Distance-Independent Inactivation of an Enhancer by the *suppressor of Hairy-wing* DNA-Binding Protein of Drosophila

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ABSTRACT

When the *gypsy* retrotransposon of Drosophila inserts between an enhancer and promoter it prevents the enhancer from activating transcription. Enhancers are blocked because the protein (SUHW) encoded by the *suppressor of Hairy-wing* [*su(Hw)*] gene binds to *gypsy*. For example, *gypsy* insertions in an 85 kilobase region between a wing margin-specific enhancer and the promoter in the *cut* gene cause a cut wing phenotype that is suppressed by *su(Hw)* mutations. A temperature-sensitive combination of mutant *su(Hw)* alleles was used to investigate the mechanism by which SUHW blocks the *cut* wing margin enhancer. By shifting from the nonpermissive to the permissive temperature and vice versa at various stages in development it was found that active SUHW is only required around pupariation when the wing margin enhancer is active to cause a cut wing phenotype. This was true whether *gypsy* was in the embryonic control region near the promoter, or in the late larval control region near the wing margin enhancer. These results indicate that SUHW must be active only when an enhancer is active to block the enhancer. Furthermore, the observations also indicate that enhancer-blocking by SUHW is reversible and that it occurs soon after binding of active SUHW to *gypsy* DNA. These results are consistent with models in which SUHW structurally interferes with enhancer-promoter interactions.

SEVERAL naturally occurring mutations in different genes of *Drosophila melanogaster* are *gypsy* retrotransposon insertions (MODOLELL, BENDER and MESELSON 1983). The phenotypes associated with *gypsy* insertions are suppressed by mutations in the *suppressor of Hairy-wing* [*su(Hw)*] gene (MODOLELL, BENDER and MESELSON 1983; RUTLEDGE *et al.* 1988), which encodes a protein (SUHW) with 12 putative zinc fingers (PARKHURST *et al.* 1988). SUHW binds a consensus DNA sequence (DORSETT 1990; SPANA and CORCES 1990) that is repeated several times downstream of the *gypsy* 5′ long terminal repeat (LTR) and the phenotypic severity of *gypsy* insertion alleles increases with the number of SUHW-binding repeats (PEIFER and BENDER 1988). It appears, therefore, that SUHW is required for most of the mutagenic effects of *gypsy*.

The SUHW bound to *gypsy* DNA alters expression of surrounding genes by at least two mechanisms. SUHW increases truncation of transcripts at the polyadenylation site in the *gypsy* 5′ LTR (DORSETT *et al.* 1989; DORSETT 1990), which results in a reduction in readthrough transcripts. SUHW also represses transcription initiation by blocking interactions between transcription control elements when positioned between the interacting control elements (GEYER, GREEN and CORCES 1990; HOLDRIDGE and DORSETT 1991; JACK *et al.* 1991; GEYER and CORCES 1992).

SUHW blocks very long distance interactions between transcription control elements in *gypsy* insertion alleles of *cut*, indicating that SUHW does not block by simple steric hindrance. The *cut* gene is a complex locus on the *X* chromosome encoding a homeobox-containing protein (BLOCHLINGER *et al.* 1988) required for differentiation of several different cell types (JOHNSON and JUDD 1979; JACK 1985; BODMER *et al.* 1987; BLOCHLINGER *et al.* 1990; LIU, MCLEOD and JACK 1991; JACK *et al.* 1991). Several different *gypsy* insertions in an 80-kb region of *cut* give rise to a cut wing phenotype (JACK 1985). The *gypsy* insertions are all downstream of a wing margin-specific transcription enhancer located 85 kb upstream of the putative promoter (JACK *et al.* 1991; Figure 1). Therefore SUHW blocks wing margin enhancer interactions with the promoter from several different positions between the enhancer and promoter, indicating that enhancer-blocking is virtually distance independent.

Because SUHW interferes with several enhancers, but only when located between them and the promoter (GEYER, GREEN and CORCES 1990; HOLDRIDGE and DORSETT 1991; JACK *et al.* 1991; GEYER and CORCES 1992), it seems very unlikely that SUHW blocks by interacting with enhancer or promoter-binding factors and more plausible that it interferes specifically with mechanisms that support long distance activation. Although these mechanisms remain

TABLE 1

cut and *suppressor of Hairy-wing* alleles

Allele	Chromosome(s)	Chromosome reference or source
ct^6	$y^2\ w^a ct^6\ f$	RUTLEDGE *et al.* (1988)
ct^{L-1}	ct^{L-1}	JACK (1985)
ct^{83h}	$In(1)FM6,\ y^{31d}\ sc^8\ w^{FM6}\ dm^+\ ct^{83h}\ B$	J. LIM, and this study
ct^{2s}	$w^{aG} ct^{2s}$	T. GERASIMOVA
$su(Hw)^2$	$su(Hw)^2\ sbd$	MODOLELL, BENDER and MESELSON (1983)
$su(Hw)^f$	$In(3LR)TM6,\ Hn^P ss^{P88}\ su(Hw)^f\ bx^{34e}\ Ubx^{P15}\ e^s$	MODOLELL, BENDER and MESELSON (1983)
$su(Hw)^{e2}$	$su(Hw)^{e2}\ bx^{34e}$	R. COYNE, M. MORTIN and M. MESELSON
	$su(Hw)^{e2}$	This study

The features of the individual alleles are described in the text.

obscure, examination of the *cut* control region suggests that in some genes, long distance activation may involve linear organization of the control elements. The *cut* enhancers are arranged in developmental order, with enhancers active during embryogenesis near the promoter (J. JACK, personal communication), and enhancers operating at later developmental stages more distal. Thus *gypsy* insertions near the promoter are embryonic lethal, insertions near the middle of the control region are larval lethal, and more distal *gypsy* insertions are viable and affect primarily the wing margin enhancer active around pupariation (JACK 1985; JACK *et al.* 1991; see Figure 1). The enhancer positioning suggests the *cut* control region is developmentally regulated.

