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The Enhancer-Blocking *suppressor of Hairy-wing* Zinc Finger Protein of *Drosophila melanogaster* Alters DNA Structure

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Insertion of the gypsy retrotransposon of *Drosophila melanogaster* into a gene control region can repress gene expression. The zinc finger protein (SUHW) encoded by the *suppressor of Hairy-wing* [*su(Hw)*] gene binds to gypsy and prevents gene enhancers from activating transcription. SUHW blocks an enhancer only when positioned between the enhancer and promoter. Although position dependent, SUHW enhancer blocking is distance independent. These properties indicate that SUHW does not interact with the transcription activator proteins that bind to enhancers. To explore if DNA distortions are involved in enhancer blocking, the ability of SUHW to alter DNA structure was examined in gel mobility assays. Indeed, SUHW induces an unusual change in the structure of the binding-site DNA. The change is not a directed DNA bend but correlates with loss of sequence-directed bends in the unbound DNA. The DNA distortion requires a SUHW protein domain not required for DNA binding, and mutant proteins that fail to alter DNA structure also fail to eliminate the sequence-directed bends. These results suggest that SUHW increases DNA flexibility. The DNA distortion is not sufficient to block enhancers, and therefore it is suggested that increased DNA flexibility may help SUHW interact and interfere with proteins that support long-distance enhancer-promoter interactions.

Insertion of a transposable element into a gene can alter gene expression in a manner that is dependent on host regulatory proteins, thereby revealing factors that regulate gene expression. For example, many mutations in *Drosophila melanogaster* are gypsy retrotransposon insertions into or adjacent to several different genes (15, 23). The mutant phenotypes associated with most gypsy insertions are suppressed by mutations in the *suppressor of Hairy-wing* [*su(Hw)*] locus (23, 30). The *su(Hw)* gene encodes a protein (SUHW) with 12 putative zinc fingers (25) that binds DNA a few hundred base pairs downstream of the gypsy 5′ long terminal repeat. The binding region contains multiple direct repeats of a consensus sequence recognized by SUHW (5, 33). Some gypsy elements have more binding sites than others, and the mutant phenotype of a *bithorax* gypsy insertion is more severe when the gypsy element has more binding sites (27). Therefore, SUHW alters gene expression.

The SUHW bound to gypsy alters gene expression in two ways. SUHW increases use of the polyadenylation site in the gypsy 5′ long terminal repeat when gypsy is downstream of a promoter (5, 7), thereby increasing truncation of gene transcripts. SUHW also blocks enhancer-promoter interactions when bound between an enhancer and a promoter (6, 10, 11, 13, 14). The SUHW-binding sites are the only part of gypsy required for enhancer blocking. For example, heat shock transcription is repressed when SUHW-binding sites are inserted in the *hsp70* promoter between the two heat shock elements or between the heat shock elements and the TATA box (13). Similarly, insertion of SUHW-binding sites at various positions in the *yellow* gene prevents different enhancers from activating gene expression (10).

The mechanism of enhancer blocking by SUHW is unknown but is likely to provide insight into how enhancers activate transcription over long distances. SUHW blocks an enhancer only when positioned between the enhancer and promoter, yet enhancer blocking is also distance independent, even for distances approaching 100 kb (6). Thus, SUHW bound just upstream of an embryonic enhancer in the *cut* locus does not interfere with that enhancer, yet it effectively blocks all upstream enhancers, including an enhancer nearly 50 kb further upstream (6, 14). Furthermore, the embryonic enhancer is effectively blocked when SUHW is bound 40 kb downstream. Therefore, it is extremely unlikely that SUHW blocks by interacting with the transcription-activating proteins that bind to enhancers. SUHW enhancer blocking is also immediate and reversible (6), indicating that blocking does not result from formation of quasi-stable heterochromatin-like structures.

An additional clue to the mechanism of enhancer blocking is the inability of SUHW to effectively block activation by the GAL4 protein in the yeast *Saccharomyces cerevisiae* when bound between *GAL4* and the promoter (19). SUHW produced in yeast is indistinguishable from *Drosophila* SUHW and is present in the yeast nucleus at high levels. The GAL4 protein activates transcription in *D. melanogaster* (8) and is blocked by bacterial LexA protein in yeast cells (4), indicating that the failure of SUHW to block GAL4 in yeast cells is not because GAL4 activates through an unusual mechanism or because GAL4 is refractory to blocking. A key difference between enhancer-promoter interactions between *S. cerevisiae* and higher eukaryotes is that activator proteins do not function when more than a few hundred base pairs away from the promoter in yeast cells. Therefore, it is plausible that SUHW blocks enhancers by interfering with proteins or chromatin structures found only in higher eukaryotes, which allows enhancers to function several kilobases away from the promoter. For example, it is possible that SUHW disturbs a processive mechanism whereby enhancers find promoters by tracking along the chromatin fiber.

The problem in long-distance activation is to bring the enhancer physically close to the promoter to allow interactions between the proteins bound to them. We considered the

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possibility that changes in the structure of the DNA between the enhancer and promoter could be involved in enhancer blocking by SUHW and explored this idea by examining the effects of SUHW on DNA structure. Gel mobility experiments indicate that SUHW induces a change in DNA structure that is not a directed DNA bend. Indeed, the change in structure correlates with the loss of DNA sequence-directed bends, consistent with the idea that SUHW increases DNA flexibility. An increase in DNA flexibility should not, by itself, interfere with enhancer-promoter interactions. Indeed, we also find that DNA distortion requires a SUHW protein domain not required for DNA binding, but comparison of the domains required for DNA distortion with those required for blocking enhancers (18) indicates that DNA distortion is not sufficient to block enhancers. We suggest that DNA flexibility may facilitate interactions between SUHW and proteins that promote enhancer-promoter interactions.

