDGKHSKP---QQRSSMRSAVKIMERTDCAICDNFDSSETLR 612
Da SRQKRPVFSACECKRHFRNTAQLRQHMDAGDESEKSGEKPQAKRSSTKVLERTDCAICDNFDSSETLR 616
Dv TRQKRASFACTECTRSFRSALLKEHLDAGDHPVKSTRAKR---SAKMIERTDCAICDNFDTTETLR 610
Dm RHIRTVEHCDPDDIFGVPEPHPSKRAK-----KDI--ESEVVPVALNTSAGSLISSQTDGNDVVVREF 673
Da KHIRSVHECDPDDIFGIQPPSAKRAKINKIPVDEE---EEEEVPVALNASSGSLISSQTDGNDVVVREF 684
Dv NHIRSVHECDPDDIFGTPEPAKRAKKTVAVAEAEQEQEDDVP-ARNTSAGSLISSQTDGNDVVVREF 679
Dm LVDEGDGAAQTITLENETYITLPLDGAIEGEQLTDEAGVKPEAKKEEAQVSPVVKKEQKSLAASLAAAI 743
Da LVDEGDGTAQTITLENETYITLPLDGEIAAEQITDE-GVKVEGQKTPPKSPVVKKEKRSKSLAASLAAAI 753
Dv LVDEGDGNAQTITDLRKLRLHPLLEGDKATES-TAETDIKAESSEKPSVSPVVKKEQKSLAASLAAAI 748
Dm ADNLEESCSDDPDSGGLTEEDILKKNVGHIDMLVDPPILKKYGNWPAEETVLCVKVINCGBDLTKG 813
Da ADNIEVPPVEEDFEGKVLTEEDILKKNVGHIDMLVDPPILKKYGNWPAEEMVLCKVINCGBDLTKG 823
Dv ADNLEPSSDDPDSGGLTEEDILKKNVGHIDMLVDPPILKKYGNWPAEETVLCVKVINCGBDLTKG 818
Dm GENYAEILDYGRMRREYCKLLFTTVIHNDISIKSLLNFPIDDVIEYVLGDQDQDQDQDQDQDQDQDQDQD 883
Da AENYAEILDYGRMRREYCKLLFTTVIHNDISIKSLLNFPIDDVIEYVLGDQDQDQDQDQDQDQDQDQDQ 886
Dv SEAYAEILDYGRMPIIL-QLLFTTVIHNDISIKSLLNFPIDDVIEYVLGDQDQDQDQDQDQDQDQDQDQ 872
Dm EEAHSVGTGETKTNEIREKPEKKEVSASEKKEIVGKAVDKDNSEEVVRENKKKPVGEQKA 944
Da ----- 944
Dv TDTREDAVESEA----- 899

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Figure 2. Sequence comparison of the *su(Hw)* protein in different *Drosophila* species. The amino acid sequence of the *su(Hw)* protein from *D. melanogaster* (Dm), *D. ananassae* (Da), and *D. virilis* (Dv) is shown. Positions with identical amino acids are denoted by an asterisk (*), whereas chemically conserved residues are underlined. The location and extent of each of the 12 zinc fingers is indicated above the amino acid sequence by thick lines. The acidic domains located in the amino- and carboxy-terminal regions of the protein are boxed. Also boxed and shaded is the region containing the leucine zipper region of the *su(Hw)* protein. The extent of the residues deleted in the *su(Hw)^{Δ100}*, *su(Hw)^{Δ7}*, *su(Hw)^{Δ283}*, and *su(Hw)^Δ* mutations is indicated by arrows above the sequence.

(compare the amino acid sequence between fingers 1 and 2). Fingers 6, 7, 8, and 9 show the highest conservation among the three species considered; in this regard, it is interesting to note that mutations found in the zinc finger region that have a strong effect on *su(Hw)* function have been mapped to the seventh finger (see below). Following the zinc finger repeats, between amino acids 624 and 861, the *su(Hw)* protein contains blocks of highly conserved sequences interspersed with regions showing very poor homology (Fig. 2). One of these conserved blocks shows striking similarity to the helix 2-coiled-coil region of basic helix-loop-helix-zipper (bHLH-Zip) proteins (Vinson and Garcia 1992), suggesting that this region of *su(Hw)* might be involved in mediating interactions with itself or other proteins (Fig. 3A). Finally, the carboxy-terminal region of *su(Hw)*, between residues 862 and 944, is quite acidic with 33% aspartic acid or glutamic acid residues. This domain is very short in the protein encoded in *D. virilis* and almost nonexistent in

