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Sequences required for enhancer blocking activity of scs are located within two nuclease-hypersensitive regions

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The *Drosophila* 87A7 heat shock locus is bordered, on the proximal and distal sides, by two special chromatin structures, scs and scs′. Each structure is characterized by two sets of nuclease-hypersensitive sites, located within moderately G/C-rich DNA, flanking an A/T-rich nuclease-resistant region. scs and scs′ have been shown to insulate a *white* reporter gene from position effects and to prevent enhancer–promoter interactions. These and other properties suggest scs and scs′ might function as chromatin domain boundaries. To identify the DNA sequences which are essential for the insulating activity of scs we used an enhancer blocking assay based on the *white* gene. Sequences capable of suppressing activation of *white* by its upstream enhancer elements reside within a 900 bp DNA fragment corresponding to the scs chromatin structure. Within this region, DNA fragments associated with the two nuclease-hypersensitive regions are essential for full enhancer blocking activity, while the central A/T-rich region is dispensable. Deletions which remove part of the hypersensitive regions result in intermediate levels of *white* activity. Insulating activity can, however, be reconstituted by multimerizing DNA fragments from either hypersensitive region. Our results suggest that the scs boundary is assembled from a discrete number of functionally redundant DNA sequences located within both hypersensitive regions and that boundaries act by decreasing the frequency of enhancer–promoter interactions. We also show that certain types of position effects, like those involved in dosage compensation, are not efficiently blocked by scs.

Key words: boundary/chromatin domain/enhancer blocking/scs/*white* gene

Introduction

The very large genomes of higher eukaryotes must be condensed into a relatively small volume inside the nucleus. This is accomplished by packaging the chromosomal DNA into a complex nucleoprotein structure, chromatin. Though this nucleoprotein stucture serves to compact the chromosome more than two orders of magnitude over B-form DNA, it is also the substrate that must be used for replication, recombination and transcription. Consequently, the structural organization of chromatin must condense the chromosome while at the same time leaving the DNA accessible as a substrate for the enzymes involved in these processes.

Much of the available evidence suggests that the 30 nm chromatin fiber is segregated into a series of discrete and topologically independent domains (Benyajatti and Worcel, 1976). This notion was first suggested by cytological studies on the 'specialized' polytene chromosomes of insects (Bridges, 1935; Alfert, 1956; Edstrom and Beerman, 1962; Ritossa, 1962; Judd *et al.*, 1972) and the lampbrush chromosomes of amphibian oocytes (Gall, 1956; Callan and Lloyd, 1960; Gall and Callan, 1962). Though the path of the chromatin fiber could not be discerned in polytene chromosomes, the characteristic and highly reproducible banding patterns suggested that each segment of the chromosome has a distinct higher order structure that is presumably determined by the underlying DNA sequence organization. In lampbrush chromosomes the chromatin fiber is organized into a series of large loops that emanate from the main axis of the chromosome. Moreover, loop formation, like the banding pattern in polytene chromosomes, appears to be dictated by the DNA sequence (Callan and Lloyd, 1960).

In addition to facilitating chromosome compaction, the higher order organization of the chromatin fiber may be of importance in the utilization of genetic information. The possible connection between chromatin domains and gene activity was initially suggested by cytological experiments which demonstrated that active and inactive genes have different higher order configurations. In lampbrush chromosomes, for example, transcriptionally active genes are located in the loops (Gall and Callan, 1962), while in *Drosophila* polytene chromosomes, transcriptional activity is often correlated with the decondensation or puffing of specific bands (Ritossa, 1962).

The organization of the chromatin fiber into discrete higher order domains requires a mechanism for segregating one domain from another. It is generally thought that there must be specialized nucleoprotein structures, domain boundaries, which serve this function. In previous studies we characterized two unusual chromatin structures from the *Drosophila* 87A7 heat shock locus, scs and scs′, which have some of the properties that might be expected for domain boundaries (Udvardy *et al.*, 1985). The 87A7 chromomere is located on the right arm of the third chromosome and contains two divergently transcribed, heat inducible *hsp70* heat shock genes separated by a short 1.4 kb intergenic spacer (Ish-Horowitz *et al.*, 1979; Goldschmidt-Clermont, 1980). The scs and scs′ elements flank the two heat shock genes and are located ~14 kb apart. When the genes are activated by heat induction, the 87A7 chromomere decondenses forming a puff, while it recondenses into a small band as the genes are turned off during recovery from heat shock. Cytological studies place the scs and scs′ elements at or near the edge of this heat shock puff, raising the possiblity that these elements may delimit the chromatin domain that decondenses upon heat

induction. Results from biochemical studies seem to be consistent with this possiblity. In chromatin digests each element is defined by a pair of nuclease-hypersensitive sites flanking an ~250–350 bp nuclease-resistant core. When the heat shock genes are activated this chromatin organization is altered (Udvardy *et al.*, 1985). This effect on the nucleoprotein structure of scs and scs′ appears to be indirect, as both elements are located several kilobases downstream from the DNA segments (the *hsp70* genes and immediate 3′ flanking spacers) whose chromatin organization is directly disrupted by RNA polymerase transcription (Han *et al.*, 1984; Udvardy and Schedl, 1984). If scs and scs′ delimit the domain of decondensation, then these indirect effects could arise from torsional strain generated by the high levels of *hsp70* gene transcription (Liu and Wang, 1987; Wu *et al.*, 1988; Tsao *et al.*, 1989) that is propagated along the chromatin fiber to the edges of the domain. Such a possibility is supported by *in vivo* mapping of the topoisomerase II sites of action in the 87A7 chromomere before and after heat induction (Udvardy and Schedl, 1993).

The genetic functions of scs and scs′ also appear to be consistent with the properties that might be expected for chromatin domain boundaries. We used two assays to test for genetic functions. In the first, we determined whether these elements could establish a domain of independent gene activity and insulate against chromosomal position effects. When DNA fragments containing scs and scs′ were placed on both sides of *white* reporter genes, we found that they protected the reporter genes from both positive and negative chromosomal position effects (Kellum and Schedl, 1991). In the second assay, we determined whether scs and scs′ had enhancer blocking activity. We found that DNA fragments containing either element could prevent enhancers from activating an *hsp70–lacZ* reporter gene when interposed between the enhancers and the *hsp70* promoter (Kellum and Schedl, 1992).

In the studies reported here, we have extended the genetic analysis of these putative chromatin domain boundaries. To define the sequences required for scs boundary function, we have used an enhancer blocking assay based on the *white* gene. This assay has permitted the identification of two short DNA sequences from scs that are essential for blocking the action of both the eye- and testis-specific *white* enhancers on the *white* promoter. These two DNA sequences correspond to the two nuclease-hypersensitive regions that define part of the scs chromatin structure. In contrast, DNA sequences spanning the nuclease-resistant core located between the two scs nuclease-hypersensitive regions appear to be dispensable for this boundary function. Our analysis of these hypersensitive regions suggests that the scs boundary may be assembled from a number of unique, but functionally redundant, elements and that these elements are in dynamic competition against enhancers for the establishment of regulatory interactions.