MATERIALS AND METHODS

SUHW protein extracts. Wild-type and mutant SUHW proteins were expressed from full-length and modified cDNA clones in *S. cerevisiae* as previously described (19). The yeast expression vector producing a protein lacking the N- and C-terminal domains and consisting primarily of the zinc finger domain (containing residues 204 to 672) is described elsewhere (19). Other truncation and deletion mutants were constructed by using the same vector and various restriction enzyme sites. The C-terminal truncations often resulted in the substitution of a few novel amino acids at the C terminus, and some internal deletions were replaced by small insertions of novel amino acids. In all cases, the junctions created in the deletion mutants were confirmed by DNA sequencing. Details of the constructions and the exact amino acid sequences of the encoded proteins are available upon request. The exact restriction sites used to make deletions can be deduced by examination of the *su(Hw)* sequence (GenBank database accession number Y00228) and the abbreviated information presented here. The protein containing residues 1 to 672 was truncated at an *Eco*RI site, and as a result of a frameshift, the protein contains an additional two amino acids at the C terminus encoded by sequences from the 3′ untranslated region of the *su(Hw)* cDNA. The protein containing residues 1 to 705 was truncated at a *Pvu*II site and contained an additional 24 amino acids at the C terminus; the mutant containing residues 1 to 725 was truncated at a *Cfr*10I site and had a 3-amino-acid tail; the truncation with residues 1 to 781 was constructed using a *Bam*HI site and has a 22-amino-acid tail; the construct containing residues 1 to 880 used a *Ple*I site and has a 22-amino-acid tail. The internal deletion lacking residues 706 to 736 was created by deleting sequences between a *Pvu*II and *Bgl*I site and resulted in an insertion of two novel amino acids; the deletion of residues 738 to 780 was accomplished with *Bgl*I and *Bam*HI and was accompanied by an insertion of six novel amino acids; residues 656 to 780 were deleted with *Ban*II and *Bam*HI; residues 706 to 724 were deleted with *Pvu*II and *Cfr*10I; residues 656 to 704 were deleted with *Ban*II to *Pvu*II; and residues 853 to 880 were deleted with *Taq*I and *Ple*I and replaced by one novel amino acid.

Whole cell extracts from *S. cerevisiae* expressing wild-type or mutant SUHW proteins were prepared as previously described (19), and SUHW DNA-binding activity was purified approximately sevenfold by phosphocellulose chromatography. Whole cell extract (approximately 70 mg of protein) was applied to a phosphocellulose column (5-ml bed volume) in chromatography buffer (CB; 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 10% glycerol, 5 μM $ZnCl_2$, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml) containing 50 mM NaCl, washed with 2 column volumes of the same buffer, and eluted stepwise with 2 column volumes each of CB containing 0.15, 0.3, 0.4, 0.6, and 0.8 M NaCl. Most of the SUHW DNA-binding activity eluted in 0.6 M NaCl and was pooled, dialyzed against 100 volumes of CB containing 50 mM NaCl, and stored at −20°C.

DNA binding reactions. Binding reaction mixtures containing the indicated DNA fragments (0.16 μg/ml, 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation) and partially purified yeast extracts (120 μg/ml) in a total volume of 25 μl were incubated at room temperature for 15 min. All reaction mixtures contained 10 mM HEPES (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 5% (vol/vol) glycerol, 40 μg each of poly(dI-dC) and poly(dA-dT) per ml, 20 μg of pGEM-1 plasmid DNA per ml, and 200 μg of bovine serum albumin per ml. The binding reaction mixtures were subjected to low-ionic-strength (22.5 mM Tris base, 22.5 mM boric acid) electrophoresis in 3.5% polyacrylamide gels for gel mobility assays or to DNase I digestion for DNase I footprinting as described elsewhere (5).

Circular permutation analysis. To generate a series of circularly permuted linear fragments of equal size containing a single SUHW-binding site at various positions, a 46-bp double-stranded oligonucleotide (5) was cloned into the *Sal*I site of plasmid pBend2 (20) (provided by S. Adhya, National Institutes of Health) polylinker, and the orientation was determined by restriction. Fragments for gel mobility experiments were generated by restriction of the resulting plasmid (pBend2-S) with various enzymes cutting the tandemly duplicated polylinker of pBend2. The DNA distortion angle was estimated from the ratio of the fastest- and slowest-migrating SUHW-fragment complexes by using an empirical equation (34). Although this calculation does not give an absolute angle without external standards analyzed under the same gel conditions, the calculated DNA distortion angles were used only to compare the effects of various SUHW mutant proteins on mobility. The gel electrophoresis conditions were identical in all experiments, and SUHW proteins with known effects were included in all experiments as controls.