D. ananassae, suggesting that it might not play an important role in *su(Hw)* function.

Analysis of mutations that affect the structure of the *su(Hw)* protein

A number of *su(Hw)* mutations were recovered by ethylmethane sulfonate (EMS) mutagenesis in a scheme described in Parkhurst et al. (1988). To determine which mutations could be used in a functional analysis of the *su(Hw)* protein, a transcriptional study of these and other known *su(Hw)* mutants was carried out. Northern analysis showed that several of the mutations tested are capable of accumulating *su(Hw)* mRNA in size and amounts comparable with wild type (Harrison 1991). This indicates that these alleles are not affected at the level of transcript accumulation and are suitable candidates for further characterization. The production of

Figure 3. Comparison of *su(Hw)* with bHLH-Zip proteins. (A) Amino acid sequence of *su(Hw)* and several proteins known to contain a functional bHLH-Zip domain. Conserved amino acids are boxed. Sequences are taken from Vinson and Garcia (1992). The position of each residue in the helix is indicated at the top of the protein sequences. (B) Diagram of a generic zinc finger. Amino acids are indicated by solid circles. The locations of the cysteine and histidine residues that interact with the Zn^{2+} ion are indicated. Also shown are specific changes responsible for the mutant phenotype in various *su(Hw)* alleles.

su(Hw) protein in these mutant lines was investigated by Western analysis of samples from adult flies of the genotype *su(Hw)/Df(3R)red-P52*. Seven of the *su(Hw)* alleles tested accumulate protein as adults in amounts comparable with wild type (Fig. 4). Of these seven, *su(Hw)^{e2}*, *su(Hw)^f*, and *su(Hw)^{E8}* produce proteins indistinguishable in size from the wild type, whereas *su(Hw)^f* and *su(Hw)^{e7}* produce smaller proteins. The *su(Hw)^{f3}* mutation does not accumulate levels of *su(Hw)* protein detectable in the Western blot shown in Figure 4, although longer exposure of the film allows the visualization of low amounts of a protein of wild type size (Harrison 1991). This mutation is caused by the insertion of the jockey transposable element into the first intron of the *su(Hw)* gene (Fig. 5). This insertion results in very low levels of *su(Hw)* RNA (Parkhurst et al. 1988) owing

to premature termination of transcription in the polyadenylation site located in the jockey element (Harrison 1991). The *su(Hw)^{f3}* mutation is fertile and suppresses the *y²* phenotype weakly (see below), suggesting that very low levels of *su(Hw)* protein can still affect the function of transcriptional enhancers in the *yellow* gene. This mutant has been included in the Western analysis to establish a correlation between levels of wild-type protein and the severity of the *su(Hw)* phenotype.

Genomic DNA was isolated from each of the mutants and subjected to PCR using three pairs of nested primers covering the entire transcribed region of the *su(Hw)* locus. The PCR products were cloned into pUC18, and several clones were sequenced to confirm the lesion in these *su(Hw)* mutants. Results of this analysis are described below and are summarized in Figure 5. Also summarized in Figure 5 is the structure of several *su(Hw)* alleles analyzed but not used in these studies.

Figure 4. Protein analysis of *su(Hw)* mutants. Protein was isolated from six adult flies, three of each sex, electrophoresed in a 7.5% polyacrylamide-SDS gel, and electroblotted to a nitrocellulose membrane. Affinity-purified polyclonal antibodies directed to a *su(Hw)*-*trpE* fusion protein were used to probe the filter. Detection of the primary antibody was carried out with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The name of each strain analyzed is indicated at top. The positions of two molecular mass markers are indicated at right.

Mutations in the zinc fingers affect the binding of *su(Hw)* to gypsy DNA

Two of the mutations that accumulate protein of normal size and abundance have been identified as amino acid substitutions in the seventh zinc finger motif. *su(Hw)^{e2}* is a G → A transition at nucleotide 2116 [see Parkhurst et al. (1988) for a description of nucleotide numbering in the *su(Hw)* gene], resulting in the replacement of an arginine by a histidine residue. This change occurs in the region of the zinc finger base, between the two histidine residues that coordinate the zinc ion (Fig. 3B). The *su(Hw)^{E8}* allele is a C → T transition at base 2109 (Parkhurst et al. 1988), resulting in the replacement of a histidine with a tyrosine residue. The lost histidine would have been one of the amino acids that coordinates zinc in the finger (Fig. 3B). These two amino acid replacements occur only two residues apart, yet the phenotypes of these mutants are extremely different (Fig. 6). *su(Hw)^{E8}* ranks among the most severe of *su(Hw)* alleles, with strong suppression and complete female sterility. Presumably, the mutant protein is rendered incapable of binding its normal recognition site. The loss of one of the

