DNA bending is a determinant of binding specificity for a *Drosophila* zinc finger protein

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The suppressor of Hairy-wing [*su(Hw)*] locus encodes a zinc finger-containing protein that binds to specific sequences of the *Drosophila* gypsy element, mediating the mutagenic effects of this retrotransposon. We carried out a detailed analysis of the *su(Hw)*–gypsy interaction using various biochemical assays. DNase I footprinting delimits a 37-bp region in the coding strand of gypsy that is protected from digestion by the binding of the *su(Hw)* protein. Specific DNA contacts involved in the interaction were determined by methylation protection analysis and by missing nucleoside experiments using hydroxyl radical. Results from these experiments indicate that the *su(Hw)* protein binds to sequences homologous to the octamer motif, which is recognized by homeo box-containing proteins in mammalian organisms. Furthermore, two DNA bends present on both sides of the binding site in the absence of protein favor the strength of this interaction.

[**Key Words:** Transcription factor; DNA bending; retrotransposon; suppressor gene]

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Mutations at the *suppressor of Hairy-wing* [*su(Hw)*] locus reverse the phenotype of gypsy-induced mutations at different loci throughout the *Drosophila* genome (Modolell et al. 1983). The *su(Hw)* gene encodes a 110,000-dalton protein that contains 12 copies of the zinc finger motif and an acidic domain with 48% Asp and Glu residues (Parkhurst et al. 1988). These two structural characteristics are typical of transcription factors. Furthermore, it has been shown that *su(Hw)* protein is localized on larval polytene chromosomes and that it has high binding specificity for sequences located in the transcribed untranslated region of the gypsy retrotransposon (Spana et al. 1988; Mazo et al. 1989). Gypsy sequences that bind *su(Hw)* protein have been implicated in the mutagenic effect of this transposable element (Geyer et al. 1988; Peifer and Bender 1988), and this effect is likely to result from the interaction of *su(Hw)* protein with transcription factors necessary for the expression of the mutant gene. This interaction may prevent the action of these transcription factors on the promoter of the gene, resulting in the mutant phenotype.

The region of gypsy that interacts with the *su(Hw)* protein contains 12 copies of a 10-bp sequence highly homologous to the octamer motif found in enhancers and promoters of many vertebrate genes separated by AT-rich spacer sequences (Spana et al. 1988; Mazo et al. 1989). The relative role of the spacer regions versus the octamer-like sites in the binding of *su(Hw)* to the gypsy element is not clear. Because octamer sites in mammalian genes are recognized by the homeo box-containing proteins Oct-1 and Oct-2 (for review, see

Schaffner 1989), the putative interaction of octamer-like sequences with a zinc finger-containing protein such as *su(Hw)* may pose an interesting paradigm to those concerned with the molecular basis of sequence recognition by DNA-binding proteins.

The interaction of a particular protein with its target DNA sequence involves the recognition of a set of steric and chemical characteristics that constitute the binding site. This recognition depends not only on hydrogen bonding between the protein and individual bases, but also on the local conformation of the DNA molecule. In particular, bending of the DNA at specific sites, determined by the base composition of the DNA in that location, has been implicated as a major determinant of protein–DNA recognition. This DNA bending has been shown to exist in the presence or absence of bound protein. Static DNA bends have been reported in the kinetoplast DNA of trypanosome mitochondrial minicircles (Marini et al. 1982; Wu and Crothers 1984; Burk-hoff and Tullius 1987), the origin of replication of bacteriophage λ (Zahn and Blattner 1985), and the SV40 origin region I (Ryder et al. 1986), whereas protein-induced bends result from the interaction of λ int (Better et al. 1982) and *cro* (Ohlendorf et al. 1982) proteins, the *Escherichia coli* CAP protein (Gartenberg and Crothers 1988) and the restriction endonuclease *EcoRI* (Frederick et al. 1984). In either case, changes in the base composition that interfere with the bending ability of the site result in a decrease in the affinity of protein–DNA interaction. For example, Gartenberg and Crothers found that mutations in the CAP-binding site that affected the mobility

of CAP–DNA complexes in polyacrylamide gels also influenced the affinity of CAP for the binding site, suggesting a direct correlation between DNA bending and binding affinity (Gartenberg and Crothers 1988).

Here, we present a detailed analysis of the interaction of the *su(Hw)* protein with its target DNA sequence in the gypsy retrotransposon. Careful mapping of the nucleotides involved in this interaction suggests that the specificity of *su(Hw)* protein–gypsy DNA recognition is attained, at least in part, through a particular local conformation of the DNA molecule as a consequence of bends present on both sides of the binding site.