Phasing analysis. To generate a series of fragments in which a single SUHW-binding site is at various rotational orientations relative to a known sequence-directed DNA bend, a series of double-stranded oligonucleotides containing three helically phased A tracts and various lengths of end spacers were cloned into the *Xba*I site of pBend2-S to generate pBend2-S-39, -41, -45, and -47, where the second number is the base pairs between the center of the A tracts and the SUHW-binding sites. The upper strands of these oligonucleotides were CTAGTTTTTGCCCGTTTTTGCCCGTTTTT, CTAGTTTTTGCCCGTTTTTGCCCGTTTTTCT, CTAGTTTTTGCCCGTTTTTGCCCGTTTTTGAGCCT, and CTAGTTTTTGCCCGTTTTTGCCCGTTTTTGCGAGCCT, respectively. The lower strands were homologous except they had CTAG on the 5′ ends and lacked CTAG on the 3′ ends. Cloning into the *Xba*I site regenerated an *Xba*I site at one end of the inserted oligonucleotides, and the orientations in which the *Xba*I site was between the A tracts and the SUHW-binding site were used. Plasmids pBend2-S-43, -45, -49, and -51 were generated by digestion of pBend2-S-39, -41, -45, and -47, respectively, with *Xba*I, filling in with Klenow polymerase and deoxynucleotides, and religation. Constructs with the opposite orientation of the SUHW-binding site were constructed in the same

manner. Fragments for gel mobility shift experiments were prepared from the resulting plasmids by digestion with *Xho*I.

RESULTS

The SUHW-binding site (Fig. 1A) is well characterized (5, 33). It consists of a 12-bp core consensus sequence flanked by AT-rich regions. Although the sequences of the AT-rich regions are not conserved between binding sites, residues in the AT-rich regions are contacted by SUHW (5, 33). In most copies of the binding site, the AT-rich regions contain short A tracts that generate intrinsic DNA bends (22). Substitution of GC for AT base pairs in the A tracts reduces the affinity of SUHW binding (33), indicating that either the AT content or the bends are important for binding. Although SUHW has not been sufficiently purified to determine whether it binds as a monomer, it contains 12 potential zinc fingers and contacts approximately 30 base pairs. Therefore, on the basis of the crystal structure of the Zif268 zinc finger protein (26), in which neighboring zinc fingers contact neighboring base triplets in the major groove, SUHW is likely to bind as a monomer. Given the extended nature of the DNA contacts, SUHW wraps almost three times around the helix, creating significant opportunity for alteration of DNA structure.

SUHW alters DNA structure. Gel mobility assays were used to determine if SUHW alters DNA structure. Distortions such as bends in the DNA helix retard the migration of linear DNA fragments, and the closer the bend is to the middle of the fragment, the greater the retardation (37). This forms the basis of the circular permutation assay, in which the mobilities of linear fragments with the putative DNA bend at different positions are compared. DNA probes for a circular permutation assay were generated by inserting a single SUHW-binding site (Fig. 1A) into a unique *Sal*I site in the pBend2 plasmid (20). Cleavage with different enzymes yields a set of DNA fragments of identical length (166 bp) with the SUHW-binding site at various positions. These probes were incubated with partially purified SUHW produced in *S. cerevisiae* (see Materials and Methods), and the SUHW-DNA complexes were separated by polyacrylamide gel electrophoresis. The complexes displayed different mobilities (Fig. 1B, first eight lanes), with the fragment containing the binding site near the center of the fragment having the lowest mobility. Mobility was highest when the binding site was at the end of the fragment. No significant differences in the mobilities of the unbound fragments were observed. This mobility pattern is observed with proteins that induce a bend in the DNA helix (34, 37), although other structural distortions can reduce electrophoretic mobility (9, 16, 17).

If it is assumed that SUHW induces a DNA bend, the angle of bending is estimated on the basis of multiple experiments to be between 60 and 70°, using an empirical formula (34). Because the circular permutation assay does not distinguish between DNA bends and other distortions, it has been suggested that the empirically determined value should be referred to as the DNA distortion angle (17). By plotting the relative mobilities of the complexes against the binding site position (Fig. 2), the distortion induced by SUHW appears to be centered in an AT-rich region 10 to 12 bp upstream of the center of the core consensus sequence (Fig. 1A). The asymmetry was reproducible in all experiments and was independent of the orientation of the binding site within the pBend2 polylinker (data not shown).

Although the experiments were performed with SUHW partially purified from *S. cerevisiae*, it is extremely unlikely that the effect on DNA structure is because of a yeast protein. The