Figure 5. Schematic map of *su(Hw)* mutations. The structure of the *su(Hw)* gene and the lesions in each of the *su(Hw)* mutations described in the text are presented diagrammatically. The shaded boxed regions on the transcript represent the sequences encoding the acidic domains; the solid spikes represent zinc finger domains; the solid circle represents the leucine zipper region. The arrow lines, above and below the representation of the *su(Hw)* transcript, delineate portions of the encoded protein that are lacking in the particular mutations. Single nucleotide substitutions resulting in missense mutations or the splice junction alteration are also indicated. Triangles labeled jockey represent insertions of foreign sequences into the *su(Hw)* gene.

ligand-binding residues has been shown to be deleterious to the function of zinc finger proteins (Blumberg et al. 1987; Redemann et al. 1988). Inability of the seventh finger to fully participate in the interaction with the recognition site may destabilize binding of the entire protein, suggesting that this finger plays a central role in the interaction of *su(Hw)* with gypsy DNA. Evidence to support this conclusion is presented below. On the other hand, *su(Hw)^{e2}* is among the mildest of the known mutations in the locus. Flies are suppressed only partially for the scored gypsy-induced mutations, and the females show no detectable reduction in fertility, suggesting that the lesion in the *su(Hw)^{e2}* mutation causes only a subtle alteration in protein function. A third mutation studied that affects the zinc finger region is *su(Hw)^f*. Sequence analysis shows that this mutation is caused by a nucleotide change from G to A at position 2377. This results in a change of a cysteine for a tyrosine residue in the tenth finger of the *su(Hw)* protein (Fig. 3B). The cysteine residue is involved in coordination with the zinc ion; therefore, the mutation should result in a change in the conformation of the finger that would severely affect its ability to interact with DNA. Nevertheless, the *su(Hw)^f* mutation does not affect female fertility and suppresses the *y²* phenotype only partially (Fig. 6), suggesting that the tenth finger does not play an essential role in the interaction between *su(Hw)* protein and gypsy DNA.

To test whether mutations in the zinc fingers affect the binding of *su(Hw)* to DNA, we have used the immu-

nolocalization of *su(Hw)* to polytene chromosomes as an assay for the ability of the mutant protein to bind DNA in vivo. When larval salivary glands are prepared from animals of the genotype *su(Hw)^{E8}/su(Hw)^V* [the latter produces no *su(Hw)* protein; see Fig. 4], no binding to the chromosomes is detected (Fig. 7). Whole-mount preparations of the mutant and wild-type salivary glands indicate that *su(Hw)* protein is present within the nucleus of both genotypes (data not shown); thus, the lack of binding is not attributable to underproduction or improper localization of the protein. A similar analysis was carried out with the *su(Hw)^{e2}* and *su(Hw)^f* mutations, which are the result of alterations in the zinc finger region that result in only weak mutant phenotypes. In agreement with this observation, immunolocalization of *su(Hw)* protein can be detected on polytene chromosomes from these two strains (Fig. 7). These two mutations show approximately wild-type levels of *su(Hw)* protein on Western blots (Fig. 4). The intensity of antibody reaction on polytene chromosomes is close to normal in *su(Hw)^{e2}* (Fig. 7), suggesting that the ability of the protein to interact with DNA is not affected severely. On the contrary, the intensity of antibody staining in *su(Hw)^f* polytene chromosomes is lower compared with that of wild type (Fig. 7), suggesting that although the *su(Hw)^f* protein can still bind to DNA, its affinity is reduced. The difference in binding between *su(Hw)^{e2}* and *su(Hw)^f* correlates with the respective abilities of these mutations to suppress the *y²* phenotype, as the *su(Hw)^f* allele reverses

Figure 6. Phenotypic effect of alterations in the structure of the *su(Hw)* protein. Shown are light micrographs of the abdomens of 3-day-old males from wild-type (CS), y^2 , and combinations of y^2 with different *su(Hw)* mutations.

the coloration of the wing and body cuticle to a greater extent than *su(Hw)*^{e2} (Fig. 6).

Deletion of the carboxy-terminal region affects su(Hw) function

Flies carrying the *su(Hw)*^l or *su(Hw)*^{e7} mutations produce *su(Hw)* protein smaller than the expected size (Fig. 4). The precise location of each mutation was determined by cloning and sequencing these alleles. Both contain nonsense mutations resulting in the premature termination of the protein product (Fig. 2). These mutants are able to accumulate protein in significant amounts; *su(Hw)*^l produces higher than normal amounts, and *su(Hw)*^{e7} produces about twofold less protein than wild type (Fig. 4). The mutation in *su(Hw)*^l is a G → T transversion at nucleotide 3318 (Parkhurst et al. 1988) and results in the loss of the terminal 149 amino acids of the protein (Figs. 2 and 5). *su(Hw)*^{e7} is a C → T transition at base 3096 and lacks the last 223 amino acids (Figs. 2 and 5). These two mutations are female fertile but differ in their phenotypic effect on y^2 . The degree of female fer-