Results

scs prevents activation of the white promoter by white upstream enhancer elements

The scs and scs′ elements were originally identified as unusual chromatin structures located near the proximal and distal limits of the 87A7 heat shock puff respectively (Udvardy *et al.*, 1985). Each element has two sets of strong nuclease-hypersensitive sites, distributed in two hypersensitive regions, surrounding a nuclease-resistant DNA segment of ~250–350 bp (Figure 3). These very strong nuclease-hypersensitive regions are sites of action for topoisomerase II following induction of the heat shock genes (Udvardy and Schedl, 1993). Flanking this central core structure are additional, weaker nuclease cleavage sites which are distributed at approximately nucleosome-length intervals. Though sequence analysis failed to reveal any large stretches of homology between scs and scs′, there were some common features of note. For both elements the nuclease-resistant core consists of very A/T-rich DNA (~75% A + T), while the major flanking nuclease-hypersensitive sites, as well as the surrounding DNA, have a more average base composition, with stretches of G/C-rich DNA (Farkas and Udvardy, 1993; Vazquez *et al.*, 1993).

In the position effect (Kellum and Schedl, 1991) and enhancer blocking assays (Kellum and Schedl, 1992), fairly large DNA fragments were tested for boundary function. Most extensively studied was a 1.7 kb scs fragment; this fragment includes not only the central core structure, but also most of the flanking weaker nuclease cleavage sites. Consequently, it was not clear from these studies which sequence elements and what chromatin structures actually contribute to the observed boundary functions (although the boundary function of scs′ was in some instances tested with a much smaller 0.5 kb fragment, the properties of this scs′ fragment were not as thoroughly analyzed). Since a much more precise definition of the critical *cis*-acting elements will be required to understand how scs and scs′ might function as domain boundaries, we undertook a detailed structure–function analysis.

While the position effect and enhancer blocking assays used in previous studies were useful in the initial tests for boundary activity, neither is suitable for a systematic analysis. Although the position–effect assay offers the convenience of a very sensitive *white* reporter and a readily visible phenotype, the influences of chromosomal position are unpredictable and a large number of transgenic lines need to be examined for each construct. The enhancer blocking assay based on the *hsp70–lacZ* reporter gene would require fewer transgenic lines, however, we thought that it might be difficult to compare directly the blocking activity of different fragments by examining β-galactosidase staining. In addition, staining for β-galactosidase would be impractical for genetic screens. For these reasons, we decided to devise a new enhancer blocking assay for boundary function based on the *white* gene (Figure 1).

The *white* gene of *Drosophila* is subject to complex stage- and tissue-specific regulation. The promoter region itself contains sequences which are capable of directing expression in the pupal and adult eye that, in the absence of significant chromosomal position effects, is ~15% of the wild-type level. Adults transgenic for a *white* mini-gene (mini-*white*) containing only this minimal promoter have yellow eyes, with somewhat darker pigmentation in males than in females as a consequence of dosage compensation. The addition of upstream enhancer elements to this *mini-white* reporter increases expression in the eye

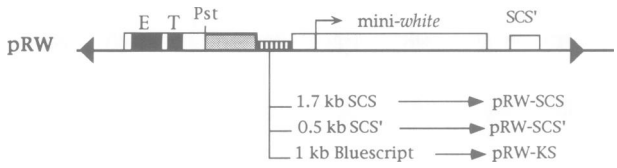

Fig. 1. Structure of the white reporter constructs. Plasmid pRW is a P element transformation vector containing the *white* transcription unit (open box) and ~1860 bp of upstream sequence. The position of the eye (E) and testis (T) enhancers is indicated by filled boxes. A polylinker was inserted at position −315 (striped box). A 500 bp scs′ element was inserted downstream of the *white* transcribed region to reduce position effects coming from the 3′ side (SCS′). P element sequences are shown as filled triangles. DNA fragments to be tested for enhancer blocking activity were cloned in the polylinker (see Materials and methods). A *Pst*I fragment (shaded box) was deleted in construct pRW-ΔPst-Scs.

to wild-type levels, which corresponds to an ~6-fold increase in the basal level of expression of the *white* minigene, giving adults which have bright red eyes. Upstream enhancers also direct expression in adult testes (Fjose *et al.*, 1984; Hazelrigg *et al.*, 1984; Levis *et al.*, 1985; Pirrotta *et al.*, 1985). In order to use the *white* gene in an enhancer blocking assay we constructed a reporter which contained the *white* promoter−*white* transcription unit separated from the upstream *white* enhancer elements by a polylinker. These polylinker sequences would permit us to insert various fragments between the enhancer and the promoter to test for enhancer blocking activity (Figure 1). To minimize variability in eye color phenotype due to position effects originating from genomic sequences located downstream of the mini-*white* reporter (Kellum and Schedl, 1991), an scs′ element was placed just beyond the 3′ end of the *white* transcription unit.

Our previous enhancer blocking experiments utilized heterologous enhancer and promoter combinations such as the *yp-1* yolk protein enhancer and the *hsp70* promoter. Hence, the first question was whether the scs and scs′ fragments are capable of blocking the potentially more specific interactions between the *white* enhancers and the *white* promoter. To address this question, we isolated transgenic lines carrying four different constructs. Two of the constructs were controls. The first was the starting reporter, pRW, while the second had a 1 kb plasmid DNA fragment (plasmid pRW-KS) inserted between the *white* enhancers and the *white* promoter. The third and fourth constructs had the 1.7 kb scs fragment or the 0.5 kb scs′ fragment respectively inserted between the enhancer and promoter (Figure 1). The starting *white* reporter gave transgenic males that typically had nearly wild-type red eyes and yellow testes. Females carrying one copy of the reporter usually showed a slightly reduced eye color compared with wild-type flies (Table I). As previously described, this may be due in part to position effects (Kellum and Schedl, 1991). Essentially the same (if not slightly higher) levels of *white* expression in the eye and testes were obtained for the second construct in which a 1 kb plasmid DNA fragment was interposed between the enhancer and the promoter (Figure 2A, Table I). This finding indicates that separating the *white* eye and testis enhancers from the promoter by a short polylinker sequence or a longer (1 kb) DNA segment has little or no effect on *white* expression.