In this report a temperature-sensitive combination of *su(Hw)* mutant alleles was used to examine whether SUHW interferes with developmental regulation of the *cut* enhancers or whether SUHW blocks the *cut* enhancers by a more direct mechanism. It was found that to block the distal wing margin enhancer, SUHW was required only around pupariation when the wing margin enhancer is active. This period was not affected by the position of the *gypsy* insertion in the control region, indicating that SUHW does not interfere with developmental regulation of the *cut* control region. The results also demonstrate that enhancer-blocking by SUHW is reversible, and that it occurs soon after binding of SUHW to *gypsy*. It is proposed that SUHW interferes directly with enhancer-promoter interactions by a general mechanism such as formation of chromatin structures incompatible with the structures that support long distance activation.

MATERIALS AND METHODS

Genetic crosses and quantitation of cut wing phenotypes: The *cut* and *su(Hw)* alleles used are listed in Table 1. Flies were raised on cornmeal, yeast and molasses medium (WIRTZ and SEMEY 1982) at the indicated temperatures. All crosses were performed in standard 25 × 95 mm glass shell vials with 5–10 males and 10–15 females per vial.

The partial cut wing phenotypes of progeny flies were quantitated by counting the number of gaps in the row of bristles along the wing margin Examples of cut wing phenotypes are shown in Figure 2. Only flies with two complete wings were scored. The number of gaps varied from none to approximately 20 per fly and were variable in position and size. When there were many wing gaps counting was slightly subjective. In practice, about 20 wing gaps per fly was the maximum number that could be reliably counted and the few flies that had slightly more gaps were rounded off to 20 gaps. Penetrance was calculated as the proportion of progeny that display one or more gaps and expressivity was calculated as the average number of wing gaps per fly among those flies displaying a phenotype.

Wing photography: Wings were flattened on glass slides in a drop of xylene and mounted in Permount after most of the xylene had evaporated. The photographs were taken using with a Zeiss Axioplan microscope at a magnification of 62.5 on Kodak Tmax film.

Mapping the ct^{83h} gypsy insertion: On the basis of phenotype the position of the *gypsy* insertion in the viable ct^{83h} allele used in most of the experiments was predicted to be near the site of *gypsy* insertion in the ct^6 allele. To confirm the position of the gypsy element in ct^{83h}, genomic DNA was prepared from 0.5 g each of $FM6,\ y^{31d}\ sc^8\ w^{FM6}\ dm^+\ ct^{83h}\ B$ and Oregon R wild-type flies as described elsewhere (LEVIS, BINGHAM and RUBIN 1982). Both genomic DNAs were restricted separately with *Eco*RI and *Hin*dIII (5 μg per reaction) and subjected to agarose gel electrophoresis and Southern blot hybridization with a radioactive probe prepared by random primer extension (SAMBROOK, FRITSCH and MANIATIS 1989) of Mct5 phage DNA (JACK 1985). This phage contains wild type *cut* sequences spanning the insertion sites of most viable gypsy insertions, including ct^6. Oregon R DNA gave the expected *Eco*RI and *Hin*dIII fragments and the ct^{83h} insertion was found to be in the same *Eco*RI and *Hin*dIII fragments (approximately 9 kb each) as the ct^6 insertion. *Gypsy* contains a single *Eco*RI site near the 3′ end, and the sizes of the *gypsy-cut Eco*RI junction fragments indicated that the ct^{83h} insertion site is within 0.2 kb of the ct^6 insertion site, and that the ct^{83h} *gypsy* element is oriented parallel to *cut*.

RESULTS

The mechanism by which the SUHW protein blocks enhancers was explored using a temperature-sensitive combination of *su(Hw)* alleles. Previous studies have not revealed whether or not the effects of SUHW are reversible, or if SUHW is required only when an enhancer is actually active to block that enhancer. We wished to determine whether SUHW blocks distal

enhancers in *cut* by interfering with developmental programming of the *cut* control region or by a more direct mechanism. For example, if SUHW blocked *cut* enhancers by interfering with a change in chromatin structure that begins near the promoter and proceeds distally as the enhancers become active in developmental order, then the developmental stages at which SUHW must be present in order to block distal enhancers should depend on the position of the *gypsy* insertion in the control region, and the effects of SUHW may not be reversible. Alternatively, if SUHW blocked distal enhancers more directly by, for example, forming particular chromatin structures incompatible with long distance communication, SUHW should be required only when an enhancer is active. These possibilities were examined using temperature shifts to determine the developmental stages at which SUHW must be active to block the distal *cut* wing margin enhancer with *gypsy* insertions at different positions.

The *su(Hw)^{e2}* allele in combination with either the *su(Hw)^{2}* or *su(Hw)^{f}* alleles displays temperature-sensitive suppression of the cut wing phenotype: To conduct these experiments it was necessary to identify mutant *su(Hw)* alleles that display temperature-sensitive suppression of the cut wing phenotype of *gypsy* insertion alleles of *cut*. When the ethyl methanesulfonate-generated *su(Hw)^{e2}* allele was isolated it was noted that in combination with the *su(Hw)^{f}* allele, *su(Hw)^{e2}* displayed temperature-sensitive suppression of the *bithorax^{34e}* (*bx^{34e}*) gypsy insertion allele (R. COYNE, unpublished observations). With *su(Hw)^{e2} bx^{34e}/ In(3LR)TM6, Hn^{P} ss^{P88} su(Hw)^{f} bx^{34e} Ubx^{P15} e^{S}* flies, the bithorax phenotype was strong at 18°, intermediate at 25° and nearly completely suppressed at 29°. After confirming this observation, *su(Hw)^{e2}* combinations with other *su(Hw)* alleles were tested for temperature-sensitive suppression of the cut wing phenotype of the viable *ct^{6}* gypsy insertion allele (Figure 1).