tility of these and other *su(Hw)* alleles was quantitated by measuring the number of eggs laid by 100 females over a period of 4 days and comparing this number to the amount of eggs laid by the *bx*^{34e} parental stock. The *su(Hw)*^l mutation has no effect on fertility, as females carrying this mutation lay the same number of eggs as the parental strain. The fertility of *su(Hw)*^{e7} could not be determined precisely, as this allele is homozygous lethal, probably because of the presence of other mutations in the same chromosome. Nevertheless, females of the genotype *su(Hw)*^{e7}/*su(Hw)*^l have clearly reduced fertility with respect to wild type. The difference in the effect of these two mutations on female fertility correlates with their effect on the phenotype of the y^2 mutation. *su(Hw)*^l is a mild mutation only capable of weakly suppressing gypsy-induced mutations (Fig. 6), whereas *su(Hw)*^{e7}/*su(Hw)*^l shows a stronger phenotype (data not shown). Deletion of carboxy-terminal *su(Hw)* sequences in either of these two mutants does not interfere with the binding of the altered protein to polytene chromosomes (data not shown, but identical to *su(Hw)*^{Δ100} in Fig. 7).

From the lesion in *su(Hw)*^l it is apparent that there are

Figure 7. Localization of mutant *su(Hw)* proteins on polytene chromosomes. Salivary glands were dissected from third-instar larvae, carrying the *su(Hw)* mutations indicated in each panel, and fixed. Samples were then incubated with *su(Hw)* affinity-purified polyclonal antibodies, followed by rhodamine-conjugated secondary antibody, and detection by epifluorescence. In each panel, the photograph at left was taken under phase contrast; the photograph at right shows the same field under fluorescence.

elements in the protein within the last 149 amino acids that are necessary for complete function of the protein, but these are not vital for critical aspects of *su(Hw)* function. The most striking structural feature within this region is a charged acidic domain, which is 33% aspartic acid or glutamic acid (Fig. 2). The *su(Hw)* proteins from *D. ananassae* and *D. virilis* lack most of this domain, suggesting that it might not play an essential role in the function of *su(Hw)*. The *su(Hw)^f* mutation is not strong, perhaps indicating that the charged region plays some auxiliary role in the activity of *su(Hw)*. The acidic domain in the amino end of the protein may be able to partially compensate for the loss of the charged region at the carboxyl terminus. An alternative and more likely possibility is that the subtle alteration in *su(Hw)* activity detected in *su(Hw)^f* is attributable to the lack of amino

acid residues 794–860, as this region of the protein is highly conserved among the *Drosophila* species analyzed (Fig. 2).

An additional 84 amino acids are lost in *su(Hw)^{e7}* beyond those deleted in *su(Hw)^f*. Whereas the amount of detectable protein is somewhat reduced in *su(Hw)^{e7}* mutants (Fig. 4), protein levels are not likely to be the cause of the phenotype. In the very weak *su(Hw)^{f3}* allele, there is much less protein than that seen in *su(Hw)^{e7}* (Fig. 4). Despite greater levels of protein in *su(Hw)^{e7}*, it has a stronger mutant phenotype than *su(Hw)^{f3}*. This would support the idea that a qualitative change in the protein is responsible for the severe phenotype associated with the *su(Hw)^{e7}* mutation. Within the extra 84 deleted amino acids between *su(Hw)^{e7}* and *su(Hw)^f* is an extended amphipathic α -helical region that shows homol-

ogy to the helix 2-coiled-coil domain of bHLH–Zip proteins (Fig. 3A), suggesting that the phenotype of the *su(Hw)^{e7}* allele is derived from a loss of this domain. To discriminate between these two possibilities, we have analyzed the effect of site-directed deletions and single amino acid substitutions in the extended leucine zipper region on the ability of the *su(Hw)* protein to reverse the mutant phenotype of *y²*.

Deletion of the amino-terminal acidic domain does not affect the mutagenic effect of su(Hw)

To further study the domains of the *su(Hw)* protein required for its function, a deletion of a small region of the *su(Hw)* gene was generated and tested in vivo. *su(Hw)^{Δ100}* is a deletion of 144 bp from bases 1074 to 1217 resulting in an in-frame deletion of the 48-amino-acid region defined as the amino-terminal acidic domain shown in Fig. 2. A similar acidic region is present in other transcriptional regulators and has been found to be essential to the transcriptional activation function in the proteins studied (Ma and Ptashne 1987; Hope et al. 1988; Berger et al. 1990). The *su(Hw)^{Δ100}* construct was introduced into flies by P element-mediated germ-line transformation. Four independently transformed lines were recovered and tested for ability to complement the *su(Hw)^V* amorphic allele. Surprisingly, all four lines were capable of rescuing the phenotypic suppression of gypsy-induced mutations associated with the *su(Hw)^V* allele (Fig. 6). The *su(Hw)* mutant genotype of the flies from two of these lines was confirmed by subjecting the transformed flies to Western blot analysis. Only the protein encoded by the *su(Hw)^{Δ100}* construct is present at wild-type levels, as determined by its slightly smaller size (Fig. 4). This mutant protein binds to polytene chromosomes with the same affinity as wild type (Fig. 7). Nevertheless, in spite of their normal behavior in the phenotypic suppression assay, females carrying the *su(Hw)^{Δ100}* mutation lay only 31% of the eggs deposited by the parental strain. We conclude that the amino-terminal acidic domain of *su(Hw)* is not essential to mediate the mutagenic effect of gypsy, but it may play some role during oogenesis. Perhaps the charged region in the carboxyl terminus of the protein is functionally redundant with the major acidic domain, explaining why deletion of either domain alone results in no effect or only a subtle phenotype.