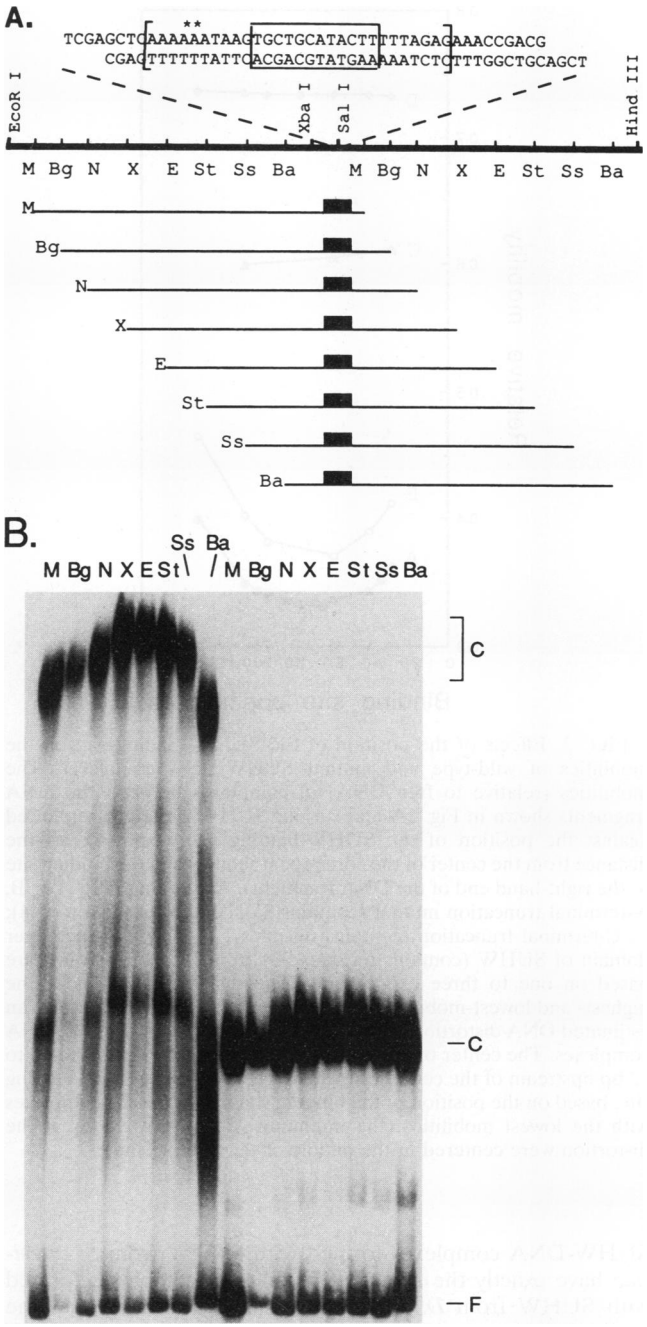

FIG. 1. Circular permutation gel mobility assay for effects of SUHW on DNA structure. (A) The shown double-stranded oligonucleotide containing a single SUHW-binding site was cloned in both orientations into the *Sal*I site between the tandem polylinker repeats of the pBend2 vector (20). The 12-bp consensus repeat conserved between different SUHW-binding sites is boxed, the residues known to be contacted by SUHW are between the brackets, and the asterisks above the sequence indicate the estimated center of the DNA distortion induced by SUHW. The 166-bp circularly permuted linear fragments were produced by restriction of various pBend2 polylinker sites: *Mlu*I (M), *Bgl*II (Bg), *Nhe*I (N), *Xho*I (X), *Eco*RV (E), *Stu*I (St), *Ssp*I (Ss), and *Bam*HI (Ba). (B) Autoradiogram of a mobility shift gel using the linear fragments with the SUHW-binding site orientation shown in panel A and either full-length SUHW (first eight lanes) or a truncated protein (last eight lanes) consisting primarily of the zinc finger domain (containing amino acids 204 to 672) partially purified from yeast cells. F, free DNA; C, SUHW-DNA complexes.

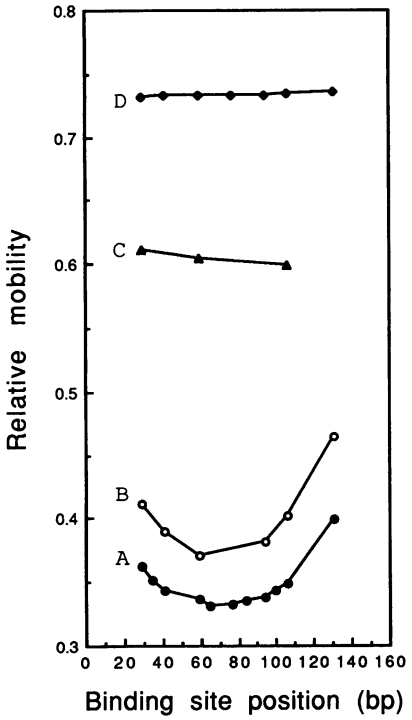

FIG. 2. Effects of the position of the SUHW-binding site on the mobilities of wild-type and mutant SUHW-DNA complexes. The mobilities (relative to free DNA) of complexes between the DNA fragments shown in Fig. 1A and various SUHW proteins are plotted against the position of the SUHW-binding site (expressed as the distance from the center of the core repeat sequence in the binding site to the right-hand end of the DNA fragment). A, full-length SUHW; B, N-terminal truncation mutant (contains SUHW residues 204 to 944); C, C-terminal truncation (contains residues 1 to 672); D, zinc finger domain of SUHW (contains residues 204 to 672). Datum points are based on one to three experiments. The relative mobilities of the highest- and lowest-mobility complexes were used to calculate (34) an estimated DNA distortion angle of 65° for the full-length SUHW DNA complexes. The center of the DNA distortion is estimated to be 10 to 12 bp upstream of the center of the core repeat of the SUHW-binding site, based on the position of the binding site in the SUHW complexes with the lowest mobilities (the minimum would be at 83 bp if the distortion were centered in the middle of the binding site).

SUHW-DNA complexes formed with SUHW from *S. cerevisiae* have exactly the same gel mobility as complexes formed with SUHW from *D. melanogaster* (19), indicating that the complex does not contain yeast proteins. Furthermore, SUHW partially purified from *D. melanogaster* has the same effect on DNA structure as SUHW produced in *S. cerevisiae* in the circular permutation assay (data not shown).

A SUHW domain not required for DNA binding is required for DNA distortion. The crystallographic structure of the Zif268 zinc finger protein indicates that it might induce only slight changes in the structure of the bound DNA (26). Given the apparent large effect of the SUHW protein, it was of interest to determine whether a protein domain other than the zinc finger domain was required for the structural alteration. Furthermore, it was also possible that the mobility anomalies were the result of a SUHW protein structure as proposed for the yeast GCN4 protein (9).