In contrast, interposing the 1.7 kb scs-containing fragment (pRW-SCS) between the *white* enhancers and promoter appears to block enhancer action. As shown in Figure 2A (see also Table I), there was a significant reduction in eye color from red to yellow in the three lines analyzed, close to that observed when no enhancer is present (Kellum and Schedl, 1991). In addition, the scs fragment completely suppresses expression of *white* in the testes (Figure 2B). The 0.5 kb scs′ fragment also shows enhancer blocking activity. However, only two of the transgenic lines showed a reduction in *white* expression comparable with that achieved with scs. The remaining lines showed only moderate (six lines) or undetectable boundary activity (one line; see Table I). This result is different from that obtained when enhancer blocking activity was assayed with the *yp-1* enhancer and the *hsp70* promoter. In this heterologous enhancer−promoter combination, no apparent differences in the enhancer blocking activity of the 1.7 kb scs fragment and the 0.5 kb scs′ fragment were detected (Kellum and Schedl, 1992). Since the end-points of this small scs′ fragment lie within the core chromatin structure (see experiments on scs below), it may lack *cis*-acting sequences required for full boundary activity. Alternatively, it is also possible that scs′ is an intrinsically weaker boundary than scs. Taken together, these results indicate that scs, and to a lesser extent scs′, are capable of interfering with the homologous interactions between *white* upstream regulatory elements and the *white* promoter. They also suggest that these interactions are probably stronger than the fortuitous interactions which generate many types of chromosomal position effects at sites of P element insertions or the heterologous interactions between the *yp-1* enhancer and the *hsp70* promoter (Kellum and Schedl, 1991, 1992). In the following sections we focus our attention on the *cis*-acting elements in scs that are required for enhancer blocking activity.

scs enhancer blocking activity is conferred by the core chromatin structure

To localize the sequences responsible for enhancer blocking activity, we inserted a series of overlapping subfragments from the 1.7 kb scs region between the enhancers and promoter of the *white* reporter construct. The location of these subfragments, together with a diagram of the chromatin organization of scs, are shown in Figure 3. Of the six subfragments initially tested (b−g), only a 0.9 kb *Pvu*II−*Pvu*II (e) fragment had enhancer blocking activity comparable with the starting 1.7 kb fragment. As illustrated in Figure 4A, the eye color phenotype of animals transgenic for the reporter construct containing the 0.9 kb *Pvu*II fragment (fly e) is yellow. A substantial reduction in *white* expression was observed in all but one of the lines analyzed. Moreover, like the full-length fragment, this *Pvu*II fragment also blocks *white* expression in testes (Table I). Since the 0.9 kb *Pvu*II fragment includes both of the major nuclease-hypersensitive regions as well as the central nuclease-resistant DNA segment, this result would localize enhancer blocking activity to the scs chromatin structure.

This suggestion is supported by analysis of the enhancer blocking activity of the other scs subfragments. On the proximal side, fragment g, which is derived from the

Table I. Eye and testis phenotypes of transformed lines

Construct	Eye					Testis			Total
	1	2	3	4	5	6	7	8	
RW	0	0	1	4	0	0	0	5	5
RWKS	0	0	0	2	1	0	1	2	3
RWSCS'	2	2	4	1	0	1	4	4	9
a (RWSCS)	3	0	0	0	0	3	0	0	3
b	0	2	1	9	2	1	2	11	14
c	0	0	0	5	0	0	3	2	5
d	1	4	2	3	0	1	7	1	9
e	11	4	0	1	0	14	1	1	16
f	0	3	4	1	0	3	4	1	8
g	0	2	1	10	3	1	6	9	16
h	0	1	5	0	0	0	4	2	6
i	0	0	1	12	6	0	2	17	19
k	1	1	1	3	1	1	1	4	6
l	0	0	1	2	0	0	0	3	3
m	0	2	4	7	0	1	4	8	13
n	2	3	0	0	0	5	0	0	5
o	0	0	4	4	0	2	5	1	8
p	5	2	1	0	0	6	1	1	8
q	0	1	0	5	0	0	0	6	6
r	4	0	0	0	0	4	0	0	4
s	1	2	1	0	0	4	0	0	4
t	0	1	2	5	0	3	5	0	8
u	4	6	4	3	0	7	6	4	17
v	1	12	0	1	0	7	6	1	14

For each construct tested (see Figures 1 and 3), the number of lines in each phenotypic class, as well as the total number of lines analyzed, are given. Eye colors are as follows: yellow (1); orange (2); dark orange-brown (3); light red (4); wild-type (5). Testis phenotypes are as follows: white or very pale (6); pale yellow (7); yellow (8).

proximal end of the 1.7 kb scs fragment and contains only a small part of the proximal nuclease-hypersensitive region, has only very low enhancer blocking activity. A similar low level of activity is shown by fragment b, which includes the entire distal nuclease-hypersensitive region, and fragment c, which contains this hypersensitive region plus the adjacent nuclease-resistant DNA segment (see fragment c in Figure 4A). On the other hand, fragment d, which contains not only the distal hypersensitive region and the nuclease-resistant central DNA segment but also a part of the proximal hypersensitive region, typically causes a readily discernable reduction in the level of *white* expression in the eye and testes. The enhancer blocking activity of fragment d is, however, still clearly less than either the full length 1.7 kb fragment (a) or the 0.9 kb *Pvu*II fragment (e). Finally, fragment f, which contains the entire proximal nuclease-hypersensitive region and extends to the proximal end of the 1.7 kb scs fragment, also has an intermediate level of activity (see Figure 4A). It is, however, somewhat more efficient in blocking the *white* enhancers than the fragments which contain only the distal nuclease-hypersensitive region (b and c). Two conclusions can be drawn from these results. First, the enhancer blocking activity of scs appears to be conferred by *cis*-acting elements localized in the core scs chromatin structure. Second, since non-overlapping fragments can have enhancer blocking activity (c and f for instance), this function is 'divisible' and must be conferred by several *cis*-acting elements.

Functional dissection of the core chromatin structure

The results described in the previous section localize enhancer blocking activity to the scs chromatin structure and suggest that several distinct *cis*-acting elements contribute to this boundary function. It was of interest to further delimit the sequences that are responsible for enhancer blocking activity.

In the first set of experiments, we tested small fragments containing different regions of the core chromatin structure for boundary function. The results of this analysis are presented in Table I and summarized in Figure 3 (fragments h–m). Splitting the 0.9 kb *Pvu*II fragment in half yields one fragment, h, which contains the distal hypersensitive sites and the A/T-rich central nuclease-resistant region, and another, k, which contains the proximal hypersensitive region. Both of these 450 bp fragments show weak but clearly discernable enhancer blocking activity. When fragment k is subdivided into two smaller fragments l and m (see Figure 3), one of the smaller fragments (m) retains some residual activity (see Table I), while the other shows no discernable activity (see fly l in Figure 4B). A complete loss of enhancer blocking activity is also observed when ~120 bp of DNA sequence corresponding to the distal hypersensitive region are removed from fragment h to give fragment i, which contains only the A/T-rich nuclease-resistant DNA segment. Instead of showing insulating activity, fragment i actually slightly increases the level of *white* expression in the eye as compared with the starting reporter construct. These results suggest that enhancer blocking activity is associated with the two G/C-rich nuclease-hypersensitive regions, rather than the A/T-rich nuclease-resistant central region.