Deletion of both acidic domains affects su(Hw) function

To examine the question of the role of the acidic domains in the function of the *su(Hw)* protein we have constructed in vitro a *su(Hw)* gene encoding a protein lacking both charged regions [i.e., a protein containing the deletions of both *su(Hw)^I* and *su(Hw)^{Δ100}*]. This altered gene was designated *su(Hw)^{NoAD}* and was inserted into the pCaSper P-element transformation vector. The resulting plasmid was then injected into preblastoderm embryos. Transformants homozygous for *su(Hw)^V* and

the transformed *su(Hw)^{NoAD}* gene express higher than wild-type levels of a protein of the expected size (Fig. 4), and this protein accumulates on polytene chromosomes at normal levels (Fig. 7). The transformed strains were then examined for their phenotypic effect on female fertility and the expression of the *y²* gene. These flies are weakly fertile (16% of the parental strain) and show a wild-type yellow phenotype (Fig. 6), indicating that the altered *su(Hw)* protein, lacking the amino-terminal acidic domain and the last 150 amino acid residues in the carboxy-terminal end of the protein, is able to suppress the gypsy-induced phenotype of *y²*. Therefore, simultaneous deletion of these two regions of *su(Hw)* results in a protein that is not functional in mediating gypsy-induced phenotypes. The lack of functionality in the *su(Hw)^{NoAD}* protein might be attributable to the simultaneous deletion of both acidic domains or to deletion of sequences adjacent to the leucine zipper domain compounded with alterations in other parts of the protein.

The leucine zipper region of su(Hw) is required for the mutagenic effect of gypsy

To show that the increased severity of the *su(Hw)^{e7}* allele with respect to *su(Hw)^I* is derived from a loss of the extended leucine zipper domain, we have generated a site-directed deletion of part of this motif. A deletion named *su(Hw)^{Δ283}* was made in vitro, removing 57 bp that encodes 19 amino acids (base pairs 3213–3269) in the region identified as a possible leucine zipper domain in the carboxy-terminal third of the protein (Fig. 2). The deletion was generated by use of an oligonucleotide that includes 15 bases on either side of the deleted sequence. This construct, containing the *su(Hw)^{Δ283}* gene, was then introduced into flies by P element-mediated transformation. Ten independently transformed lines of the *su(Hw)^{Δ283}* construct were recovered. Flies transformed with *su(Hw)^{Δ283}* and homozygous for the *su(Hw)^V* null allele accumulate normal amounts of *su(Hw)* protein that is able to bind to polytene chromosomes with the same affinity as wild type (Fig. 7). Nevertheless, these flies are weakly fertile, laying only 2% of the number of eggs deposited by the parental strain, and reverse the *y²* phenotype completely (Fig. 6). Only the leucine zipper region is disrupted by the 19-amino-acid deletion in *su(Hw)^{Δ283}*, yet the effect is similar in severity to the null phenotype of *su(Hw)^V*. These results suggest that the putative extended leucine zipper domain is a region of functional importance within the *su(Hw)* protein.

To further establish that the mutant phenotype of the *su(Hw)^{Δ283}* protein is attributable to a lack in the functionality of the leucine zipper domain, we analyzed the effect of specific point mutations affecting this region. Several amino acids are conserved in the same relative positions among *su(Hw)* and bHLH–Zip proteins, interspersed with less conserved sequences (Fig. 3A). It is expected that if the sequence preservation is attributable to a maintenance in the function of the putative leucine zipper motif, changes that affect conserved amino acids would have an effect on *su(Hw)* function, whereas mu-