The 120-kDa SUHW protein contains three general domains (Fig. 3). The 12 potential zinc fingers are clustered in the middle between flanking hydrophilic N- and C-terminal domains. To map the domains required for alteration of DNA structure, the circularly permuted DNA probes were incubated with partially purified deletion mutant SUHW proteins produced in yeast cells, and the complexes were separated by polyacrylamide gel electrophoresis. All mutant proteins containing the zinc finger domain bound DNA, and mutant protein lacking only the N-terminal domain gave the nearly the same DNA distortion angle as did the wild type (Fig. 2 and 3). However, all mutant proteins lacking the C-terminal domain did not alter DNA structure (Fig. 3), including the mutant lacking both the N- and C-terminal domains and consisting primarily of the zinc finger domain (Fig. 1 to 3).

The C-terminal residues required for the effect on DNA structure were mapped by testing several C-terminal truncation and internal deletion mutant proteins (Fig. 3). Although comparison of C-terminal truncation mutants suggested that amino acid residues between 705 to 781 of SUHW are required to alter DNA structure, internal deletions of residues in this region did not result in loss of the ability to alter DNA structure (Fig. 3). Therefore, we conclude that either the C-terminal domain contains redundant subdomains or only a certain minimum amount of the primarily acidic C terminus is required to alter DNA structure.

To examine the possibility that SUHW proteins lacking the C-terminal domain fail to alter DNA structure because they might bind differently to DNA, we compared the DNase I footprints of the full-length protein and the protein consisting primarily of the zinc finger DNA-binding domain (containing residues 204 to 672). The footprints were indistinguishable (Fig. 4), suggesting that the zinc finger domain alone contacts the same DNA sequences as the full-length protein. There were short sequences in the binding site that were not digested by DNase I even in the absence of SUHW, and differences in these regions would therefore not be detected. However, the same extract and salt concentrations were required to obtain equivalent protections with the two proteins, indicating that the binding affinity was not significantly altered by the loss of the ability to alter DNA structure.

SUHW alters A-tract bends in the binding site. The circular permutation assay indicated that SUHW alters DNA structure and that it requires a protein domain not involved in DNA binding, although the possibility that the observed mobility effects are due to protein structure was not entirely ruled out. To determine if the anomalous mobility in the circular permutation assay is the result of a directed DNA bend, a phasing analysis (39) was performed in which the SUHW-binding site was placed at different helical rotations relative to a DNA sequence-directed bend. If the alteration in DNA structure is a directed bend, then when it is rotationally aligned so that it is in the same direction relative to the helical axis as the known bend, the effect on mobility will be increased, but if it is rotationally aligned such that it is in the opposite direction as the known bend, then the effects of the two bends on gel mobility will cancel each other out. Therefore, phasing analysis provides a sensitive method for distinguishing between directed DNA bends and other types of structural alterations (16, 17, 31, 32, 39).

We prepared a set of DNA probes that contained a SUHW-binding site separated by short spacers of variable length from three A tracts in phase with each other (Fig. 5A). The phased A tracts are estimated to create a total bend of approximately 50 to 60° (34). The spacer lengths varied over one turn of the helix to place the binding site in various helical rotations with the phased A-tract bend. Complexes of SUHW with these probes were subjected to polyacrylamide gel electrophoresis