In combination with *su(Hw)^{2}* or *su(Hw)^{f}*, *su(Hw)^{e2}* displayed temperature-sensitive suppression of *ct^{6}* (Table 2). The *su(Hw)^{2}* allele is a female-sterile insertion allele (PARKHURST *et al.* 1988). The *su(Hw)^{f}* allele complements the female-sterility of *su(Hw)^{2}* and is a point mutation in one of the zinc fingers (HOOVER *et al.* 1992). The *su(Hw)^{2}/su(Hw)^{f}* heteroallelic combination, which strongly suppresses *gypsy* insertion alleles (RUTLEDGE *et al.* 1988), reduces SUHW DNA-binding activity to around 20% of wild-type levels (DORSETT 1990). At 18° virtually all of the *su(Hw)^{e2}/su(Hw)^{2}* flies and approximately 40% of the *su(Hw)^{e2}/su(Hw)^{f}* flies displayed a partial cut wing phenotype, similar to the phenotype in Figure 2E. This phenotype is much less severe than that observed with wild-type *su(Hw)* and *gypsy* insertion alleles of *cut* (Figure 2B). At 29° suppression of the cut wing phenotype of *ct^{6}* was virtually complete, with approximately 90% of the flies displaying wild-type wing phenotypes with both the *su(Hw)^{e2}/su(Hw)^{2}* and *su(Hw)^{e2}/su(Hw)^{f}* allele combinations. At 25° the wing phenotypes with both of these combinations were similar to those observed at 18°, although the penetrance was somewhat less than at 18°. In controls, the cut wing phenotype in *ct^{6}* flies with wild type *su(Hw)* and the suppressed cut wing phenotype in *ct^{6}* flies either homozygous for *su(Hw)^{2}* or heteroallelic for *su(Hw)^{2}* and *su(Hw)^{f}* were unaffected by temperature (not shown).

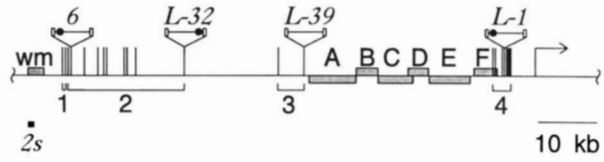

FIGURE 1.—The *cut* locus control region. The putative transcription start site based on cDNA sequences (BLOCHLINGER *et al.* 1988; J. JACK, personal communication) is indicated by the angled arrow to the right, and the positions of independent *gypsy* insertions with a cut wing phenotype are indicated by vertical lines (after JACK 1985). In some cases the *gypsy* element and the allele designation are shown, with open boxes indicating the *gypsy* long terminal repeats (LTRs) and filled circles indicating the SUHW-binding region when the *gypsy* element orientation is known. The restriction fragment containing the wing margin enhancer (JACK *et al.* 1991) is indicated by the shaded box labeled "wm," and restriction fragments known to contain one or more enhancers driving *cut* expression in specific embryonic tissues (J. JACK, personal communication) are labeled "A" through "F." The region deleted in the *ct^{2s}* deficiency is indicated underneath by a filled box (after MOGILA *et al.* 1992). The *gypsy* insertions are classified into four phenotypic classes. Class 1 insertions are viable and display only a cut wing phenotype and class 2 insertions are viable and display cut wing and vibrissae phenotypes (JOHNSON and JUDD 1979; JACK 1985). Class 3 insertions are homozygous and hemizygous larval lethal and fail to complement the phenotypes of class 1 and class 2 insertions (JACK 1985). Class 4 insertions are embryonic lethal with phenotypes in several embryonic tissues (JACK 1985; BODMER *et al.* 1987; BLOCHLINGER *et al.* 1990; LIU, MCCLEOD and JACK 1991; J. JACK, personal communication) and fail to complement the phenotypes of the other three classes. The site of the *ct^{83h} gypsy* insertion (J. LIM, personal communication) that occurred in the *In(1)FM6, y^{31d} sc^{8} w^{FM6} dm^{+} B* chromosome (KIDD 1966) was mapped to within 0.2 kb of the *ct^{6}* insertion site, and the *ct^{83h} gypsy* element is in the same orientation as the *ct^{6}* gypsy element.

TABLE 2

Temperature-sensitive suppression of *ct^{6}*

Temperature	Penetrance of cut wing phenotype			
	n^{a}	*su(Hw)^{e2}/su(Hw)^{2}*	n	*su(Hw)^{e2}/su(Hw)^{f}*
18°	65	0.98 ± 0.02[b]	63	0.41 ± 0.06
25°	62	0.68 ± 0.03	33	0.30 ± 0.08
29°	57	0.09 ± 0.04	70	0.13 ± 0.04

su(Hw)^{e2} bx^{34e} males were crossed to *y^{2} w^{a} ct^{6} f, su(Hw)^{2} sbd/ In(3LR)TM6, Hn^{P} ss^{P88} su(Hw)^{f} bx^{34e} Ubx^{P15} e^{S}* females at the indicated temperatures and the proportion of male progeny of the indicated *su(Hw)* genotype with a cut wing phenotype (penetrance) was determined.

[a] Number of progeny scored.

[b] Error values are standard deviations of the mean.