Results

The su(Hw) protein can interact with individual octamer-like repeats

Preliminary analysis of the interaction of *su(Hw)* protein with gypsy DNA by DNase I footprinting experiments indicated that this protein protects a 340-bp region from nuclease digestion (Spana et al. 1988). The sequence of this region, located in the transcribed untranslated portion of gypsy, is depicted in Figure 1A. Homologies to the octamer site, which are repeated 12 times throughout the area, are indicated by boxes; the different copies of the octamer-like motif are flanked by AT-rich sequences. Given the structure of this region, it seems possible that each of the octamer-like repeats represents a unit of interaction or a binding site for the *su(Hw)* protein. Nevertheless, protection of this region from digestion is not uniform, but it is possible to differentiate between alternating areas of weak and strong protection. These areas of unequal protection do not overlap individual octamer-like repeats, but rather they extend over varying lengths of gypsy DNA (Spana et al. 1988). One possible explanation for this situation is that each area of protection does not represent a binding site, but is composed of several individual binding sites, represented by octamer-like repeats, that show similar affinities for *su(Hw)*. The weak or strong protection would then be determined by peculiarities in the DNA structure of individual repeats. An alternative explanation is that the unequal protection is determined by the differential accessibility of specific regions to *su(Hw)* protein due to some global conformation of the DNA in the area as a consequence of the repetitive nature of this region.

The first question we sought to answer was whether the characteristic alternation of weak and strong interaction of *su(Hw)* with gypsy sequences is dictated by the structure of the region as a whole or whether it is a property of individual copies of the repeated motif. *su(Hw)* protein partially purified from an overproducing *Drosophila* cell line (see Materials and methods) was used to carry out footprinting analysis of an oligonucleotide extending from residue 671 to 775 (plasmid pFR3-1). Using the entire 340-bp region for the analysis, this DNA fragment was found previously to contain a weakly protected region that includes two octamer-like repeats and strongly protected sequences encompassing one oc-

Figure 1. DNA sequence of the *su(Hw)* binding site in the gypsy retrotransposon. (A) Structure of the binding site showing the 12 copies of the octamer-like repeat (boxed regions). Numbering of nucleotides is as described by Marlor et al. (1986). (B) Structure of an individual binding site and surrounding regions. Boxed sequences are homologous to the octamer motif. Sequences protected by *su(Hw)* from DNase I digestion are indicated on the top and bottom of the sequence for the coding and noncoding strands, respectively. Asterisks denote nucleotides involved in the interaction, as deduced from missing nucleoside experiments, for the coding (top) and noncoding (bottom) strands. Similarly, the "+" symbol denotes G residues determined to contact the *su(Hw)* protein by methylation interference experiments. Finally, vertical arrows indicate nucleotides with decreased sensitivity to hydroxyl radical as a consequence of DNA bending.

tamer-like repeat (Spana et al. 1988). Figure 2 shows the result of this experiment. The *su(Hw)* protein binds with higher affinity to sequences extending from nucleotide 726 to 764 on the noncoding strand, whereas the affinity of the interaction is lower for sequences located between nucleotides 683 and 726 (Fig. 2A). The extent and location of these two areas has been marked by open and filled boxes adjacent to the footprints to respectively denote weakly and strongly protected regions. A similar experiment was carried out with the coding strand (Fig. 2B); protection of this strand by *su(Hw)* from DNase I digestion is weaker than for the noncoding strand, and a footprint can be seen clearly only in the region between nucleotides 728 and 764, corresponding to the strongly protected region in the other strand.

These experiments suggest that the pattern of weak and strong protection is not a property of the region of gypsy that interacts with *su(Hw)* as a whole, but can be reproduced in a DNA fragment containing only one weak and one strong protected area. In fact, the limits of the footprinted region and the strength of the interaction are the same when oligonucleotides containing only the weakly or strongly protected regions shown in Figure 2 are used as substrates for the protection experiments (data not shown). These results indicate that the weak or strong binding characteristics of the protein–DNA interaction are properties of the specific sequence of indi-

Figure 2. DNase I footprinting of the *su(Hw)* binding site. Six micrograms of plasmid pFR3-1 were digested with *Eco*RI or *Hind*III and filled in with [32 P]dNTP to label the noncoding (A) or coding (B) strands, respectively. The DNA was incubated with *su(Hw)* protein and digested with DNase I as described in Materials and methods, and then electrophoresed on an 8% polyacrylamide-urea sequencing gel. In each panel, lane 1 contains Maxam–Gilbert G + A sequencing reactions, lane 2 contains free DNA digested with DNase I, and lane 3 contains DNA-*su(Hw)* protein complex incubated with DNase I. An open bar adjacent to the gel in A indicates a region of light protection extending from nucleotide 683 to nucleotide 725 (see Fig. 1), whereas a solid bar denotes strongly protected sequences extending from nucleotide 726 to nucleotide 764. The solid bar in B indicates a weakly protected region extending from nucleotide 728 to nucleotide 765 on the coding strand that corresponds to the strongly protected sequences in the noncoding strand.

vidual binding sites and thereby validate the use of short oligonucleotides containing particular regions of protection to study the interaction of *su(Hw)* with gypsy DNA. Furthermore, because the strongly protected region includes only one octamer-like repeat plus adjacent sequences, these results suggest that this minimum region of protection may represent a unit of interaction between *su(Hw)* and the gypsy element. In the sections below, we describe additional experiments designed to more accurately establish the boundaries of a binding site using the strongly protected region shown in Figure 2. The extent of this region on both coding and noncoding DNA strands of the gypsy element is depicted in Figure 1B.