In the second set of experiments, we generated internal deletions in either the starting 1.7 kb scs fragment or in the 0.9 kb *Pvu*II fragment (Figure 3, fragments n–r). One set of deletions removed a 400 bp restriction fragment corresponding approximately to the central A/T-rich

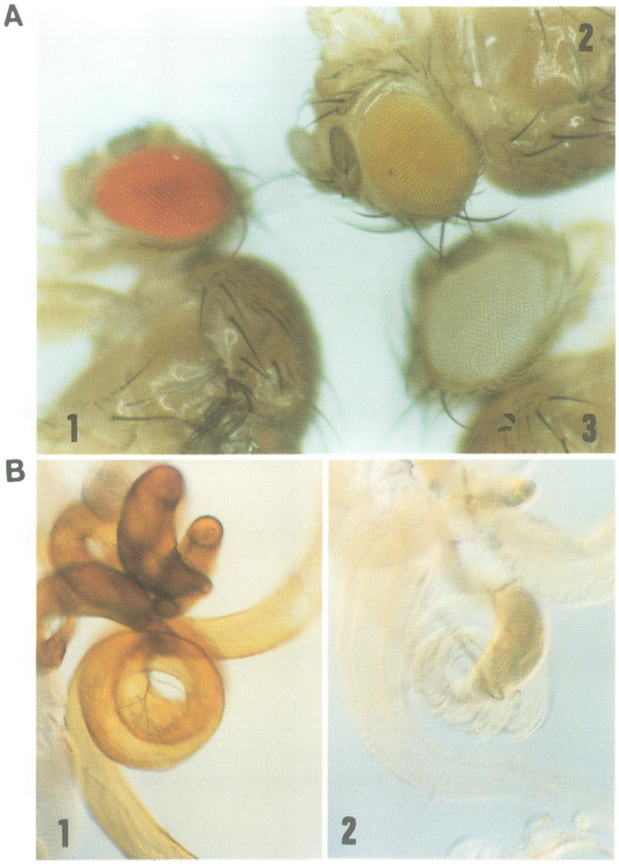

Fig. 2. Enhancer blocking activity of scs. (**A**) Eyes from young females transformed with pRW-KS (1), pRW-SCS (2) or from the *white* recipient strain (3) are shown. (**B**) Testes of 7-day-old males from a line carrying either pRW-KS (1) or pRW-SCS (2) are shown. All transformed flies were heterozygous for the P elements.

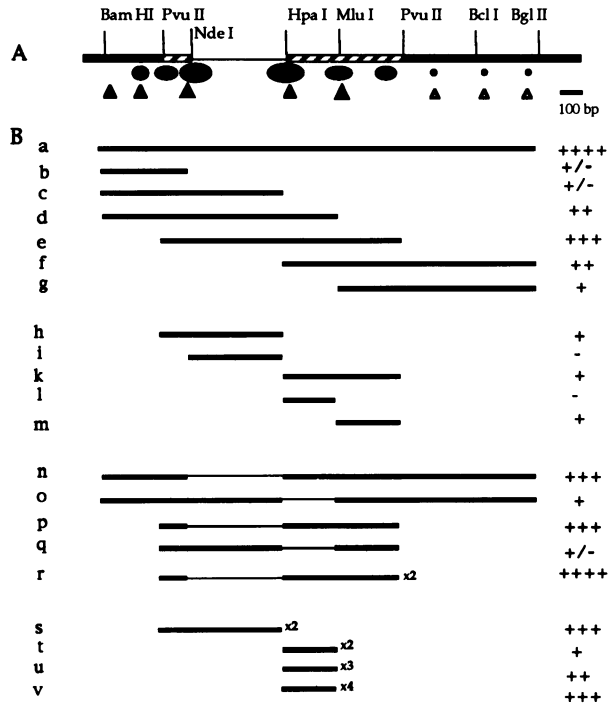

Fig. 3. Map of scs and summary of the deletion analysis. (**A**) A restriction map of the 1.7 kb scs region (*Bam*HI−*Bgl*II) is shown. G/C-rich sequences are indicated by thick lines, while the A/T-rich *Nde*I−*Hpa*I fragment is shown as a thin line. Nuclease-hypersensitive sites are represented by filled ellipses, with larger ellipses indicating stronger hypersensitive sites. Constitutive (open triangles) and heat shock-inducible topoisomerase II sites (filled triangles) are also indicated. Restriction fragments with boundary activity are hatched. (**B**) The different restriction fragments tested for enhancer-blocking activity are shown. Internal deletions are represented as lighter lines. ×2, ×3 and ×4 denote the presence of two, three and four copies of the fragment respectively. A summary of the enhancer blocking activity of the different fragments is given on the right side (− not detectable; +/− faint; + low; ++ moderate; +++ good; ++++ complete blocking). See Table I for a detailed phenotypic analysis.

nuclease-resistant DNA segment (*Nde*I−*Hpa*I fragment, constructs n and p). As shown in Figure 4A (see also Table I), this deletion had little impact on the enhancer blocking activity of either the original 1.7 kb scs fragment or the 0.9 kb *Pvu*II fragment. Most transgenic lines carrying these constructs showed an enhancer blocking activity very similar to the corresponding undeleted fragments. Some lines, however, had a very slight increase in *white* expression in the eye (light orange color instead of yellow), while two lines carrying the smallest deletion construct (p) showed some expression of *white* in the testes. This could indicate that the sequences involved in enhancer blocking might extend just beyond the *Nde*I and *Hpa*I restriction sites used to generate the deletions. In this context, it is interesting that a duplication of fragment p completely suppressed enhancer-mediated activation of *white* in both the eyes and testes in all four lines obtained (fragment r), thus behaving like the original 1.7 kb scs.

While the nuclease-resistant DNA segment can be deleted without significantly impairing enhancer blocking activity, this is not true for the nuclease-hypersensitive sequences. For instance, deletions which remove part of the proximal hypersensitive region, fragments o and q, show a greatly reduced enhancer blocking activity (compare constructs o and q with constructs n and p in Figure 4A and Table I). The enhancer blocking activity of these fragments was similar to that of fragments that contained only the distal hypersensitive region (e.g. b, c and h) or part of the proximal hypersensitive region (g). These findings argue that the proximal and distal scs nuclease-hypersensitive regions are required, and are also sufficient, for enhancer blocking activity. Within these two regions, three DNA fragments (*Pvu*II−*Nde*I, *Hpa*I−*Mlu*I and *Mlu*I−*Pvu*II of ~120, 220 and 220 bp respectively) seem to contribute to a very similar extent to the enhancer blocking activity. In contrast, the A/T-rich intervening region is dispensable.

Multimerization of the hypersensitive regions can recreate a boundary

The results presented in the previous sections would be compatible with two simple models for scs boundary function. In the first, full boundary activity would require a combination of functionally distinct *cis*-acting elements present in the distal and proximal nuclease-hypersensitive regions. In this case, a boundary composed of *cis*-acting elements from one of the nuclease-hypersensitive regions would have at most only partial enhancer blocking activity, even when these particular elements were reiterated. In the second model, boundary activity would be constituted from several different *cis* elements that have essentially