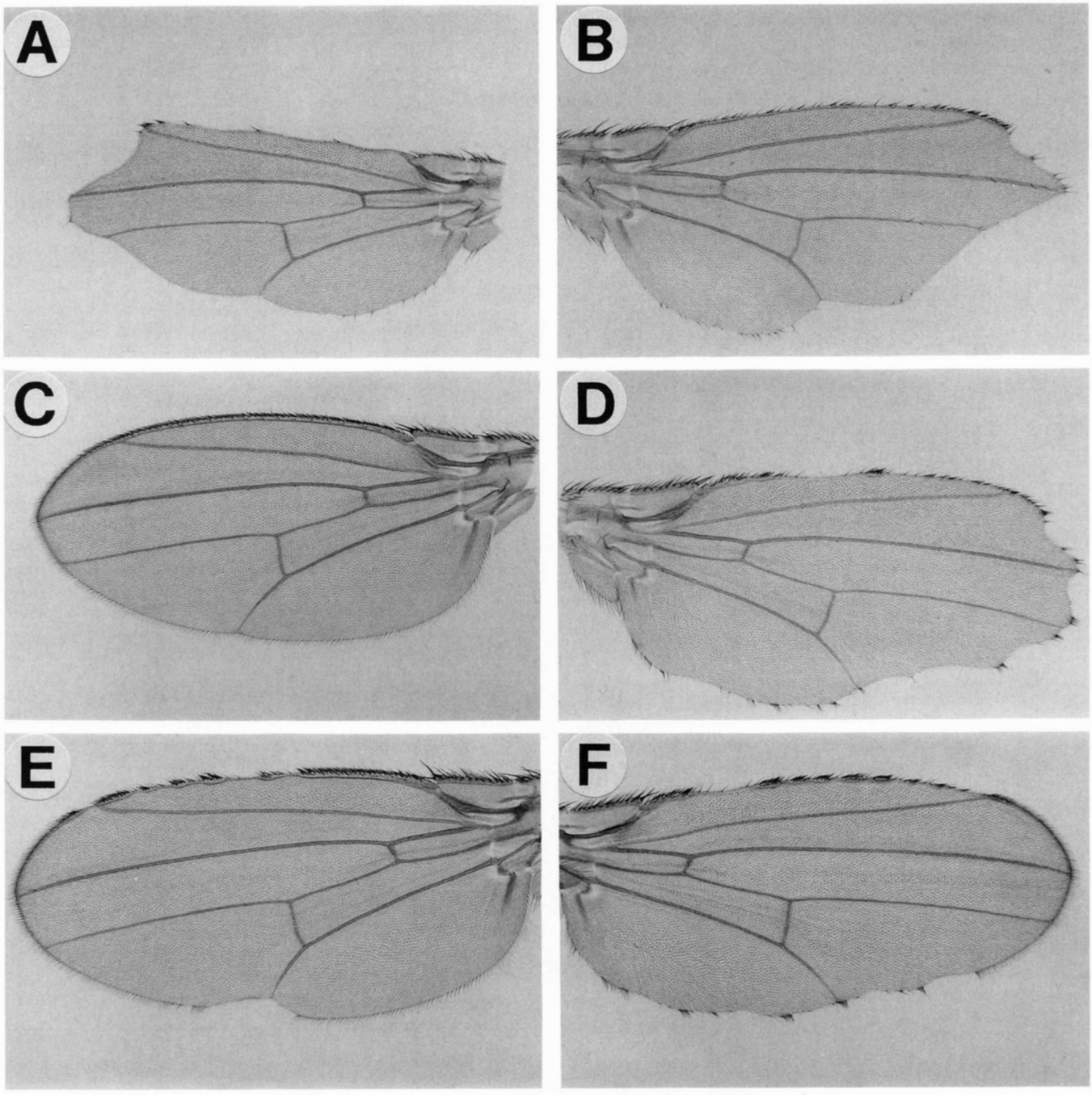

FIGURE 2.—Cut wing phenotypes. (A) ct^{2s} male (25°); the phenotype is not affected by temperature and is not suppressed by *su(Hw)* mutations. (B) ct^{83h} female (25°); the phenotype is identical to other *gypsy* insertion alleles, is unaffected by temperature and completely suppressed by $su(Hw)^2/su(Hw)^f$. (C) ct^{L-1}/ct^{2s}; $su(Hw)^{f2}/su(Hw)^2$ (29°); the phenotype is identical to wild type and the ct^{L-1}/ct^{2s}; $su(Hw)^2/su(Hw)^f$ phenotype. (D) ct^{83h}; $su(Hw)^{f2}$ female (25°); the phenotype is the same at 18° but no flies with this genotype eclose at 29°. (E) ct^{83h}/ct^{2s}; $su(Hw)^{f2}/su(Hw)^2$ (18°); penetrance was high and the expressivity variable although always less severe than with homozygous $su(Hw)^{f2}$. (F) ct^{L-1}/ct^{2s}; $su(Hw)^{f2}/su(Hw)^2$ (18°); penetrance was high and expressivity variable. Complete genotypes of the flies producing the wings shown here can be deduced from the information in Table 1.

Attempts to determine the temperature sensitivity of suppression by homozygous $su(Hw)^{f2}$ were unsuccessful. The cut wing phenotype of *gypsy* insertion alleles with homozygous $su(Hw)^{f2}$ at 18° and 25° (Figure 2D) was stronger than with the heteroallelic *su(Hw)* combinations and fully penetrant, but homozygous $su(Hw)^{f2}$ progeny were not recovered from cultures raised at 29°. Even when bx^{34e} was crossed off the $su(Hw)^{f2}$ chromosome, no flies with a *gypsy* insertion *cut* allele and homozygous $su(Hw)^{f2}$ were recovered at 29°.

Despite the inability to test the temperature-sensitivity of homozygous $su(Hw)^{f2}$, we postulate that $su(Hw)^{f2}$ is the temperature-sensitive allele because no

allelic combinations lacking $su(Hw)^{e2}$ displayed temperature-sensitive suppression. The basis for the temperature-sensitivity is unknown, although the sequence of $su(Hw)^{e2}$ indicates that the protein it encodes has an amino acid substitution in one of the zinc fingers (V. G. CORCES, personal communication). It is plausible, therefore, that DNA binding is affected by the temperature.

SUHW is required around pupariation to block the *cut* wing margin enhancer: The developmental stages at which SUHW must be active to block the wing margin enhancer were determined by shifting *cut* mutant flies with the $su(Hw)^{e2}/su(Hw)^{2}$ heteroallelic combination from 18° (high SUHW enhancer-blocking activity) to 29° (low SUHW enhancer-blocking activity) and vice versa at various times in development. Two *gypsy* insertion alleles were compared, one with the insertion near the promoter and the other with the insertion near the wing margin enhancer. The ct^{L-1} allele is an embryonic lethal insertion near the promoter (Figure 1) and ct^{83h} is a viable allele identical in phenotype to ct^{6}. The position of the insertion in ct^{83h} was mapped by Southern blot hybridization and found to be very close to the ct^{6} insertion site and the wing margin enhancer (Figure 1).