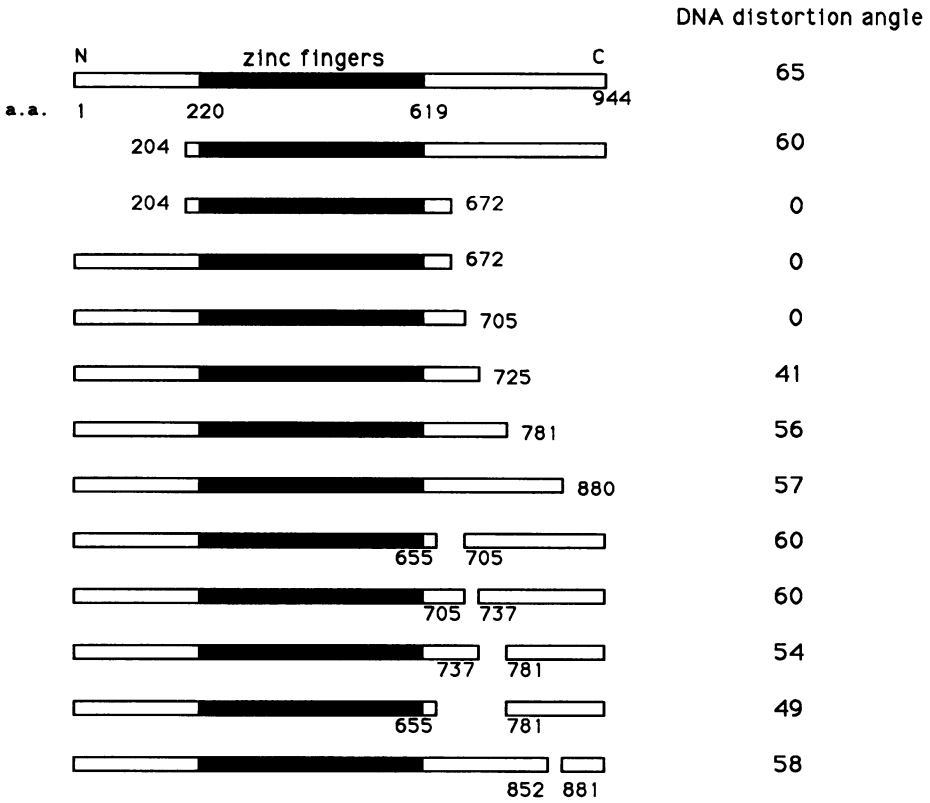

FIG. 3. Mapping the SUHW domains required to alter DNA structure. At the top is a map of the full-length protein, with the N terminus on the left and the domain containing the 12 potential zinc fingers indicated by the shaded portion. Maps of the residues remaining in the various truncation and internal deletion mutants are shown below. The calculated DNA distortion angles (in degrees) based on one to three experiments are shown on the right. All experiments included a protein with a known effect, usually the full-length protein, as a control. The distortion angle calculated for the full-length protein varied less than 8% from the mean in independent experiments. The distortion angles were not normalized to known DNA bend angles and therefore should be used only as a means of comparing the relative gel mobility anomalies induced by the various SUHW proteins.

and found to have the same mobilities, regardless of the length of the spacer between the A tracts and the SUHW-binding site (Fig. 5B). In contrast, the unbound probes varied in mobility, displaying phasing between the A tracts in the binding site with the external A tracts. Loss of phasing between the binding-site A tracts and the external A tracts in the SUHW complexes was also observed when a similar series of fragments with the binding site in the opposite orientation was tested (data not shown).

The two binding site A tracts are separated by approximately one helical turn and are on opposite strands (Fig. 1A), and no overall bend was detected in the unbound site by the circular permutation assay (Fig. 1B). However, phasing between the internal and external A tracts in the unbound DNA fragments is expected because the end-to-end distances of the DNA molecules vary with the different helical orientations of the binding site to the external A-tract bend (Fig. 5A).

The loss of A-tract phasing in the SUHW complexes correlates with the DNA distortion induced by SUHW. Thus, mutant proteins that do not appear to alter DNA structure by the circular permutation assay, such as the protein consisting primarily of the zinc finger domain, produce complexes with mobilities proportional to the free probe mobilities in the phasing assay (Fig. 5C and data not shown). This finding indicates that the A tracts in the binding site can still phase with the external A tracts even when bound by protein. Phasing between internal and external A tracts was also detected in complexes with the protein truncated at amino acid 705, the highest-molecular-weight mutant tested that does not alter DNA structure, and was not observed with the protein truncated at amino acid 781, the lowest-molecular-weight C-terminal truncation that gives a mobility anomaly similar to that of wild-type SUHW (data not shown). Therefore, the inability to detect A-tract phasing in wild-type SUHW complexes is not an inability to resolve mobility differences, because these two mutant protein complexes have similar intermediate mobilities.

The phasing experiments indicate that SUHW does not induce a directed DNA bend that can phase with an A-tract-directed bend. They also indicate that when SUHW binds, it somehow prevents the A-tract bends in the binding site from phasing with the external A-tract bends. Because the loss of phasing between the internal and external A tracts correlates with the ability of the SUHW proteins to cause anomalous mobilities in the circular permutation assay, we conclude that the altered mobilities in the circular permutation assay involve an alteration of DNA structure.

SUHW may increase DNA flexibility. The effects of SUHW on DNA structure can be explained by an increase in DNA flexibility in the binding site. Increased flexibility would allow the bound site to bend in several directions, thereby causing anomalous mobility in the circular permutation assay. Further-

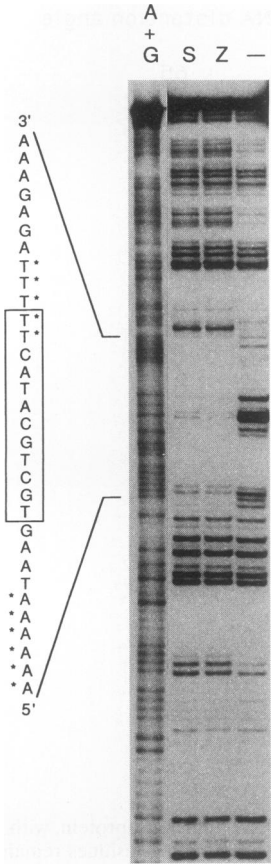

FIG. 4. DNase I footprints of full-length SUHW and the zinc finger domain. Shown is an autoradiogram of a denaturing polyacrylamide gel used to separate the products of DNase I digestion of SUHW-DNA complexes. The binding reaction mixtures contained a 0.16-μg/ml concentration of a 5′-end-labeled 326-bp fragment from the bx^{34e} gypsy element (BaBx [5]) that contains multiple SUHW-binding sites and a 120-μg/ml concentration of partially purified yeast extract containing full-length SUHW (S) or a truncated protein (Z) consisting primarily of the zinc finger domain (containing residues 204 to 672). The other lanes are a no-extract control (−) and a Maxam-Gilbert sequencing reaction (A+G) for alignment.