A detailed analysis of specific nucleotides involved in the interaction with su(Hw)

The maximum extent of a binding site for *su(Hw)* as de-

finied by DNase I footprinting experiments can be specified on the basis of the results shown in Figure 2A for the strongly protected region. This binding site expands from nucleotide 726 to nucleotide 764 on the noncoding strand (Fig. 1B). Because digestion by DNase I shows some sequence specificity, the limits of this binding site represent an overestimate. Although a large footprinted region results from this digestion, it is likely that all nucleotides within the protected area are not involved or necessary for the interaction.

To determine the particular bases within the binding site that are important for the DNA interaction with *su(Hw)* protein, we carried out missing nucleoside experiments using hydroxyl radical (Hayes and Tullius 1989). This reagent causes random cleavage of the DNA phosphodiester backbone by reaction with the deoxyribose residue, resulting in a one-base gap on average per DNA molecule. The ability of *su(Hw)* to bind the gapped DNA was then assayed by gel mobility shift, and the bound DNA present in the slow migrating band containing the gypsy-*su(Hw)* protein complex was isolated and analyzed on a sequencing gel. The results of this experiment are shown in Figure 3A for the coding strand and in Figure 3B for the noncoding strand. Those DNA molecules in which a nucleoside important for the interaction with *su(Hw)* has been removed are unable to bind the protein, causing the absence of the corresponding band in the sequencing gel. Therefore, each band missing in lane 3, Figure 3, A and B, denotes a nucleotide that is important for the binding of *su(Hw)* to gypsy DNA; those bases have been indicated with asterisks in Figure 1B. It is apparent from these results that the sequence involved in the interaction is centered around the octamer-like repeat and extends on both sides of this motif. But the binding site defined by this type of approach does not extend as far as that determined by DNase I footprinting, suggesting that the bases involved in the actual interaction are a subset of those protected from nuclease digestion.

Definition of the binding site by methylation interference experiments

The missing nucleoside experiments described above define the bases that are important for the binding interaction, but not necessarily those that make contact with the bound protein. To define which residues within the binding site are actually contacted by the *su(Hw)* protein during the formation of a complex, we carried out methylation interference experiments in which both G and A residues were methylated with dimethyl sulfate. The effect of this modification on the interaction with *su(Hw)* was then tested by gel mobility-shift analysis. The bound and unbound DNA present in the slow and fast moving bands, respectively, were eluted and subjected to electrophoresis on a polyacrylamide-urea gel. The results of these experiments for the coding and noncoding strands are shown in Figure 4, A and B, respectively. The intensity of the bands corresponding to A residues, methylated at the N-3 position, which faces

Figure 3. Missing nucleoside experiment. Six micrograms of plasmid pFR3-1 were digested with *Hind*III or *Eco*RI, and the coding (A) or noncoding (B) strands were labeled with [32 P]dNTP. The DNA was then digested with hydroxyl radical as described in Materials and methods and used in a gel mobility-shift assay. The DNA present in the slow migrating band was isolated and electrophoresed on an 8% polyacrylamide-urea gel. In each panel, lane 1 contains a G + A sequencing ladder, lane 2 represents the hydroxyl radical control digestion of naked DNA, and lane 3 contains the bound fraction from the missing nucleoside experiment. Sequences written adjacent to each gel denote those nucleosides missing from the *su(Hw)*-bound DNA and corresponding to low intensity or absent bands in the gel.

the minor groove, is the same in the bound and unbound fractions of DNA. This suggests that these bases are not involved in direct contacts with *su(Hw)* and that this protein probably does not interact with gypsy through the minor groove of the DNA helix. On the other hand, the intensity of the bands corresponding to guanines located at positions 743, 746, and 758 on the coding strand, as well as those located at 747 and 751 on the noncoding strand, is decreased in the lane containing protein-bound relative to the unbound DNA. These results suggest that these particular G residues participate in contacts with *su(Hw)* because their methylation interferes with binding of the protein. Furthermore, since G residues are methylated at the N-7 position, which faces the major groove, contacts of *su(Hw)* protein with gypsy DNA most likely take place through the major groove of the DNA helix.

DNA bends at the *su(Hw)* binding site

There is an apparent discrepancy between results obtained by methylation interference experiments and those obtained by the missing nucleoside approach with regard to defining which DNA bases contact the *su(Hw)*

protein. Whereas nucleotides 733 to 759 on the coding strand are necessary for the binding of *su(Hw)* protein to its target sequence as judged from results of the missing nucleoside experiment, only a few specific purines within this region seem to actually contact the protein during the formation of a complex. This is particularly obvious, for example, in the case of A residues 733 to 738 on the coding strand and 753 to 757 on the noncoding strand, whose interaction with *su(Hw)* is not appreciably affected by methylation. This discrepancy can be explained IF the *su(Hw)* protein contacts these nucleotides in the major groove and this interaction would not be affected by the presence of a methyl group in the minor groove, thereby explaining the discrepancy. An alternative explanation is that these A residues are not involved in direct hydrogen bonding with *su(Hw)*, but rather they contribute to a certain conformation of the DNA that can be recognized by the *su(Hw)* protein and that is essential for the binding.