The ct^{83h} allele was used instead of ct^{6} because it was isolated in an *FM6* balancer chromosome (J. LIM, personal communication). This allowed construction of a stable stock with ct^{L-1}/ct^{83h} females. By crossing these females to males with the viable ct^{2s} allele, it is possible to compare the wing margin phenotypes of the two *gypsy* insertions in the ct^{L-1}/ct^{2s} and ct^{83h}/ct^{2s} sibling females. The ct^{2s} allele is a small *P* element-generated deficiency that complements the lethal phenotypes of ct^{L-1}, but does not complement the cut wing phenotype of *gypsy* insertion alleles because it lacks the wing margin enhancer (MOGILA *et al.* 1992; Figure 1). As expected, ct^{2s} itself has a very strong cut wing phenotype (Figure 2A) that is not suppressed by *su(Hw)* mutations.

Crosses generating ct^{L-1}/ct^{2s}; $su(Hw)^{e2}/su(Hw)^{2}$ and ct^{83h}/ct^{2s}; $su(Hw)^{e2}/su(Hw)^{2}$ progeny (Table 3) were set up at both 18° and 29°, and the parents were transferred to new vials every 12–24 hr. This gave rise to progeny populations that were relatively synchronized, with greater than 75% of the progeny in a single vial eclosing in a 2–3 day period. The few vials displaying poor synchrony were discarded. Cultures were transferred from 29° to 18° or from 18° to 29° at various days after the start of egg-laying, and the cut wing phenotypes of the ct^{L-1}/ct^{2s}; $su(Hw)^{e2}/su(Hw)^{2}$ and ct^{83h}/ct^{2s}; $su(Hw)^{e2}/su(Hw)^{2}$ adult progeny were scored (for simplicity these two types of progeny will hereafter be referred to only by the *cut gypsy* insertion allele they contain). Both the proportion of flies that displayed a cut wing phenotype (penetrance) and the severity of the cut wing phenotype (expressivity) in those flies displaying a phenotype were determined. Expressivity was quantitated by counting the number of gaps in the row of bristles along the wing margin (see MATERIALS AND METHODS). Examples of the cut wing phenotypes observed are shown in Figure 2. The standard deviations of the expressivity values tended to be large because the ranges of values were broad and the distributions were non-Gaussian. The data collected from several crosses are presented in Table 3 and selected aspects are analyzed in graphic forms in Figure 3.

At 29° development was twice as fast as at 18°. The peak of eclosion was day 18 after the start of egg-laying at 18° and day 9 at 29°. The peak of pupariation, determined by counting pupae in sample vials, was between days 5 and 6 at 29° and days 10–12 at 18°. The ct^{L-1} progeny tended to eclose slightly earlier than the ct^{83h} progeny. For example, at 18°, 33% of the ct^{83h} progeny and 45% of the ct^{L-1} progeny eclosed on day 18, while 27% of the ct^{83h} and 16% of the ct^{L-1} progeny eclosed on day 19. This slight difference occurred in all crosses, but proved to be small relative to the time period that SUHW must be active to block the wing margin enhancer.

As expected, penetrance of the cut wing phenotype was high in progeny raised at 18° from egg-laying to eclosion and low in progeny raised at 29° from egg-laying to eclosion (Table 3). The penetrance was also low when cultures were shifted from 18° to 29° as late as 9 days after egg-laying, indicating that inactivation of SUHW prior to pupariation was sufficient to allow the wing margin enhancer to function (Table 3 and Figure 3A). This was true with both ct^{L-1} and ct^{83h}. If the cultures were shifted from 18° to 29° during the peak of pupariation at 10–12 days after the start of egg-laying, intermediate penetrance values were observed. If, however, the cultures were shifted after pupariation, on days 14 and 16, the penetrance was high, indicating that it was too late in development to inactivate SUHW and allow the wing margin enhancer to function.

The period at which SUHW must be active to cause a cut wing phenotype is the stage at which wing margin enhancer-dependent expression of *cut* is required for proper differentiation of the wing margin cells (JACK *et al.* 1991). Expression of Cut protein in the wing margin progenitor cells begins just prior to pupariation and the progenitor cells begin to differentiate 16- to 20 hr postpupariation at 25°. If *cut* is not expressed in the wing margin, the progenitor cells begin to die 20–24 hr postpupariation, leading to a cut wing phenotype.

The same critical period for SUHW activity was observed when cultures were shifted from 29° to 18°. Thus, the cut wing phenotype was highly penetrant

TABLE 3

Determination of the critical period for SUHW-mediated blocking of the wing margin enhancer in the ct^{L-1} and ct^{83h} *gypsy* insertions by temperature shift of $su(Hw)^{e2}/su\text{Hw}^2$ *flies*