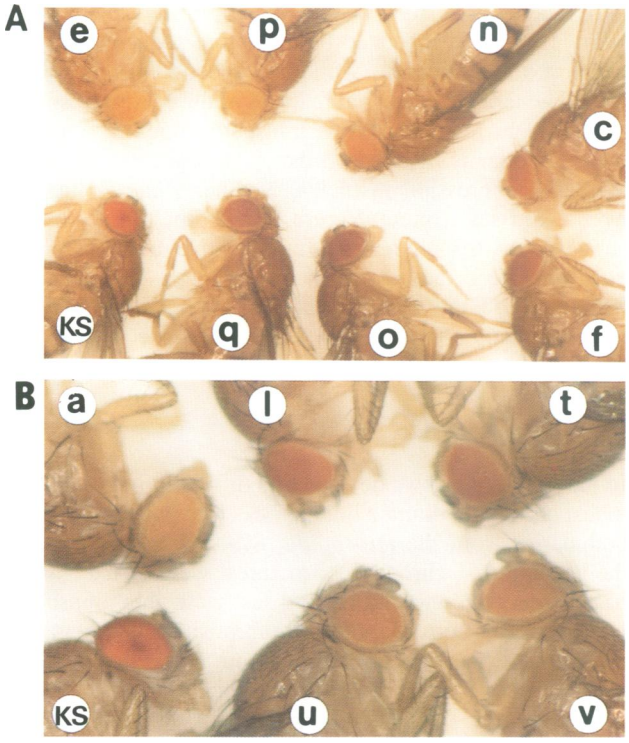

Fig. 4. Enhancer blocking activity of scs fragments. The eyes of young females heterozygous for the transgenes are shown. Letters correspond to the fragments shown in Figure 3. (**A**) Lines carrying different scs subfragments or deletions. (**B**) Lines carrying 1, 2, 3 or 4 copies of the distal hypersensitive site (fragment *Hpa*I−*Mlu*I, l, t, u and v respectively). Flies transformed with pRW-SCS (a) or pRW-KS (KS) are also shown.

equivalent functions. In this model boundary function would be 'additive' and, at least to a first approximation, would depend on the number of *cis*-acting elements. In this case, a boundary composed of *cis*-acting elements from one of the nuclease-hypersensitive regions or even a part of the nuclease-hypersensitive region could have nearly full activity if there were a sufficient number of copies of these elements.

To test whether a boundary could be reconstituted from one of its subfragments, we generated multimers of fragment l, which contains a part of the proximal scs nuclease-hypersensitive region (constructs t−v, Figures 3 and 4B). Although *cis*-acting elements in fragment l are critical for full boundary function (see, for example, the blocking activity of fragments o and q above), this fragment has no detectable enhancer blocking activity on its own (see l in Figure 4 B). As can be seen in Figure 4B, multimerization of this fragment partially restores enhancer blocking activity. While one copy has no activity, partial activity is observed when fragment l is duplicated (construct t). When the array contains three copies of fragment l, the enhancer blocking activity is much like that exhibited by the 500 bp scs′ fragment. About one-fourth of the lines show a reduction in *white* expression comparable with that observed with the 0.9 kb *Pvu*II fragment, while in the remaining lines only moderate to low enhancer blocking activity is observed. When the array contains four copies of fragment l (construct v), the blocking activity is slightly increased as compared with three copies. Moreover, a much more homogeneous response is observed, since 12 out of 14 lines show an orange eye color. Quite similar results are obtained when fragment h, which contains the distal hypersensitive region plus the A/T-rich nuclease-resistant DNA region, is dimerized (Figure 3, construct s). While a single copy of fragment h has only very low activity in the eye and testis (see Table I), the enhancer blocking activity of the dimer is comparable with that observed with four copies of fragment l. These results show that sequences from both hypersensitive regions (fragments *Pvu*II−*Nde*I and *Hpa*I−*Pvu*II, see Figure 3) are able to direct the assembly of a functional boundary, suggesting that the two hypersensitive regions are functionally equivalent. However, efficient enhancer blocking can only be achieved when both regions, or multimers of each region, are present.

Boundary function can be partially overcome by altering the enhancer

The enhancer blocking activity of fragments derived from the scs region varies depending upon which sequences they contain. When the fragments include the two major scs hypersensitive regions, they appear to almost completely suppress enhancer-dependent activation of *white*—the eye (and testis) color phenotype is comparable with that obtained for a *white* mini-gene lacking the *white* enhancer, i.e. shielded from position effects by scs and scs′ (Kellum and Schedl, 1991). In contrast, when critical sequences are deleted, enhancer−promoter interactions are partially or completely restored. These findings suggested that there might be strong competition between the ability of the enhancer to establish productive interactions with the *white* promoter and the ability of the boundary to block such interactions. Therefore, it would be of interest to determine whether the ability of scs to block enhancer−promoter interactions depends upon the relative strength of the enhancer. For this purpose we generated a construct, RW-ΔPst-SCS, in which about 530 bp of intervening sequences located between the *white* upstream enhancers and the *white* promoter region were deleted from construct pRW-SCS (Figure 1; see Materials and methods). This region is thought to contain a target for a putative repressor of the *white* gene, suppressor of white-spotted (*su-w*^{sp}) (Davison *et al.*, 1985; Hazelrigg, 1987). Deletions in this region, as well as mutations in the putative repressor gene, have been shown to increase expression of several *white* alleles. Five independent lines carrying this construct were isolated. Heterozygous females from all five lines showed a light orange eye color phenotype, as is illustrated for three of the lines in Figure 5A. This eye color is comparable with that observed in transgenic lines carrying two copies of the RW-SCS construct, which has an intact *white* enhancer. In addition, three out of the five lines showed a light yellow pigmentation in the testis. These findings would suggest that the boundary activity of scs can be partially overcome by altering the properties of the enhancer.

scs elements do not interfere with dosage compensation

The X-linked *white* gene is normally subject to dosage compensation and its expression is increased in male flies. *white* transgenes inserted back into the fly genome retain their ability to respond to the dosage compensation system

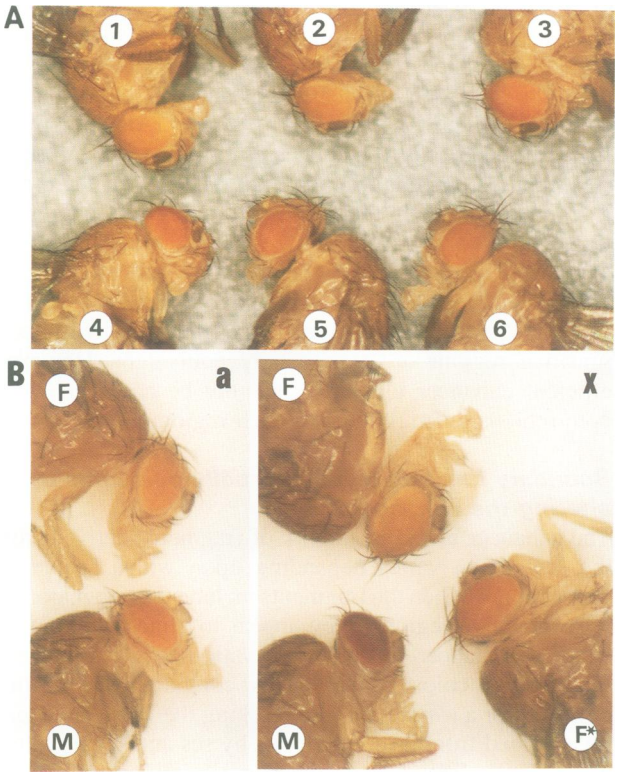

Fig. 5. Effects of enhancer alterations and dosage compensation. (**A**) Females transformed with plasmid pRW-SCS (1–3) or with plasmid pRW-ΔPst-SCS (4–6) are shown. All females are heterozygous for the constructs and come from independent lines, except for 2 and 3, which are a heterozygote and a homozygote from the same line respectively. (**B**) Flies with an insertion of construct e on the X chromosome (x, right) or on an autosome (a, left) are shown. Sexes are indicated with the symbols M for male and F for female. All flies carry one copy of the transgene, except for the one labeled F*, which is a homozygous female.