tations in other residues would not have a phenotypic effect. To test this hypothesis we made two different mutations in vitro affecting one conserved and one non-conserved amino acid, respectively. The first mutation is a change in nucleotides 3256–3258 from CTT to AAA. This results in a change in leucine at position 775 (marked with an asterisk in Fig. 3A) to lysine. This construct was named *su(Hw)^{L775K}* and was introduced into the *Drosophila* germ line by P element-mediated transformation. Flies homozygous for *su(Hw)^V* and any one of four different *su(Hw)^{L775K}* transformants accumulate normal levels of the *su(Hw)^{L775K}* protein (data not shown). These flies show the same level of fertility as the parental strain and contain normal levels of *su(Hw)* protein but strongly suppress the *y²* phenotype in a manner indistinguishable from *su(Hw)^{Δ283}* [data not shown; the cuticle phenotype is identical to that of *su(Hw)^{Δ283}* in Fig. 6]. The second mutation analyzed consists of an alteration in nucleotide 3226 from a G to an A. This changes the aspartate residue at position 765 to asparagine (marked with an asterisk in Fig. 3A). This construct was named *su(Hw)^{D765N}* and introduced into the germ line as described above. Transformants homozygous for the *su(Hw)^V* mutation and carrying this insertion are unable to suppress the *y²* phenotype (data not shown; the phenotype is identical to that of *y²* in Fig. 6) and therefore show wild-type *su(Hw)* function, indicating that the change from aspartate to asparagine does not affect the functionality of *su(Hw)*. These results suggest that the region of *su(Hw)* affected by the *su(Hw)^{Δ283}* and *su(Hw)^{L775K}* mutations is structurally and functionally equivalent in mediating protein interactions to the leucine zipper motif and that this region plays a crucial role in *su(Hw)* function.

Discussion

The repressive effect of the *su(Hw)* protein on enhancer function shows an interesting directionality: Only enhancers located distally from the promoter with respect to the position of the *su(Hw)*-binding region are affected by the presence of this protein (Corces and Geyer 1991; Jack et al. 1991; Geyer and Corces 1992). This directional effect offers some clues as to the mechanism by which *su(Hw)* represses enhancer action, suggesting that *su(Hw)* acts either by interfering with DNA looping that allows transcription factors bound to enhancers to interact with the transcription complex, by interfering with the tracking process of these factors towards the promoter, or by establishing chromatin domains of independent gene activity that insulate DNA sequences within a domain from neighboring regions (Geyer and Corces 1992; Roseman et al. 1993). Several structural domains of the *su(Hw)* protein seem to have important roles in eliciting gypsy-induced mutant phenotypes and during oogenesis. In general, there is a direct correlation between the degree of female sterility of a particular *su(Hw)* allele and its ability to suppress gypsy-induced mutations. The zinc fingers are necessary for DNA binding, suggesting that the interaction of *su(Hw)* with gypsy

DNA is also a prerequisite for its effect on upstream enhancers. It is interesting to note that the three mutations analyzed affecting zinc finger structure are clustered in a specific subset of zinc fingers, suggesting that not all 12 fingers of the *su(Hw)* protein are involved in DNA sequence recognition. This is supported by the varying degree of sequence conservation of the different fingers among the *su(Hw)* protein of three different *Drosophila* species; fingers 6, 7, 8, and 9 show 100% identity or similarity in all three species examined, whereas the rest of the fingers contain several nonconserved amino acids. This relative degree of conservation correlates with the importance of the various fingers in DNA binding as judged by the nature of the zinc fingers affected by random mutagenesis of the *su(Hw)* gene.

A second structural domain identified as functionally important in this study is composed of the two acidic regions located in the amino- and carboxy-terminal ends of the *su(Hw)* protein. Several results suggest that these domains might act in conjunction and that they are important for *su(Hw)* function. Deletion of the amino-terminal acidic domain from the *su(Hw)* protein of *D. melanogaster* has no major consequence on the mutagenic effect of this protein. In addition, analysis of the sequence of the *su(Hw)* protein in various *Drosophila* species indicates that the carboxy-terminal acidic domain is missing in *D. ananassae* and is shortened considerably in *D. virilis*; the weak effects observed in the *su(Hw)^I* mutation, which is missing this region of the protein, are probably attributable to additional sequences that affect the integrity of the leucine zipper region. These two lines of evidence suggest a nonessential role for the acidic domains of the *su(Hw)* protein. Nevertheless, deletion of both regions simultaneously renders the protein nonfunctional, suggesting that the acidic domains have a functional role, but each one can substitute for the other in mediating the *yellow* mutant phenotype induced by the insertion of the gypsy element. The functional interchangeability of the acidic domains also suggests that they might act synergistically and therefore might be located in close proximity to each other in the mature *su(Hw)* protein. The normal role of the acidic domains in *su(Hw)* function has not been determined, but it might be related to the process of transcriptional activation characteristic of transcription factors such as GAL4, GCN4, and VP16 (Gill and Ptashne 1988; Hope et al. 1988; Cress and Triezenberg 1991).