One such conformational alteration, induced by AT-tracts similar to those flanking the *su(Hw)* binding site

Figure 4. Methylation interference analysis of the *su(Hw)*-gypsy interaction. Plasmid pFR3-1 was digested with *Hind*III or *Eco*RI, and the coding (A) or noncoding (B) strands were labeled with [32 P]dNTP. The DNA was then treated with DMS and used in a gel mobility-shift assay with *su(Hw)* protein. The bound and free DNAs were purified from the slow and fast migrating bands, respectively, and electrophoresed on an 8% polyacrylamide gel. Lane 1 contains a Maxam-Gilbert G sequencing reaction, lane 2 contains the free DNA from the gel mobility-shift experiment, and lane 3 contains *su(Hw)*-bound DNA. Asterisks denote bands of decreased cutting in the methylated DNA. Sequences adjacent to the gels denote the area of interest, with "+" symbols indicating G residues affected by DMS treatment, and "-" symbols indicating purines not affected.

(see Fig. 1B), is that caused by bending of the DNA helix (Koo et al. 1986; Burkhoff and Tullius 1987). To investigate the possibility that changes in the DNA curvature take place in the *su(Hw)* binding site, we assayed the pattern of digestion of this DNA by hydroxyl radical. This agent cuts the DNA backbone nearly equally at each position, but narrowing of the minor groove in an A tract results in bending of the DNA and is reflected by a progressively decreased cutting frequency through that region (Burkhoff and Tullius 1987). This reduction in hydroxyl radical cleavage in regions of the DNA helix in which the minor groove is compressed can be used to map sequences that are bending. An end-labeled oligonucleotide extending from nucleotide 671 to nucleotide 775 (plasmid pFR3-1) was digested with hydroxyl radical and the resulting DNA fragments were analyzed by electrophoresis on a sequencing gel. The result of this experiment is shown for the noncoding strand in Figure 5A, where regions of decreased cutting are marked by arrows. This pattern of light and intense digestion can be seen better in a densitometer scan of the autoradiograph in which the sequence of the DNA has been overimposed on each of the peaks corresponding to a single gel band (Fig. 6A). The octamer-like sequence in this

Figure 5. DNA bending at the *su(Hw)* binding site. Plasmids pFR3-1 (A) and pFR3-3 (B) were digested with *EcoRI* and labeled with [32 P]dNTP. The DNAs were then treated with hydroxyl radical and electrophoresed as described in Materials and methods. Lane 1 contains naked DNA treated with hydroxyl radical, lane 2 contains untreated DNA, and lane 3 contains a Maxam–Gilbert G + A sequencing reaction. Arrows indicate regions of decreased cutting corresponding to bends. The location of the L and R bends is indicated in Fig. 1B. The top arrow indicates a bend in a region of the DNA not altered by the *in vitro* mutagenesis.

Figure 6. Densitometer scans of the hydroxyl radical digestions. The autoradiographs shown in Fig. 5, A and B, were scanned with a Joyce-Loebel Chromoscan-3 densitometer, and the results are shown in A and B, respectively. Letters on top of each peak denote the sequence of the *su(Hw)* binding site in the respective plasmid. The location of the L and R bends in plasmid pFR3-1 is indicated in A. The position where these bends should be located in plasmid pFR3-3 is indicated in B.

binding site is flanked by two regions of decreased hydroxyl radical cutting, indicating that the minor groove in these regions is compressed, and the DNA is bending into the minor groove. These two regions for this particular binding site have been designated R (right) bend, and L (left) bend; their location in the binding site is shown in Figure 1B, where the A residues with decreased cutting by hydroxyl radical are indicated by arrows. Similar regions of DNA bending have been mapped adjacent to all octamer-like repeats present in the 340-bp region of gypsy that interacts with *su(Hw)* shown in Figure 1A (data not shown).

DNA bending is important for the interaction of *su(Hw)* with gypsy DNA *in vitro*

In view of the results obtained above, we decided to test if the changes in DNA conformation correlated with DNA bending at the A tracts, located on both sides of the octamer-like sequence, play an important role in the recognition of this site by *su(Hw)* protein. To accomplish this goal, we made changes in the DNA of the A tracts by synthesizing oligonucleotides in which these sequences have been altered. To minimize other effects and ensure that these changes would affect only the bendability of the DNA but not the specific interactions between the altered bases and the *su(Hw)* protein, we made transitional mutations, that is, the T residues at

positions 734 and 735 were changed for C, and the A residues at 754 and 755 were changed for G. A synthetic oligonucleotide containing these various altered sequences (plasmid pFR3-3) was then end-labeled and subjected to hydroxyl radical cleavage. Figure 5B shows the cutting pattern for the noncoding strand. It can be seen that, out of the three areas of decreased digestion shown in Figure 5A, the middle and bottom regions indicated by the two lower arrows in panel 5A, corresponding to the L and R bends respectively, are not visible in the mutated oligonucleotide (Fig. 5B), whereas the top region of decreased cutting corresponding to a DNA bend located between nucleotides 772 and 779, is still present. Figure 6B shows the result of a scanning of the autoradiograph where this effect can be better appreciated. The cutting modulation in the regions where the L and R bends are present in the wild-type DNA is more uniform in the mutant oligonucleotide, suggesting that these sequences are more accessible to hydroxyl radical attack. Therefore, the minor groove in these two regions is not compressed, and the DNA is no longer bending as a result of the sequence alterations introduced.