Temperature and day	Cut wing phenotype					
	ct^{L-1}			ct^{83h}		
	n[a]	Penetrance[b]	Expressivity[c]	n	Penetrance	Expressivity
18° to 29°						
2	130	0.13 ± 0.03[d]	1.1 ± 0.2	142	0.19 ± 0.03	1.2 ± 0.4
3	78	0.05 ± 0.03	1.0 ± 0.0	54	0.02 ± 0.02	1.0 ± 0.0
4	135	0.05 ± 0.02	1.1 ± 0.3	108	0.05 ± 0.02	1.2 ± 0.4
5	138	0.13 ± 0.03	1.1 ± 0.3	147	0.24 ± 0.04	1.3 ± 0.6
6	84	0.04 ± 0.02	1.0 ± 0.0	85	0.02 ± 0.02	1.0 ± 0.0
7	95	0.07 ± 0.03	1.0 ± 0.0	110	0.08 ± 0.03	1.1 ± 0.3
8	105	0.12 ± 0.03	1.2 ± 0.4	85	0.25 ± 0.05	1.2 ± 0.4
9	142	0.11 ± 0.03	1.3 ± 0.6	155	0.26 ± 0.04	1.2 ± 0.5
10	80	0.73 ± 0.05	4.8 ± 3.4	109	0.62 ± 0.05	3.5 ± 3.0
12	97	0.52 ± 0.05	5.4 ± 4.6	93	0.38 ± 0.02	4.0 ± 3.5
14	84	1.00 ± 0.00	13.0 ± 4.6	69	0.97 ± 0.02	8.6 ± 3.5
EC	152	0.99 ± 0.01	9.2 ± 4.2	142	1.00 ± 0.00	6.8 ± 3.9
29° to 18°						
1	145	1.00 ± 0.00	10.8 ± 4.2	145	0.99 ± 0.01	6.7 ± 2.8
2	57	1.00 ± 0.00	12.0 ± 3.6	42	1.00 ± 0.00	7.6 ± 2.6
3	48	0.96 ± 0.03	8.2 ± 3.9	45	0.93 ± 0.04	4.9 ± 2.7
4	77	0.97 ± 0.02	7.9 ± 4.0	70	0.94 ± 0.03	4.0 ± 2.5
5	133	0.63 ± 0.04	7.3 ± 4.2	113	0.71 ± 0.04	4.8 ± 2.9
6	52	0.31 ± 0.06	4.5 ± 3.3	50	0.32 ± 0.07	2.8 ± 1.7
7	64	0.08 ± 0.03	1.0 ± 0.0	56	0.09 ± 0.04	1.0 ± 0.0
EC	229	0.06 ± 0.02	1.0 ± 0.0	184	0.09 ± 0.02	1.2 ± 0.5

$w^{aG}ct^{2s}$; $su(Hw)^{e2}$ males were crossed to $ct^{L-1}/In(1)FM6$, y^{31d} sc^8 w^{FM6} dm^+ ct^{83h} B; $su(Hw)^2$ $sbd/In(3LR)TM6$, Hn^P ss^{P88} $su(Hw)^f$ bx^{34e} Ubx^{P15} e^s females at the indicated temperature and allowed to lay eggs for 12–24 hr before transferring to new vials. Cultures were transferred to the indicated temperature on the indicated day after the start of egg-laying and kept at the new temperature until all progeny had eclosed. EC indicates that the culture was kept at the original temperature until eclosion. The $su(Hw)^{e2}/su(Hw)^2$ progeny with the indicated *cut* allele heterozygous with ct^{2s} were scored for the cut wing phenotype; the composite values for all eclosing progeny are presented.

[a] Number of progeny scored.

[b] Proportion of flies displaying cut wing phenotype.

[c] Average number of wing margin gaps per fly among flies with cut wing phenotype.

[d] Error values are standard deviations of the mean.

when cultures were shifted to 18° as late as 4 days after the start of egg-laying at 29°, but an intermediate penetrance was observed when cultures were shifted to 18° at days 5 and 6, during the peak of pupariation (Figure 3A). Cultures shifted to 18° later in development were almost fully suppressed, with a low penetrance. The same dependence on the timing of the temperature shift was seen with both the ct^{L-1} and ct^{83h} alleles. Small scale experiments with the ct^6 allele further confirmed that the critical period is around pupariation (not shown).

The product of the penetrance and expressivity of the cut wing phenotype is a measure of the average phenotypic severity among a mutant population. When the products of the penetrance and expressivity of the the two gypsy insertion alleles from all temperature shift time points are plotted against each other, the ct^{L-1} phenotype is consistently 50% stronger than the ct^{83h} phenotype (Figure 3B). The difference in phenotype between ct^{L-1} and ct^{83h} may reflect differences in the quality or number of SUHW binding sites in the *gypsy* elements because differences in SUHW binding sites can affect the strength of the cut wing phenotype (HOOVER *et al.* 1992). However, because the ct^{L-1} phenotype is stronger than the ct^{83h} phenotype by the same factor at all time points, it appears that the two alleles react identically to the temperature shifts at all stages.

Another way to look for subtle differences between the two *gypsy* insertion alleles is to compare the phenotypes of progeny eclosing on successive days in those crosses in which the temperature shift occurred during the critical period around pupariation. Because the crosses were synchronized simply by restricting the time of egg-laying to a 12–24-hr period, the progeny in a given vial are at slightly different stages of development. Therefore, when a cross is shifted during the critical period, the phenotype of the progeny eclosing on successive days may be different because they were at different stages when the temperature shift occurred. Indeed, this was observed when a sufficient number of progeny eclosed on successive days to give accurate penetrance values. Thus, when crosses were shifted from 18° to 29° during the