A third functional domain identified in the *su(Hw)* protein is defined by the *su(Hw)^{Δ283}* allele. This mutation deletes 19 amino acid residues in a region that shows homology to the leucine zipper motif characteristic of some DNA-binding proteins (Landschulz et al. 1988). The homology is higher to proteins of the bHLH–Zip family of transcription factors such as Myc, Max, AP-4, and FIP (Murre et al. 1989; Carr and Sharp 1990; Blackwood and Eisenman 1991; Blancar and Rutter 1992). These proteins typically contain a basic region that binds DNA followed by a HLH motif and, immediately adjacent, a coiled-coil region similar to the leucine zipper sequence of C/EBP (Vinson and Garcia 1992). The

su(Hw) protein has considerable homology to the second helix and the coiled-coil region of these proteins (Fig. 3), showing perfect conservation of residues in the d position, with the sole exception of the second leucine in the coiled-coil region that is changed to an aspartate. This position is also variable in other members of the family; for example, the leucine has been substituted by a methionine in l-Myc and a histidine in Max. In addition to the conserved amino acids in Fig. 3, the *su(Hw)* protein shows a high degree of homology to other members of the family in other residues of the bHLH–Zip motif. For example, helix 2 contains conserved hydrophobic amino acids in the e, g, and a positions that are located on four contiguous spokes of an α -helical wheel projection, creating a side of the helix that is highly hydrophobic and might be important for dimerization (Vinson and Garcia 1992). In addition, the e and g positions of the coiled-coil region contain conserved hydrophilic amino acids that have been implicated in regulating the specificity of dimerization in basic leucine zipper (bZIP) proteins by interhelical interactions (Cohen and Parry 1990; O'Shea et al. 1991). The functionality of the *su(Hw)* leucine zipper region is not only supported by the high degree of homology to members of the bHLH–Zip family but also by results of the mutational analysis carried out in this region of the protein. In particular, substitution of a conserved leucine for a lysine in the last d position renders the *su(Hw)* protein nonfunctional, whereas a mutation affecting an aspartate residue in a nonconserved a position of the coiled-coil region has no effect on *su(Hw)* function (Fig. 3). These results suggest that the essential role of this region of *su(Hw)* in eliciting a *yellow* phenotype is attributable to its function as a leucine zipper domain. This domain might not be involved in the dimerization of *su(Hw)*, an event that is necessary in bHLH–Zip proteins to bind DNA, as *su(Hw)* contains zinc fingers that interact with the DNA instead of the basic region of bHLH–Zip proteins. In support of this contention, the *su(Hw)* protein migrates as a monomer in gel filtration columns (D. Gdula and V. Corces, unpubl.), and mutations affecting the leucine zipper region of *su(Hw)* do not affect DNA binding. Instead, this region might be involved in interactions with other proteins that cooperate with *su(Hw)* in the transcription of gypsy and other cellular genes.

The question then arises as to the specific role of this region in mediating gypsy-induced phenotypes. The repressive effect of *su(Hw)* on enhancer function might involve a direct interaction between the *su(Hw)* protein and transcription factors present in enhancers of the *yellow* gene, resulting in the inability of these transcription factors to interact with the promoter. If this is the case, the results discussed above suggest that this interaction is mediated by the acidic and/or leucine zipper domains of the *su(Hw)* protein, as deletion of either domain results in the reversion of the *yellow* mutant phenotype. But as a role for the leucine zipper region in mediating *su(Hw)*-induced phenotypes, one has to assume that the affected transcription factors also contain leucine zipper motifs. Although this could be possible for *yellow* and

the few other *Drosophila* genes affected by gypsy insertions, it could not explain that the *su(Hw)*-binding region protects *white* expression from chromosomal position effects, independent of the chromosomal location of the *white* gene (Roseman et al. 1993). This lack of specificity suggests that the leucine zipper region might interact with a second protein that in turn mediates the negative effect of *su(Hw)* on enhancer function. An alternative explanation for the repressive effect of *su(Hw)* on gene expression could be that the binding of *su(Hw)* to gypsy DNA causes directional changes in chromatin structure by establishing boundaries between higher order domains of gene activity (Geyer and Corces 1992; Roseman et al. 1993). The need for acidic and leucine zipper domains for this function is not clear, but one can postulate that these regions of *su(Hw)* interact with other chromosomal proteins necessary for the formation of chromatin domains.

Materials and methods

Isolation and enzymology of nucleic acids

Isolation of plasmid DNA, construction and screening of λ libraries, labeling of DNA, and enzymology of nucleic acids were carried out by standard procedures (Maniatis et al. 1989). Genomic DNA from *Drosophila* adults was prepared as described by Parkhurst and Corces (1985). Total RNA for Northern analysis was isolated by homogenization in 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA, and 0.5% SDS, followed by phenol extraction and ethanol precipitation. Poly(A)⁺ RNA was selected by chromatography on oligo(dT)-cellulose. Southern and Northern analyses were done as described by Parkhurst and Corces (1985). DNA sequence analysis was performed by dideoxy chain-termination methodology (Sanger et al. 1977). P element-mediated transformation was carried out as described by Rubin and Spradling (1982), using the *white* gene as a selectable marker in the CaSpeR vector (Pirrotta et al. 1985).