To test if the lack of the specific DNA conformation of the binding site that results from both bends contributes to the affinity of *su(Hw)* for gypsy DNA, we carried out DNase I footprinting experiments with both wild-type and mutant oligonucleotides containing the binding site. Figure 7 shows the result of this experiment. Binding of *su(Hw)* to the oligonucleotide carrying

Figure 7. DNase I footprinting of wild-type and mutant binding sites. Plasmids pFR3-1 (A) and pFR3-3 (B) were digested with *Eco*RI, end-labeled with [32 P]dNTP, incubated with *su(Hw)* protein and digested with DNase I. The DNA was then electrophoresed on a polyacrylamide-urea sequencing gel. In both panels, lane 1 contains a Maxam–Gilbert G + A sequencing reaction, lane 2 contains naked DNA digested with DNase I, and lane 3 contains DNA incubated with *su(Hw)* and digested with DNase I. Bars adjacent to the gels denote regions of protection from digestion by DNase I.

the wild-type sequence of gypsy, which contains a bending site on either side of the octamer-like repeat, strongly protects the DNA from nuclease digestion (Fig. 7A). To the contrary, interaction between this protein and the mutated oligonucleotide, which lacks both DNA bends, is decreased to the point that protection is barely detectable (Fig. 7B). These results suggest that DNA bending represents an important contribution to the affinity of the interaction between *su(Hw)* protein and its target sequence on gypsy DNA.

To quantitate to a first approximation the effect of DNA bending on the ability of *su(Hw)* protein to interact with the binding site, we carried out gel mobility-shift experiments with this protein and oligonucleotides extending from positions 671 to 776 containing the wild-type and mutated sequences. Figure 8 shows the result of this experiment. Increasing amounts of partially purified *su(Hw)* protein were incubated with a labeled oligonucleotide corresponding to the normal sequence of gypsy (Fig. 8A), or one in which both bends have been eliminated by the A→G and T→C transitions described above. Simple examination of the autoradiographs indicates a difference in the affinity of the protein for both oligonucleotides that correlates with the results of the footprinting experiments. This difference was quantitated in a first approximation by scanning the gels shown in Figure 8 and determining the ration of the values representing the affinities of *su(Hw)* protein for wild-type and mutant DNA. The result of this analysis indicates that base changes that interfere with the ability of the binding site to bend on both sides of the octamer-like sequence result in a 3.5-fold decrease in the affinity of *su(Hw)* protein for the binding site.

In vivo significance of DNA bending at the *su(Hw)* binding site

The 340-bp region of gypsy, containing twelve binding sites, that interacts with *su(Hw)* (Fig. 1A), is able to elicit the same y^2 phenotype as the complete gypsy element when inserted in the same location of the *yellow* gene (P.K. Geyer and V.G. Corces, in prep.). To test the biological significance of the 3.5-fold reduction in the affinity of *su(Hw)* protein for its binding site when DNA bends on both sides of the octamer-like repeat are removed, we analyzed the ability of these sequences to induce mutant phenotypes at the *yellow* locus using the same type of assay. An oligonucleotide containing three binding sites and extending from nucleotide 671 to 775 (plasmid pFR3-1) was dimerized to construct a DNA fragment containing six binding sites as a consequence of the tandem repetition of the first three (see Materials and methods). A parallel approach using the mutant oligonucleotide contained in plasmid pFR3-3 was used to build a DNA fragment containing two tandem repeats with six binding sites in which two of these sites contain mutations that interfere with their ability to bend. Because the mutant oligonucleotide in pFR3-3 contains two weakly protected sites [and therefore has a low affinity for *su(Hw)*] and a site that is strongly protected in

Figure 8. Quantitative gel mobility-shift analysis. Plasmids pFR3-1 (A) and pFR3-3 (B) were digested with *Eco*RI, end-labeled with [32 P]dNTP, and digested with *Nde*I. The DNA fragment containing gypsy sequences was purified through an agarose gel, incubated with *su(Hw)* protein, and electrophoresed on a 5% polyacrylamide gel. Lanes in A and B contain increasing amounts of *su(Hw)* extract, from no protein in lane 1 to 12 μ l in lane 9; lanes with the same numbers in A and B contain the same amount of protein. C represents the protein-DNA complex, and D represents the free DNA. The band located on top of D in B represents contaminating pUC18 DNA.

wild-type DNA but shows low affinity for *su(Hw)* due to the mutations that affect bending, the final construct contains six sites with low affinity for *su(Hw)*. Both DNA fragments constructed in this fashion were then introduced in the 5' region of the *yellow* gene. The resulting plasmid was then cloned into the Carnegie 20 transformation vector and injected into *y^{-ac-}; ry⁵⁰⁶* preblastoderm embryos (Rubin and Spradling 1982, 1983). Transformants were selected for the *ry⁺* phenotype.