(Hazelrigg *et al.*, 1984; Levis *et al.*, 1985; Pirrotta *et al.*, 1985; Hazelrigg, 1987). Sequences able to increase expression of *white* autosomal transgenes in males have been mapped to the promoter proximal region of *white*. In the course of our work, over 200 independent lines carying different *white* constructs were generated. The vast majority of these transgenes was dosage compensated. Autosomal inserts gave rise to heterozygous males which had darker eyes than heterozygous females. Heterozygous males, however, were generally lighter than females homozygous for the same construct. On the other hand, X-linked transgenes usually gave rise to hemizygous males which had eyes comparable with, or often substantially darker than females homozygous for the same insert. Moreover, these males were generally darker than males with autosomal copies of similar inserts, indicating that the dosage compensation system was hyperactivating X-linked inserts in males (transformants from a typical X-linked line and a typical autosomal line are shown in Figure 5B). It has been shown previously that autosomal genes, which normally are not dosage compensated, may become subject to hyperactivation in males when re-inserted in the X chromosome by P element-mediated transformation (Scholnick *et al.*, 1983; Spradling and Rubin, 1983). Thus, the increased levels of expression of X-linked *white* transgenes in males may be the result of two factors. The first is the male-specific elevation in *white* expression mediated by *cis*-acting sequences in the *white* promoter region, while the second is a 'position effect' generated by special features of the X chromosomal environment in males. Most transgenic lines in which a *white* reporter is inserted on the X chromosome show an increased expression in males, whether *white* is flanked by scs and scs′ (line e, Figure 5B) or not. These results suggest that scs elements, although able to block most position effects acting on the *white* gene (Kellum and Schedl, 1991), are unable to interfere with those generated by the dosage compensation system and specific for the male X chromosome.

Discussion

In previous studies we devised two different *in vivo* assays to test chromatin domain boundaries. Two putative domain boundaries from the *Drosophila* 87A7 heat shock locus, the scs and scs′ chromatin structures, were tested. In the first assay, we found that scs and scs′ were able to insulate *white* reporter constructs from both positive and negative euchromatic position effects (Kellum and Schedl, 1991). In the second assay, we found that DNA fragments containing the scs and scs′ chromatin structures could block interactions between the *yp-1* yolk protein enhancer and the heterologous *hsp70* promoter (Kellum and Schedl, 1992). The smallest scs fragment used in these studies was a 1.7 kb *Bam*HI–*Bgl*II restriction fragment. It contains not only the core scs chromatin structure (two major hypersensitive sites flanking a central A/T-rich nuclease-resistant region), but also a series of minor hypersensitive sites which are spaced at nucleosome-length intervals and extend over several hundred base pairs. In the studies reported here we have attempted to define the sequences in this 1.7 kb fragment that are required for enhancer blocking activity. To facilitate this analysis, we used a *white* enhancer–*white* promoter construct for the enhancer blocking assay.

Several findings are of interest. First, enhancer blocking activity maps to the DNA sequences that encompass the core scs chromatin structure. In contrast, flanking sequences have little or no activity. Second, within this core structure, the *cis*-acting elements that are critical for function appear to be located in the two nuclease-hypersensitive regions. Both hypersensitive regions appear to be essential, since deletions in either of them can substantially reduce or even eliminate enhancer blocking activity. Third, the central A/T-rich nuclease-resistant region does not appear to be required for enhancer blocking activity. scs fragments containing only the two nuclease-hypersensitive regions appear to retain most of the activity. In contrast, when the central A/T-rich region is placed between the *white* enhancer and promoter, it appears to slightly increase enhancer–promoter interactions, rather than reduce or block them. One possible explanation is that if this A/T-rich DNA region is a favored site for nucleosome formation it could act as a hinge, bringing sequences on either side into proximity, as has been shown for the *Drosophila hsp26* gene (Thomas and Elgin, 1988). Fourth, the two nuclease-hypersensitive regions appear to contain *cis*-acting elements that are, at least to some extent, functionally redundant. Although neither hypersensitive

region shows full activity on its own, enhancer blocking activity can be reconstituted by multimerizing small restriction fragments containing sequences from one or the other hypersensitive region. Moreover, the effectiveness of such a reconstituted 'boundary' appears to depend upon the number of multimerized fragments. This finding would suggest that it is possible to assemble an enhancer blocking boundary by bringing together multiple copies of one or perhaps a few proteins that interact with sequences in these small restriction fragments. In this respect, these multimerized scs fragments appear to be analogous to the reiterated binding sites for the *su(Hw)* protein found in *gypsy* retrotransposons. Like the multimerized scs subfragments, the enhancer blocking activity of *gypsy* also appears to be correlated with the number of binding sites for *su(Hw)* (Geyer *et al.*, 1988; Peifer and Bender, 1988; Smith and Corces, 1992). On the other hand, the two scs hypersensitive regions do not contain any obvious reiterated sequences. Hence, the normal scs boundary is most probably assembled from several different proteins that may have distinct sequence specificities, but otherwise overlapping, if not similar, functional properties. In this context, it should be pointed out that scs does not contain consensus *su(Hw)* binding sites and we have been unable to observe any effect of *su(Hw)* mutations on the function of scs (J.Vazquez, J.Gausz and P.Schedl, unpublished data). Thus, scs probably uses other, as yet unidentified, proteins. The *white* reporter system described here should provide a convenient tool for genetic identification of genes whose products are important for the enhancer blocking activity of scs. Furthermore, the localization of enhancer blocking activity to relatively small DNA sequences should be useful in the identification of proteins which bind to scs and are involved in its function.

The mechanism by which scs or other regulatory boundaries [the *su(Hw)* binding sites of the *gypsy* transposon and chicken globin 5′ element] are able to block interactions between enhancers and promoters is unclear (Holdridge and Dorsett, 1991; Kellum and Schedl, 1991, 1992; Geyer and Corces, 1992; Chung *et al.*, 1993; Dorsett, 1993; Roseman *et al.*, 1993). In the enhancer blocking assay, all of these elements appear to function as boundaries, rather than conventional silencers, since they must be interposed between the enhancer and the promoter in order to attenuate enhancer action. In fact, when they flank these elements, they seem to optimize enhancer–promoter interaction by insulating the enhancer and promoter from the effects of extraneous regulatory elements in the surrounding environment (Kellum and Schedl, 1991; Roseman *et al.*, 1993). These findings would appear to rule out mechanisms in which boundary elements either directly inactivate the enhancer or short-circuit its interaction with the promoter by providing an alternative target or trap. Similarly, these elements do not seem to exert their regulatory effects by interfering with promoter activity.