DNA amplification by PCR and site-specific in vitro mutagenesis

Genomic DNAs were subjected to the PCR to amplify sequences from the *su(Hw)* region (Saiki et al. 1985; Mullis and Faloona 1987). Three pairs of primers containing restriction sites were used to amplify overlapping fragments covering the entire transcribed region of *su(Hw)*. Amplified DNA was digested and cloned by standard techniques (Maniatis et al. 1989).

Small deletions within the coding region of *su(Hw)* were directed in vitro by a 30-base oligonucleotide containing the sequences flanking the desired deletion using the Amersham in vitro mutagenesis system based on methods described in Sayers et al. (1988). Both the *su(Hw)* ^{Δ 283} and *su(Hw)* ^{Δ 100} plasmids were constructed using this approach. Generated deletions were used to replace fragments of CaSpeR 5.2, a P-element construct containing a minimum of sequence necessary for the complete phenotypic rescue of *su(Hw)* mutants. The *su(Hw)* ^{Δ 100} plasmid was generated by replacing a 541-bp *Bsu*361 fragment (from base 678 to 1219) with the fragment deleting the acidic domain (missing from base 1074 to 1217). The *su(Hw)* ^{Δ 283} plasmid was generated by replacing a 1.95-kb *Xba*I–*Bam*HI fragment (1325–3272) by the 1.6-kb cDNA fragment containing a deletion in the bHLH–Zip region (from nucleotide 3213 to 3269). The *su(Hw)*^{L775K} mutation was constructed as follows. Two oligonucleotides

were used for mutagenesis by PCR. The first oligonucleotide, GTGCGTGAATTCCTCGTGGACGAAG, contains an *EcoRI* site 5' to the leucine zipper domain; the second one, GGTG-GATCCACTAGCTATACAATTTTTACCCACA, contains a *BamHI* site 3' to the domain and three nucleotide changes that result in a codon change of leucine to lysine. The resulting PCR fragment was digested with *EcoRI* and *BamHI* and ligated into the *EcoRI*–*BamHI* sites of pBSuHw, which contains a full-length *su(Hw)* cDNA. This plasmid was then digested with *BamHI* and *XbaI*, and the 1.6-kb fragment containing the mutation was used to replace the homologous wild-type fragment from plasmid pCaSpeR 5.2. The presence of the mutation was confirmed by DNA sequencing. The *su(Hw)*^{D765N} mutation was constructed using a similar strategy, but the 3' primer GGTG-GATCCACTAGCATATCAATAAGTTTACCCACATTCTCTTTGAGCTTTATGTTCTCCTCAGTTAG was used for PCR. This primer contains one nucleotide change with respect to the wild-type DNA that results in a substitution of aspartic acid with asparagine. Both plasmids were injected into preblastoderm embryos as described above.

Immunoblotting analysis

Protein was prepared by homogenization of six animals in hot 60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.7 M β -mercaptoethanol, followed by boiling for 10 min. Debris was precipitated by centrifugation, and the supernatant was subjected to polyacrylamide gel electrophoresis as described by Laemmli (1970). Protein was electroblotted to a nitrocellulose membrane in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol (Towbin et al. 1979). Membranes were blocked and incubated with affinity-purified *su(Hw)* antibodies (Spana et al. 1988). Staining was performed by addition of substrate solution (0.1 M Tris-HCl (pH 9.6), 0.1 M NaCl, 5 mM MgCl₂, 0.25 mM nitro blue tetrazolium, and 0.25 mM 5-bromo-4-chloro-3-indolyl phosphate). Filters were developed with the ECL kit from Amersham and visualized with Kodak X-ray film.

Immunofluorescence analysis

Salivary glands from third-instar larvae were dissected in Cohen's buffer (Cohen and Gotchel 1971), incubated for 10 min., and fixed in 100 mM NaCl, 2 mM KCl, 2% NP-40, 2% formaldehyde, and 10 mM sodium phosphate (pH 7.0) for 15 min. The glands were then transferred to 45% acetic acid for 10–45 min and squashed (Heller et al. 1986). Slides were incubated with affinity-purified antibodies to *su(Hw)* at 4°C overnight, washed in 10 mM Tris-HCl (pH 7.6), 0.12 M NaCl, and incubated with rhodamine isothiocyanate-conjugated secondary antibody for 3 hr at room temperature. Slides were then mounted in glycerol and analyzed by fluorescence microscopy.

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