Flies transformed with a *yellow* gene containing the wild-type DNA fragment show a phenotype intermediate between *y²* and wild-type, that is, the coloration of the bristles is normal, but the wings and the cuticle of the thorax and abdomen are slightly darker than *y²*. The amount of pigment in these flies is similar to that described for the *y^{76d28}* mutant (Geyer et al. 1990). To the contrary, flies transformed with the mutant DNA fragment, in which the ability of the DNA to bend at the two strong sites present has been abolished, are indistinguishable from wild-type (data not shown). These results indicate that the decrease in the affinity of *su(Hw)* for its

binding site when no bends are present translates into a reduction in the ability of this same DNA to cause a mutant phenotype at the *yellow* locus. Therefore, the results observed in vitro correlate with in vivo effects of the *su(Hw)* protein, suggesting that a 3.5-fold reduction in the in vitro-measured affinity has biological relevance.

Discussion

The *su(Hw)* protein binds to specific sequences of the gypsy element and through this interaction is responsible for the mutagenic effect of this retrotransposon (Harrison et al. 1989). The region of gypsy that binds *su(Hw)* is 340 bp long and contains multiple copies of a sequence very similar to the octamer repeat (Spana et al. 1988; Mazo et al. 1989) found in enhancer and promoter elements of various higher eukaryotic genes (Bohmann et al. 1987; Rosales et al. 1987). The results presented in this manuscript suggest that *su(Hw)* indeed interacts with specific bases of the octamer-like motif and that each of these repeats represent one unit of interaction or a binding site for the *su(Hw)* protein. The definition of a binding site was facilitated by the existence of regions of light and strong protection to digestion by DNase I within the 340 bp of gypsy sequence that interacts with *su(Hw)*. This characteristic pattern of protection is reproduced when oligonucleotides that contain only one region of weak protection and its adjacent strongly protected neighbor are used. These results suggest that the alternation of weakly and strongly protected areas is not a property of some global and specific structure of the 340-bp region as a whole, but rather it must represent peculiarities in the DNA sequence of individual areas that allow high or low affinity interactions of *su(Hw)* with each of these regions. Each of these units of weak or strong interaction must correspond to a binding site or a group of consecutive sites with similar affinity for *su(Hw)*. The repetitive nature of the sequences located within the 340-bp region, together with the minimum size of a strongly protected region flanked by weakly protected ones, suggest that a binding site should be formed by an octamer-like repeat plus some of the adjacent sequences.

The apparent boundaries for each individual binding site depend on the method of study employed to analyze the interaction. DNase I digestion experiments delimit a 37-bp protected region centered around an octamer-like site and extending on each side to include the adjacent AT-rich regions to the next octamer-like site. A binding site defined with these limits raises the question of whether each octamer-like site has the ability to interact with *su(Hw)*, because binding to a site would preclude, for steric reasons, the interaction with adjacent ones. Nevertheless, the size of the binding site as determined by DNase I protection experiments is probably an overestimate because poor digestion of the AT-rich regions adjacent to the octamer-like sites makes the assignment of the exact boundaries of the binding site ambiguous. To overcome this problem, we used the

missing nucleoside technique to determine the exact nucleotides involved in the interaction of *su(Hw)* with gypsy DNA. This experimental approach, described first by Brunelle and Schleif (1987) with DNase I, has been modified to make use of hydroxyl radical to randomly remove a nucleoside from the DNA backbone (Hayes and Tullius 1989; Chalepakidis and Beato 1989). Results from these experiments suggest that the extent of the binding site, defined as the nucleotides necessary for the binding of *su(Hw)* protein to gypsy sequences, is shorter than suggested by nuclease protection experiments. The binding site is composed of the octamer-like motif and adjacent bases extending halfway to the next octamer-like site on either side. This allows the existence of a complete binding site per octamer-like repeat and suggests the presence of 12 such units per copy of gypsy DNA.

The octamer motif present in vertebrate genes such as the histone H2B promoter, the immunoglobulin IgH enhancer, or the SV40 enhancer, is recognized by the Oct-1 and Oct-2 proteins (Schaffner 1989). These two transcription factors contain DNA binding domains homologous to the homeo box and a second conserved domain designated the POU-specific box (Sturm et al. 1988; Müller et al. 1988; Clerc et al. 1988; Scheidereit et al. 1988). Methylation interference experiments confirm that indeed *su(Hw)* protein interacts with the octamer-like motif through at least several G residues present on both DNA strands of this repeat. These results suggest that both helix–turn–helix and zinc finger proteins are capable of recognizing very similar sequences. This recognition may take place through similar structural motifs, because the α -helices of helix–turn–helix proteins make contacts with the major groove (Pabo and Sauer 1984), and NMR studies of zinc fingers have indicated the presence of α -helices that have been proposed to take part in protein–DNA contacts (Lee et al. 1989). In support of this, the methylation interference experiments also suggest that *su(Hw)* makes contacts with the DNA helix through the major groove as has been shown for other zinc finger proteins such as TFIIB and SP1 (Sakonju and Brown 1982; Gidoni et al. 1984).