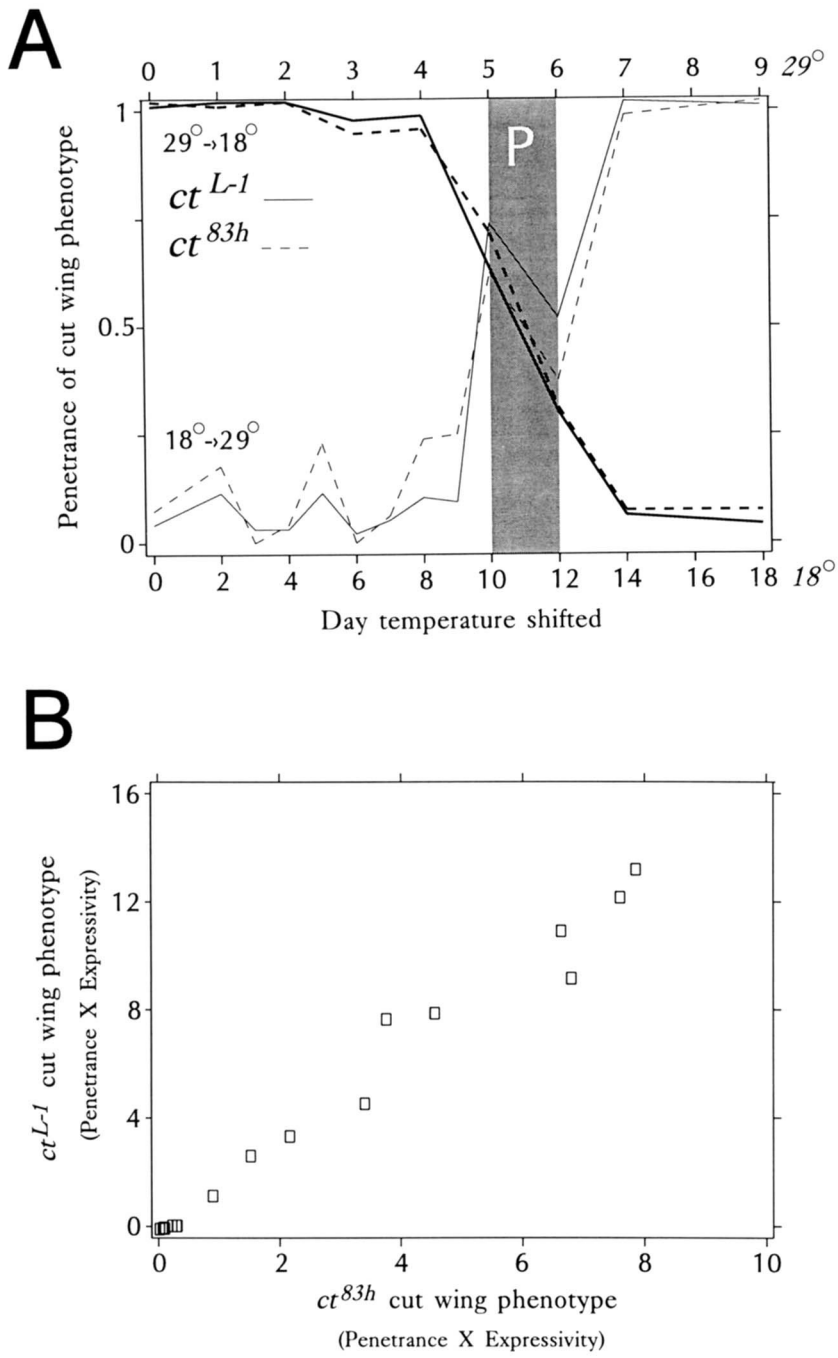

FIGURE 3.—Determination of the critical period for *su(Hw)*-mediated blocking of the *cut* wing margin enhancer and comparison of the ct^{L-1} and ct^{83h} *gypsy* insertion alleles. The crosses, temperature shifts, and genotypes scored are described in the text and in Table 3. Both panels are derived from data listed in Table 3 but the error values are not plotted. (A) Dependence of the penetrance of the cut wing phenotype in ct^{L-1}/ct^{2s}; $su(Hw)^{f2}/su(Hw)^2$ (ct^{L-1}) and ct^{83h}/ct^{2s}; $su(Hw)^{f2}/su(Hw)^2$ (ct^{83h}) flies on the time of temperature shift. The time of temperature shift is given in days after the start of egg-laying for cultures started at 29° (top axis) and 18° (bottom axis). The shaded box labeled "P" indicates the time of peak pupariation. The penetrance values obtained when cultures were kept at 18° until eclosion were plotted as the 29° to 18° day 0 shift and day 18 18° to 29° shift. The penetrance values obtained with cultures kept at 29° until eclosion were plotted as the 18° to 29° day 0 shift and day 9 29° to 18° shift. (B) Comparison of the strengths of the ct^{L-1} and ct^{83h} cut wing phenotypes. The cut wing phenotypes of the two alleles, expressed as the product of the penetrance and expressivity, from each of the temperature shift time points are plotted against each other, revealing a linear relationship in which the ct^{L-1} phenotype is 50% stronger than the ct^{83h} phenotype.

critical period (10–12 days after the start of egg-laying) the penetrance of the cut wing phenotype of both the ct^{L-1} and ct^{83h} progeny was greater in flies eclosing earlier than in flies eclosing one day later (Table 4). The shift in penetrance is in the expected direction because flies eclosing later were at an earlier stage in development when the shift to 29° occurred, and therefore will have more time for the wing margin enhancer to function. Furthermore, although for both of the *gypsy* alleles there was a significant difference in the penetrance between the two successive days of eclosion, the penetrance values observed with the two *gypsy* alleles on a given day of eclosion were not significantly different from each other. This was also true when flies were shifted from 29° to 18° during the critical period (Table 4). Therefore, these observations strongly indicate that the period that SUHW must be present to block the *cut* wing margin enhancer is identical for the two *gypsy* insertion alleles, even though the insertions are in very different positions in the control region.

DISCUSSION

The SUHW protein that binds the *gypsy* retrotransposon prevents upstream activators from activating

TABLE 4

Changes in the wing margin phenotype on successive days of eclosion with cultures temperature-shifted during the critical period for blocking the wing margin enhancer

Temperature	Day shifted	Day eclosed	Penetrance of cut wing phenotype			
			n^a	ct^{L-1}	n	ct^{83h}
18° to 29°	10	14	59	0.80 ± 0.05[b]	57	0.82 ± 0.05
		15	14	0.29 ± 0.12	48	0.38 ± 0.07
	12	16	51	0.73 ± 0.06	22	0.77 ± 0.09
		17	33	0.28 ± 0.08	38	0.26 ± 0.07
29° to 18°	5	14	60	0.45 ± 0.06	35	0.54 ± 0.08
		15	25	0.96 ± 0.04	29	1.00 ± 0.00

The crosses, temperature shifts, and progeny scored are described in Table 3; the data presented here contributed to composite values presented in Table 3.

[a] Number of progeny scored.

[b] Error values are standard deviations of the mean.

transcription when *gypsy* has inserted between the activator and promoter (GEYER, GREEN and CORCES 1990; HOLDRIDGE and DORSETT 1991; JACK *et al.* 1991; GEYER and CORCES 1992). The experiments reported here show that SUHW must be active only around pupariation to block the *cut* locus wing margin enhancer. Because this is the developmental stage at which the wing margin enhancer drives *cut* expression (JACK *et al.* 1991), it appears that to block an enhancer, SUHW must be active only when the enhancer is active.