Although these boundaries do not appear to antagonize directly enhancer elements, enhancer blocking seems to involve some sort of competition between these elements and the establishment of productive enhancer–promoter interactions. This is suggested by the range of enhancer blocking activities exhibited by many of these scs deletions, by the multimerized scs fragments and by the presumably incomplete 0.5 kb scs′ fragment. Partial

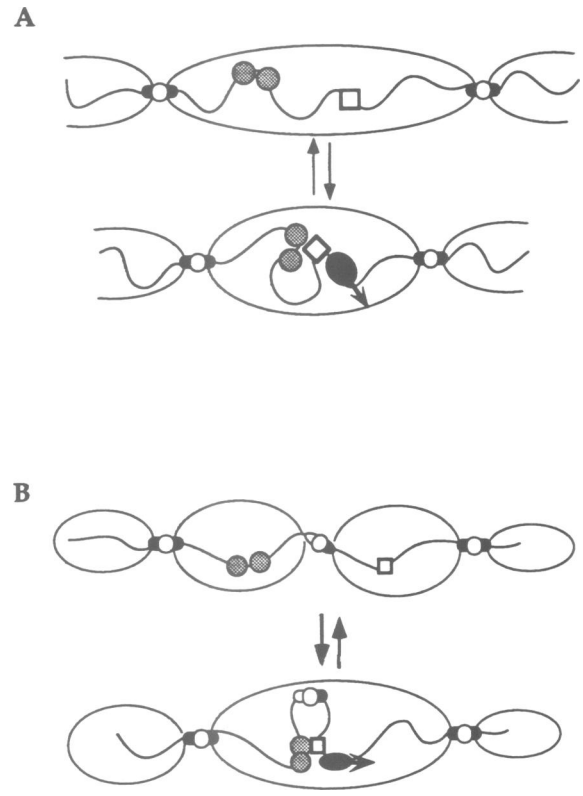

Fig. 6. A model for boundary function. The organization of chromatin into domains is depicted in (**A**). Within a domain, shown as an ellipse, the chromatin fiber is able to fold freely, allowing regulatory elements to interact. These conformational changes do not affect adjacent domains, which are insulated by the presence of functional boundaries (bi-color structures). When a boundary is inserted between an enhancer and a promoter (**B**) it will segregate them into two distinct domains, preventing regulatory interactions from occurring. In the case of a weak or incomplete boundary, depicted here, the two domains may transiently merge, allowing regulatory interactions to occur. This may also happen in the case of a stronger enhancer–promoter interaction.

boundary activity is also observed when the number of *su(Hw)* binding sites is reduced or when there is only one copy of the chicken β-globin element. In all these cases, it must be supposed that the enhancer is able to overcome the boundary and interact with the promoter, triggering expression (see Figure 6). In addition, the frequency of these enhancer–promoter interactions appears to be dependent upon properties of both the boundary and the enhancer. When the boundary activity of scs is reduced by the removal of key *cis*-acting elements, there is a concomitant increase in the level of expression of *white*. Conversely, the enhancer blocking activity of an intact scs element can be partially overcome by altering features of the enhancer (see construct RW-ΔPst-SCS, Figure 5A). It seems likely that the ability of the enhancer to compete with the boundary reflects the avidity of the enhancer's interactions with the promoter. This is suggested by the difference in the enhancer blocking activity of the 0.5 kb scs′ fragment when assayed using the heterologous *yp-1* enhancer–*hsp70* promoter combination versus the homologous *white* enhancer–promoter. Although the *yp-1* yolk protein enhancer (Brenan *et al.*, 1982; Garabedian *et al.*, 1985, 1986), which drives high levels of transcription of the yolk protein genes, is probably a much stronger

enhancer than the tissue-specific enhancers of the *white* gene (Fjose *et al.*, 1984), scs′ was more efficient in the context of the *yp-1* enhancer–*hsp70* promoter–*lacZ* reporter. Thus, the ability of an enhancer to overcome the presence of a boundary might reflect the specificity of the enhancer–promoter interaction, rather than the enhancer's absolute strength. Finally, as illustrated by our results on dosage compensation, not all types of regulatory interactions are inhibited by scs. *white* transgenes located on the X chromosome are characterized by a higher level of expression in males. Although there are sequences in the promoter region of *white* which are able to confer dosage compensation to *white* transgenes, it is clear from our results that some specific features of the X chromosome affect the level of expression of *white*. It has been shown that autosomal genes may become subject to dosage compensation when integrated in the X chromosome (Scholnick *et al.*, 1983; Spradling and Rubin, 1983), suggesting some sort of X chromosomal position effect. These influences can act on the *white* promoter even when the mini-*white* reporter is flanked by scs and scs′, suggesting that they are of a different nature than enhancer–promoter interactions or most euchromatic position effects. Dosage compensation has been correlated with the presence of distinctly modified histone isoforms (Turner *et al.*, 1992; Bone *et al.*, 1994) and with the presence on the chromosome of a protein with potential helicase activity (Kuroda *et al.*, 1991). On the other hand, it is generally accepted that enhancers exert their regulatory function by establishing protein–protein contacts with promoters through a looping mechanism (Ptashne, 1988). Thus, the ability of scs to interfere specifically with a subset of regulatory interactions may reflect differences in the mechanisms involved.

The competition between the establishment of productive enhancer–promoter interactions and the blocking of these interactions by boundary elements appears to be a relatively dynamic and perhaps continuous process. This is suggested by considering the eye color phenotypes observed in our experiments as compared with those found when the choice between alternative states becomes 'permanently' fixed over several cell generations. In our experiments, deletions which reduce enhancer blocking cause a rather uniform increase in the level of *white* expression throughout the eye and we do not observe the clonally inherited phenotypes typically found for an 'all-or-none' decision such as the heterochromatin-induced position effect variegation (reviewed in Reuter and Spierer, 1992). This result would argue that the switching between alternate states—the formation of productive enhancer–promoter interactions and the blocking of these interactions by the boundaries—occurs over a time scale that may be significantly shorter than a cell generation. These observations also argue against models in which enhancers establish stable interactions with promoters, but rather suggest that promoter–enhancer interaction is a transient, dynamic process, with the level of activation being directly related to the frequency of these interactions (see Figure 6). In this context, boundaries might act by lowering the frequency of interaction between regulatory elements.

How do boundaries compete with the establishment of enhancer–promoter interactions? We have previously suggested that the scs-like chromatin structures might function by delimiting domains of chromatin assembly, promoting the folding of chromatin domains in a manner which maximizes interactions between regulatory elements residing within one domain and minimizes those between regulatory elements located in different domains (see Figure 6A). These boundaries could direct the folding of the chromatin fiber, either by interacting with each other or by interacting with components of the nuclear matrix or membrane. In either case, it would be the competition between these interactions and enhancer–promoter interactions (Figure 6B) which would determine the efficacy of enhancer blocking. Given the results described here, these domains must be considered as dynamic rather than static structures, capable of interconverting between alternate topological organizations. The frequency of such interconversions presumably depends in part upon the properties of the boundary elements and in part on the properties of the enhancers and their cognate promoters. This view of higher order chromatin structure would be compatible with the very rapid transitions in topological organization and levels of chromatin compaction that are known to occur during such processes as transcription and replication.