In addition to this protein–DNA recognition based on hydrogen bond interactions, *su(Hw)* also recognizes a specific conformation of the binding site that is determined by the bending of DNA sequences located on both sides of the octamer-like repeat. This ability of DNA sequences adjacent to the octamer-like motif to bend can be detected by the decreased susceptibility of these sequences to cutting by hydroxyl radical; bending of A tracts causes a narrowing of the minor groove that results in lower accessibility to hydroxyl radical (Burkhoff and Tullius 1987). Because the A tracts are located on opposite strands but separated by ~ 1.5 helix turns, both bends are located on the same face of the DNA helix. Therefore, the bends occurring at A tracts on both sides of the octamer-like repeat and their effect should be additive, giving the DNA a curve through this region (Koo et al. 1986). This curvature could perhaps allow additional interactions of the *su(Hw)* protein with specific

bases in the binding site. For example, the guanine at position 759 is located on the other side of the bend with respect to the binding site; because two G residues in the octamer-like site in addition to the one at position 759 are contacted by *su(Hw)*, bending of the DNA located in between may allow distant bases such as the G at 759, to be contacted by the protein. The importance of this bending in the interaction of *su(Hw)* protein with gypsy sequences is demonstrated by experiments that alter the sequence composition of the A tracts and therefore interfere with the ability of these sequences to bend. Oligonucleotides containing these altered sequences show a 3.5-fold decrease in their affinity for *su(Hw)*, a change of similar magnitude to that observed for the CAP binding protein when changes that interfere with bending are introduced in its target sequence (Gartenberg and Crothers 1988). This 3.5-fold reduction in the affinity of *su(Hw)* for its binding site has biological relevance in the whole fly, because the altered site is not able to induce a mutant phenotype when inserted into the 5' region of the *yellow* gene. These results therefore suggest that both global conformation and specific DNA–protein contacts contribute to the definition of the binding site and the recognition of a specific sequence by the *su(Hw)* protein in the live cell.

Materials and methods

Preparation of *su(Hw)* from S2M3 cells

Nuclear extracts were made from S2M3 cells stably transformed with the plasmid pTC-*suHw* that overexpress *su(Hw)* protein when exposed to copper ions (Spana et al. 1988). Extracts were made following the procedure of Wu (1984) with the following changes: the EDTA was omitted and ZnCl_2 was added to 100 μM . Extracts were further purified by chromatography over a heparin-agarose column. Protein retained on the column was eluted with a step gradient using 0.2, 0.4, 0.6, 0.8, and 1.0 M KCl. Fractions were assayed for *su(Hw)* binding activity using a gel mobility-shift assay (Fried and Crothers 1981; Garner and Revzin 1981). Fractions containing *su(Hw)* binding activity were pooled, diluted to 0.1 M KCl, and passed over a DNA-cellulose column. Retained protein was eluted with a step gradient using 0.2, 0.4, 0.6, 0.8, and 1.0 M KCl. Fractions containing *su(Hw)* binding activity, as determined by gel mobility-shift analysis, were pooled, dialyzed against 0.1 M KCl, and stored at -70°C . Electrophoresis of this preparation on a polyacrylamide gel, followed by visualization of the proteins using silver staining, suggest that *su(Hw)* protein constitutes 15% of the total protein present in the extract.

Construction of probes used for binding experiments

A 73-bp oligonucleotide extending from nucleotide 713 to 775 and containing sequences of the second and third gypsy repeats was synthesized with *SalI* and *SphI* overhangs. The complementary strand was synthesized in the same fashion and both strands were annealed and cloned into the *SalI* and *SphI* sites of pUC18, with the *SalI* site adjacent to the 5' end of the gypsy coding strand. This construct was designated p2/3. An oligonucleotide of 113 bp extending from nucleotide 671 to 775 and representing the first three gypsy repeats was synthesized with *BamHI* and *SalI* overhangs. The complementary strand was

also synthesized and both strands were annealed. The resulting DNA fragment was cloned into the *Bam*HI and *Sal*I sites of the pUC18 vector with the *Bam*HI site adjacent to the 5' end of the gypsy coding strand and was designated pFR3-1. A second oligonucleotide also of 113 bp designated pFR3-3 was synthesized and cloned into the *Bam*HI and *Sal*I sites of pUC18 following the same strategy. pFR3-3 is identical to pFR3-1 except for the following changes; the two A tracts surrounding the third gypsy repeat were disrupted by substitution of the two T residues at positions 734 and 735 for Cs and the A residues at 754 and 755 for Gs.

DNA binding experiments

Plasmids containing the appropriate oligonucleotides were digested with restriction enzymes (see figure legends for particular details) and DNA fragments containing 5' overhangs were end-labeled with [³²P]dNTP and the Klenow fragment of *Escherichia coli* polymerase (Maniatis et al. 1982). All DNA binding reactions were carried out in 15 mM HEPES (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 100 μM ZnCl₂, 5 mM DTT, 10% glycerol, and 5 μg of poly[d(I-C)]. DNase I footprinting reactions were done by digestion of 1 ng of labeled fragment with 50 ng of DNase I for 40 sec in a reaction volume of 50 μl, in the presence or absence of 20–50 μg of protein extract. The digested products were separated on an 8% polyacrylamide-urea sequencing gel.