The mechanism by which SUHW blocks enhancers is unknown. Because SUHW blocks several enhancers, but only when positioned between the enhancers and their promoters, it seems unlikely that SUHW blocks by interacting with enhancer- or promoter-binding factors. Indeed, in the *cut* locus *gypsy* insertions do not affect enhancers located within a few kilobases downstream of the insertion site, yet they block enhancers that are several kilobases upstream (*e.g.*, ct^{L-39}, Figure 1). It is more plausible that SUHW specifically interferes with mechanisms that support long distance enhancer-promoter communication. The existence of such mechanisms as separate from the process of transcription activation is indicated by the lack of long distance activation in yeast even though yeast activators such as GAL4 can activate transcription in Drosophila (FISCHER *et al.* 1988). Consistent with this idea, SUHW does not block activation by GAL4 in yeast even though SUHW enters the nucleus and binds DNA (J. KIM, B. SHEN and D. DORSETT, unpublished results).

The experiments reported here examine the mechanism by which SUHW blocks long distance activation in the *cut* locus. The *cut* enhancers are organized in developmental order, with enhancers active during embryogenesis proximal to the promoter, and enhancers active at later stages in development located more distally. This organization suggests that there may be a shift in the structure of the upstream region that begins near the promoter and proceeds distally during development as enhancers become active. For example, it could be imagined that the chromatin shifts from a "closed" to an "open" conformation to allow the enhancer-binding factors access to the enhancers. If SUHW blocked this shift in chromatin structure then it would be expected to prevent distal enhancers from becoming active. However, it would also be expected that the developmental stages at which SUHW must be active to block a distal enhancer would depend on the position of the *gypsy* insertion. Because the developmental stage SUHW was required to block the *cut* wing margin enhancer was the same when *gypsy* was near the promoter and when it was near the enhancer, it is very unlikely that SUHW interferes with developmental programming of the *cut* locus.

The results presented also provide evidence that the effects of SUHW are reversible. When flies were shifted from 18° to 29° 9 days after the start of egg-laying, they displayed little cut wing phenotype, indicating that the wing margin enhancer is not blocked during pupariation. However, with the ct^{L-1} allele, in which the *gypsy* insertion is near the promoter, SUHW was active and presumably blocking the embryonic and larval enhancers during the nine days prior to the temperature shift. Therefore SUHW did not permanently alter the ability of the upstream region to function. Furthermore, the same critical period for wing margin enhancer-blocking was observed with the ct^{83h} allele, in which the embryonic and larval enhancers were not blocked. If any of the embryonic enhancers are active in the cells destined to become wing margin cells, this implies that the ability of SUHW to block the wing margin enhancer is not affected by whether or not any of the embryonic enhancers were active.

It can also be concluded that the effects of SUHW

occur soon after SUHW becomes active. If SUHW must act for a significant period of time before an enhancer can be effectively blocked, then a shift from 29° to 18° would have to be made before the critical period to block the wing margin enhancer. Because the critical period determined by shifting the temperature from 29° to 18° is the same as the critical period determined by shifting from 18° to 29°, and because this period corresponds to the stage that the wing margin enhancer is active, it is likely that the enhancer is blocked as soon as active SUHW is present, and that the enhancers become functional as soon as SUHW becomes inactive. It is unlikely, therefore, that SUHW induces quasi-stable structures such as heterochromatin.

It has been proposed that SUHW blocks enhancers by acting as a roadblock to linear diffusion of factors along the DNA from enhancers to promoters (GEYER and CORCES 1992). The results presented here, indicating that the effects of SUHW are reversible, immediate, and independent of the prior activity of the control region separating the enhancer and promoter, are consistent with this model. However, other mechanisms may be more attractive because there are as yet no eukaryotic examples of an enhancer-binding factor that diffuses along the DNA to the promoter to activate transcription. Indeed, it has been shown that when an enhancer is on one DNA molecule, and a promoter on another, a protein bridge linking the two DNA molecules allows activation of transcription (MÜLLER, SOGO and SCHAFFNER 1989).

If factors do not slide from the enhancer to the promoter, the problem of long distance activation is how to bring the enhancer with its bound factor into close physical proximity of the promoter. For example, this could be accomplished by compacting the DNA between the enhancer and promoter with a specific chromatin structure. If SUHW were to interfere with formation of this structure, it would interfere with enhancer-promoter interactions. If the chromatin between SUHW and the promoter remains compactable, only enhancers distal to SUHW would be affected. It is also feasible that SUHW links the bound DNA to the nuclear matrix or the nuclear envelope and forms the base of a DNA loop. In this case, enhancers located distally to SUHW would be in a separate loop from the promoter and perhaps sterically or topologically constrained from interacting with the promoter. With either of these mechanisms SUHW would affect only distal enhancers. Furthermore, in both cases SUHW need only be active when the enhancer is active, and blocking would be immediate and reversible. Although other mechanisms are imaginable, the observations presented here are consistent with models in which SUHW induces an immediate and reversible change in DNA and/or chromatin structure that interferes with enhancer-promoter communication.

A role for SUHW in chromatin structure is consistent with a phenotype associated with some *su(Hw)* alleles. In addition to suppressing *gypsy* insertion alleles, strong *su(Hw)* alleles (LINDSLEY and ZIMM 1992) and a null *su(Hw)* allele (HARRISON, MORTIN and CORCES 1992) are female-sterile, which correlates with a failure of nurse cell polytene chromosomes to decondense after polytenization (KLUG, BODENSTEIN and KING 1968). Although it is unknown if the effect on chromosome structure is direct, SUHW binds several sites in the Drosophila genome that do not contain *gypsy* (SPANA, HARRISON and CORCES 1988). It is plausible that the genomic binding sites are involved in regulating chromosome structure, and also that they provide barriers that prevent the control elements of a gene from regulating a neighboring gene. Indeed, the singular ability of SUHW to block long distance activation suggests that SUHW may partially protect the *gypsy* promoter against control elements that neighbor insertion sites.

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