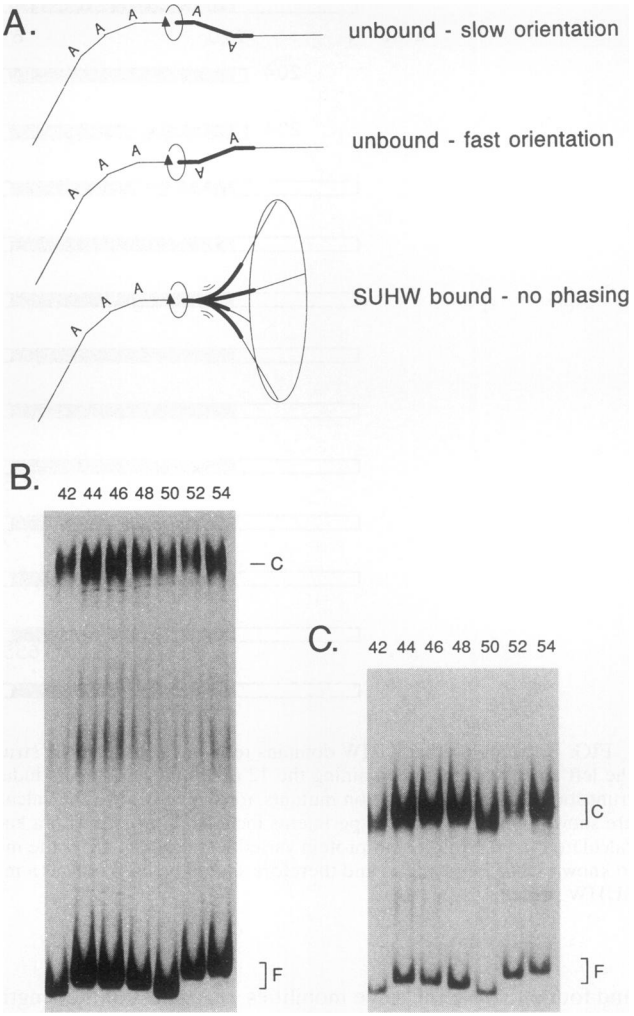

FIG. 5. Phasing between the SUHW-binding site and external A tracts. (A) Schematic diagrams of linear fragments generated from pBend2 constructs containing a single SUHW-binding site (heavy line) and a series of phased A tracts that generate a directed DNA bend of approximately 50 to 60°. The two A tracts in the SUHW-binding site are shown, and the variable spacer used to alter the rotational alignment of the SUHW-binding site and the external A tracts is indicated by the circular arrow. The top diagram is a fragment in which the SUHW-binding-site A tract closest to the external A tracts is in phase with the external A tracts, and just below is a fragment in which the closest binding-site A tract is maximally out of phase. The bottom diagram illustrates that if SUHW allowed the binding site to flex in several different directions, it would prevent phasing between the external A-tract bend and either the SUHW-induced flexure or the internal binding-site A tracts. (B) Autoradiogram of a mobility shift gel used to separate complexes formed between full-length SUHW and fragments with various spacers between the external A-tract bend and the SUHW-binding site. The number of base pairs between the center of the external A tracts and the center of the core repeat in the SUHW-binding site is indicated at the top of each lane. F, free probe DNA; C, SUHW-DNA complexes. (C) Autoradiogram of a mobility shift gel using the zinc finger domain of SUHW (residues 204 to 672) and the same fragments as in panel B.

more, because the complexes could bend in several directions, the SUHW-induced DNA flexure would not phase with external A-tract bends (Fig. 5A), and A tracts internal to the binding site would also not phase with external A tracts when flexible DNA separates them from the external A tracts. Consistent with this hypothesis, it has very recently been demonstrated that localized DNA flexibility resulting from unpaired bases gives a mobility anomaly in the circular permutation assay but does not phase with neighboring A-tract bends (15a).

Because the loss of phasing between the internal and external A tracts was observed with both orientations of the binding site, the increased flexibility cannot be at just one point in the binding site. For example, we considered the possibility that an increase in flexibility occurred just in the A tract that maps closest to the apparent center of the DNA distortion determined by the circular permutation analysis. Phasing would not occur when the flexible A tract was between the external bend and the inflexible A tract. However, in the other orientation of the binding site, the inflexible A tract would

phase with the external bend even when bound to SUHW. Indeed, because the two binding site A-tract bends almost cancel each other out in the unbound site, the phasing between the external A tracts and the inflexible A tract should demonstrate a greater amplitude upon protein binding. However, both binding-site orientations demonstrated a loss of phasing upon SUHW binding, indicating that increased DNA flexibility occurs within both binding-site A tracts and perhaps in the entire binding site.

It is possible that DNA flexure is caused by melting of the AT-rich regions in the binding site, which should generate single-stranded DNA. We used several methods, including $KMnO_4$ oxidation (2), dimethyl sulfate-S1 nuclease treatment (1), and P1 nuclease digestion (3), but failed to detect single-stranded DNA (data not shown). It is likely, however, that even if single-stranded DNA were present, it would be protected from these reagents by SUHW. Indeed, this region is protected from hydroxy radicals by SUHW (33).

DISCUSSION

Effect of SUHW on DNA structure. The SUHW zinc finger protein encoded by the *su(Hw)* gene binds to gypsy retrotransposon insertions and, by an unknown mechanism, prevents enhancers that are promoter distal to the gypsy insertions from activating transcription (6, 10, 11, 13, 14). Investigation of the mechanism by which SUHW blocks enhancers should increase knowledge of how enhancers function. Because SUHW blocks several different enhancers in a distance-independent manner, but only when positioned between the enhancer and promoter, it can be inferred that SUHW does not interact with activator proteins that bind to enhancers. We prefer the alternative idea that SUHW interferes with proteins or chromatin structures specific to higher eukaryotes that support long-distance communication between enhancer- and promoter-binding proteins. This would explain why SUHW blocks a 90-kb interaction between the wing margin enhancer and the promoter in the *cut* gene (6, 14) yet fails to block a short-range interaction in yeast cells (19). One factor that we thought might be involved in SUHW enhancer blocking is an alteration in DNA structure that is incompatible with enhancer-promoter interactions. Therefore, we tested whether SUHW alters DNA structure using gel mobility shift assays. Although we find that SUHW alters DNA structure, the change appears to be an increase in DNA flexibility, which by itself should not interfere with long-distance enhancer-promoter interactions.