For the dimethylsulfate (DMS) interference experiments, 300,000 dpm of end-labeled fragment was treated with DMS according to the procedure of Maxam and Gilbert (1980). The methylated DNA was then used in five gel mobility-shift assays (Fried and Crothers 1981; Garner and Revzin 1981). The binding reactions were carried out in a 25 μl volume and contained 5 μg of protein extract. After incubation at 4°C for 10 min, the reactions were loaded on a 5% polyacrylamide gel and the complexes fractionated in 1 × TBE buffer (89 mM Tris-borate, 89 mM borate, and 3 mM EDTA) at 15 V/cm. Free and complexed probes were eluted from the gel into 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS (Maxam and Gilbert 1980). The eluted DNA was precipitated by addition of 10 μg of tRNA and two volumes of ethanol. The samples were resuspended in 200 μl of 0.3 M sodium acetate, 0.1 M EDTA and reprecipitated by addition of 30 μl of 1% cetyl-trimethyl ammonium bromide (CTAB) at room temperature. The CTAB precipitated DNA was reprecipitated twice with ethanol after resuspension in 0.3 M sodium acetate. Precipitation with CTAB was done to remove contaminating polyacrylamide (Sturm et al. 1987). The samples were resuspended in 100 μl of freshly diluted 1 M piperidine and incubated at 90°C for 15 min. The piperidine was removed by lyophilization. Equal amounts of counts of the free and bound probes were fractionated on a 8% polyacrylamide-urea sequencing gel.

Missing nucleoside experiments were performed using hydroxyl radical-treated DNA as described by Hayes and Tullius (1989). DNA was prepared and treated with hydroxyl radical as described below. The gapped DNA was used in gel mobility-shift assays following the procedure outlined above under the DMS interference experiment. Equal amounts of counts of free and complexed probe were fractionated on a 8% polyacrylamide-urea sequencing gel.

Quantitative gel mobility-shift assays were carried out in a 25-μl reaction volume; the amount of protein added varied and is given in the figure legend. The binding reactions were incubated at 4°C for 10 min and the free and complexed probes were fractionated on a 5% polyacrylamide gel in 1 × TBE buffer. The gel was preelectrophoresed at 15 V/cm for 1 hr, and then loaded

and run at 15 V/cm. The gels were dried before autoradiography. Autoradiographs of the gels were scanned on a Eikonix 1412 CCD camera (Brenowitz et al. 1986).

Hydroxyl radical analysis of DNA bending

Plasmid DNA for the hydroxyl radical reactions was isolated from *E. coli* using a modification of the cleared lysate procedure as outlined by Burkhoff and Tullius (1987). For the final purification step, the plasmid was chromatographed in 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.2 M NaCl, on a Sephacryl S-500 column (1.6 × 40 cm; 1 ml/min) using a Pharmacia FPLC system. Purification was carried out in this manner to avoid introducing nicks into the DNA when exposed to ethidium bromide. The reactions were carried out in 100 μl of binding buffer minus the glycerol with 50,000–70,000 cpm of end-labeled probe. Solutions of 20 mM Fe(II), and 0.03% H₂O₂ were made fresh. To make the Fe(II)-EDTA, equal volumes of the 20 mM Fe(II) and 40 mM EDTA were mixed. To cut the probe with hydroxyl radical, 10 μl of Fe(II)-EDTA, 10 μl of 0.03% H₂O₂, and 5 μl of 20 mM sodium ascorbate were added to the reaction tube and the reaction carried out for 30 sec at room temperature. The reaction was quenched by addition of 10 μl of 0.1 M thiourea, 10 μl of 3 M sodium acetate and 10 μg of tRNA. Two volumes of ethanol were added and the DNA precipitated at –70°C for 10 min. The precipitated DNA was resuspended in loading buffer (Maxam and Gilbert 1980) and the hydroxyl radical cleavage products fractionated on an 8% polyacrylamide-urea sequencing gel. The gels were dried before autoradiography. The autoradiographs were scanned using a Joyce-Loebel Chromoscan-3 densitometer.

Drosophila germ line transformation

Plasmids for transformation of *Drosophila* embryos were made as follows. Plasmid pFR3-1 was cut with *Bam*HI and the overhang filled in with Klenow fragment, then cut with *Hind*III and the resulting 120-bp fragment containing nucleotides 671 to 775 of gypsy isolated. This fragment was then cloned into pFR3-1 that was first cut with *Sal*I, the 5' overhang filled in and then cut with *Hind*III. The resulting clone contained a tandem duplication of nucleotides 671 to 775 of gypsy and therefore 6 copies of the octamer-like repeat. An *Eco*RI–*Hind*III fragment containing this duplication was cloned into the *yellow* gene contained in the plasmid pD-2873 (Geyer and Corces 1987) at the *Eco*47III site located 893 bp upstream from the start site of transcription of the *yellow* gene. A *Sal*I fragment containing this modified *yellow* gene was cloned into the *Sal*I site of the P element transformation vector Carnegie 20 (Rubin and Spradling 1983). A *yellow* gene construct containing mutated *su(Hw)* binding sites was created the same way using pFR3-3 instead of pFR3-1. P element-mediated germ line transformation was performed following the procedure of Rubin and Spradling (1982) using the *rosy* eye color as a selection for transformants.

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