Materials and methods

Plasmids

To generate the reporter plasmid pRW, an *Eco*RI–*Bam*HI scs′ fragment of about 500 bp (Kellum and Schedl, 1991) was cloned in the unique *Spe*I site of the P element vector pW8 (Klemenz *et al.*, 1987) downstream of the *white* transcription unit. The *Eco*RI–*Cla*I fragment corresponding to the 5′ half of *white* driven by an *hsp70* promoter was replaced by the corresponding *Eco*RI–*Cla*I fragment from pCaSpeR (Pirrotta, 1988). The resulting hybrid plasmid, p*white*-scs′, contains a *white* mini-gene plus *white* promoter sequences up to an *Spe*I site at approximately position −315 (O'Hare *et al.*, 1984; Steller and Pirrotta, 1985). The *white* upstream regulatory region from position −1860 to −315, corresponding to a *Hinc*II–*Spe*I restriction fragment, was subcloned into the *Cla*I site of the pKS$^-$ vector plasmid (Stratagene). The whole pKS$^-$ polylinker (*Kpn*I–*Sac*II) containing the *white* upstream region was then cloned into a *Kpn*I–*Eco*RI digest of p*white*-scs′. The resulting plasmid, pRegulatory-*white*-scs′ (pRW) contains a *white* mini-gene plus *white* upstream sequences up to position −1860 into which a fragment of pKS polylinker (*Cla*I–*Sac*II) has been inserted in the *Spe*I site located at position −315. This polylinker contains two unique restriction sites, *Xba*I and *Not*I, which were used for the insertion of DNA fragments. Plasmids pRW-SCS, pRW-SCS′ and pRW-KS were obtained by inserting a 1.7 kb *Bam*HI–*Bgl*II fragment containing the whole scs region (Udvardy *et al.*, 1985; Kellum and Schedl, 1991), a 500 bp *Eco*RI–*Bam*HI scs′ fragment or a *Pvu*II–*Sca*I fragment of about 970 bp from pKS$^-$ (Stratagene) respectively into the *Xba*I site of pRW. Additional constructs were obtained by cloning different restriction fragments from scs (see Figure 3), after purification on 1–2% agarose gels, into either the *Xba*I or *Not*I restriction sites. To obtain the internally deleted fragments (Figure 3, fragments n–q), the *Bam*HI–*Bgl*II scs fragment was subcloned into Bluescript (pKS$^-$; Stratagene) digested with either *Nde*I/*Hpa*I or *Hpa*I/*Mlu*I, filled-in with DNA polymerase I (Klenow fragment; Boehringer Manheim) and religated. Either the 1.7 kb fragment or the internal 0.9 kb *Pvu*II–*Pvu*II fragment was then transferred to pRW. Multiple insertions were detected by restriction enzyme digestion. The orientation of the fragments in the multiple insertions was not determined. In most other cases, the orientation of the different scs fragments is the same (5′-*Bam*HI–*Bgl*II-3′). In a few cases where both orientations were analyzed, similar results were obtained (data not shown). Plasmid pRW-ΔPst was obtained by cutting pRW with *Pst*I and religation. This removes *white* upstream sequences from position −315 (polylinker) to −845 (O'Hare *et al.*, 1984). The 1.7 kb *Bam*HI–*Bgl*II scs fragment was cloned in the *Not*I site of pRW-ΔPst to give pRW-ΔPst-SCS. A similar construct carrying a *Bam*HI deletion (sequence −315 to −750) gave rise to flies with eyes similar to those of flies transformed with pRW-SCS (M.Muller, K.Hagstrom and P.Schedl,

unpublished data), suggesting that the darker eye color of pRW-ΔPst-SCS lines results from deletion of specific sequences in the *white* upstream region (−750 to −845).

Establishment of transformed lines

Reporter plasmids were injected into w^{1118} embryos (Spradling and Rubin, 1982), prior to pole cell formation, at a concentration of 400 μg/ml, together with 100 μg/ml helper plasmid pUChsΔ2-3wc (Tomlinson *et al.*, 1988). Survivors were collected and mated individually to w^{1118}. Transformed progeny was selected on the basis of pigmented eyes. Standard crosses to w^{1118} were used to allocate the inserts to either the X chromosome or to the autosomes. In a number of cases, dominantly marked balanced lines were used to allocate the inserts to specific autosomes. Homozygous lines were established either by inbreeding, using eye color as a marker for homozygosity, or by using balancer lines. To assess copy number, as well as the integrity of the transformed plasmids, genomic DNA was isolated from each independent line, digested with *Eco*RI and *Sal*I, electrophoresed on 1% agarose gels and transfered to nitrocellulose filters in 20× SSC (Southern, 1975). Filters were hybridized to radiolabeled pRW and bands corresponding to the inserts visualized by autoradiography. Lines showing heterochromatic position effect variegation or obvious euchromatic position effects (patterned expression) were not used.

Eye color and testis pigmentation

Flies were raised at 20–22°C. All estimations of boundary activity were based on at least two sets of eye color determinations by visual inspection under the dissecting microscope, using 2–4-day-old females heterozygous for the transgene or as otherwise indicated. Heterozygous or hemizygous 7–10-day-old males were dissected in Ringer solution and the color of the testis was immediately determined.

To measure the amount of eye pigment, fly heads were collected under the dissecting microscope after freezing the flies in dry ice and vortexing for about 5 s at maximum speed. Heads were homogenized in 1 ml of a 1:1 mix of ethanol and 1 N HCl. Pigment was extracted for 2 h at room temperature and debris were removed by centrifugation in an Eppendorf microfuge. Absorbance was read at 480 nm, using extract from w^{1118} flies as a control. For intermediate-to-dark eye colors, a good correlation was observed between the OD readings and the eye color as observed under the dissecting microscope. For pale eye colors, however, the OD readings were unreliable and could not be used for quantitative analysis. To compare the enhancer blocking activity of different constructs (Figure 3 and Table I), numbers were attributed to each eye color (yellow = 1 to wild-type = 5, see Table I) and the eye color of all lines carrying a given construct was averaged. The symbols in Figure 3 can be interpreted as follows: ++++ yellow, complete blocking; +++ yellow−orange, good blocking; ++ orange−brown, intermediate blocking; + light red, low activity; +/− red eye color, very low activity (but present in some lines); − red−wt, no evidence of boundary activity. In ambiguous cases, testis pigmentation was also used to rate different constructs.

Photography

Eyes of 2–4-day-old transformed females carrying one copy of the reprorter construct were photographed using a Nikon HFX-IIA stereomicroscope fitted with an FX-35WA camera and light meter. Illumination was from a Dolan Jenner Fiber Lite A 200 fiber optic light source. Kodak Ektar 100 negative color film was used. Testes of 7–10-day-old males were dissected in *Drosophila* Ringer solution, mounted in glycerol and immediately photographed using a Nikon microphot SA microscope fitted with an FX-35DX camera and AFX-DX exposure unit on Kodak Ektar 100 film.

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