Although it has not been proven that the change in DNA structure induced by SUHW is an increase in DNA flexibility, it is difficult to imagine other distortions that could explain the presence of a gel mobility anomaly in the circular permutation assay and the lack of a directed DNA bend in the phasing analysis. Furthermore, it has recently been demonstrated that DNA flexibility resulting from unpaired bases behaves like a bend in the circular permutation assay but does not phase with external directed DNA bends (15a). DNA flexibility has also been proposed to explain the effects of a mutant Fos-Jun heterodimer on DNA structure (16, 17). This proposal is also based on a mobility anomaly in the circular permutation assay and a lack of phasing with external A-tract bends. In the case of SUHW, however, the loss of preexisting A-tract bends in the binding site provides additional strong evidence for an increase in DNA flexibility. The loss of the A-tract bends also provides proof that the DNA is distorted and therefore that the mobility anomaly is not entirely an effect of protein structure as suggested for the GCN4 protein (9). Because phasing analysis is not used routinely, it is feasible that other DNA-binding proteins thought to bend DNA actually increase DNA flexibility.

The role of the C-terminal domain of SUHW in altering DNA structure. We find that distortion of DNA structure by SUHW protein requires most of the C-terminal domain, which is not required for DNA binding (Fig. 3). There are at least two other proteins that induce DNA distortions by using a domain not essential for binding DNA. Limited proteolysis of the TUF yeast regulatory factor generates protein fragments that bind DNA as well as the complete protein but do not alter DNA structure (36). Similar to SUHW, TUF induces an asymmetric flexure center, but it is unknown if the distortion is a directed bend. The bacteriophage ϕ29 p4 protein contains a 12-amino-acid basic domain that increases DNA flexure without changing the binding affinity (29). With both the TUF and p4 proteins, however, it is believed that the domains required for DNA flexure contact DNA. In contrast, the SUHW domain required for DNA flexure does not alter the DNase I footprint (Fig. 4) and therefore might function indirectly by altering the conformation of the zinc finger domain.

Because much of the SUHW C-terminal domain is rich in acidic residues, it is also feasible that DNA distortion involves electrostatic repulsion between the C-terminal domain and the phosphate groups in the DNA. In this case, it might be expected that the distortion angle would vary with the number of acidic residues, but in the series of truncated proteins, the ability to alter DNA structure is lost suddenly rather than gradually (Fig. 3). The sudden loss might suggest that the structural distortion requires a critical mass of acidic residues. However, the N-terminal domain of SUHW also contains a large cluster of acidic residues (25) that is equivalent to the cluster left in some of the C-terminal truncations that alter DNA structure, yet the N-terminal region has no effect on DNA structure (Fig. 3). Therefore, DNA distortion is likely to require more than simple electrostatic repulsion. Furthermore, if the C-terminal domain has a fixed orientation relative to the DNA-binding domain of SUHW, simple electrostatic repulsion would be expected to cause a bend with a fixed angle relative to the DNA axis, but the DNA distortion induced by SUHW does not phase with external A-tract bends (Fig. 5).

The role of DNA flexibility in blocking enhancers. Although increased DNA flexibility might be expected to aid long-distance enhancer-promoter interactions, it is also possible that by facilitating the appropriate protein-protein interactions, increased flexibility could assist formation of a structure that antagonizes enhancer-promoter interactions. Increased flexibility could assist structure formation by bringing DNA-bound proteins and sites on DNA into contact with each other, similar to the way DNA bends aid initiation of DNA replication (21, 24, 38), site-specific recombination (12, 28), and transcription activation (reviewed in reference 35). Increased DNA flexibility could also help SUHW interact and interfere with factors that assist long-distance enhancer-promoter interactions.

We have attempted to examine the role of the DNA distortion induced by SUHW in enhancer blocking by comparing the protein domains required for DNA distortion with the domains required for blocking. In the C-terminal domain, only deletions affecting residues between 737 to 880 dramatically reduce the ability of SUHW to block enhancers in the *yellow*, *cut*, and *Ultrabithorax* genes (18). Deletions affecting only this region, however, do not significantly reduce the ability of SUHW to alter DNA structure (Fig. 3). Indeed, because most of the C-terminal domain must be deleted to prevent DNA distortion, a protein lacking the ability to alter DNA structure cannot be made without also deleting the domain important

for enhancer blocking. Therefore, it cannot be determined if DNA distortion is required for full enhancer-blocking activity. However, the mutant SUHW proteins with reduced enhancer-blocking activity that retain the ability to distort DNA indicate that enhancer-blocking requires more than DNA distortion. Indeed, the domain important for enhancer blocking contains a potential leucine zipper (25), suggesting that it might be involved in protein-protein interactions. It is plausible, therefore, that increased DNA flexibility facilitates interactions between SUHW and proteins involved in long-distance enhancer-promoter interactions.

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