Deletion of an Insulator Element by the Mutation facet-strawberry in Drosophila melanogaster

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Manuscript received November 17, 1999
Accepted for publication April 4, 2000

ABSTRACT

Eukaryotic chromosomes are thought to be subdivided into a series of structurally and functionally independent units. Critical to this hypothesis is the identification of insulator or boundary elements that delimit chromosomal domains. The properties of a Notch mutation, facet-strawberry (faswb), suggest that this small deletion disrupts such a boundary element. faswb is located in the interband separating polytene band 3C7, which contains Notch, from the distal band 3C6. The faswb mutation alters the structural organization of the chromosome by deleting the interband and fusing 3C7 with 3C6. Genetic studies also suggest that faswb compromises the functional autonomy of Notch by allowing the locus to become sensitive to chromosomal position effects and can block enhancer-promoter interactions. Moreover, we find that insulating activity is dependent on sequences deleted in faswb. These results provide evidence that the element defined by the faswb mutation corresponds to an insulator.

INSULATORS are DNA sequences that confer position-independent expression to reporter genes and prevent or attenuate enhancer-promoter interactions in a position-dependent manner in transgenic assays (Kelkum and Schedl 1991, 1992; Geyer and Corces 1992; Dorsett 1993). Such elements have been identified in a variety of species, including vertebrates, and may function to modulate gene expression during development. Insulators have also been proposed to be part of chromosomal domain boundaries that would define independent domains of gene regulation and perhaps chromosome organization (reviewed in Geyer 1997; Kelkum and Elgin 1998; Bell and Fel senfeld 1999; Gerasimova and Corces 1999; Udvardy 1999). Some of the best-characterized insulator elements include the gypsy transposon, the scs and scs’ elements from the 87A7 heat-shock locus, and the fab-7 element of the bithorax complex in Drosophila, as well as the 5’ hypersensitive region of the chicken β-globin locus control region (LCR).

Transgenic assays have been used to define some of the DNA sequences required for insulator function, and a number of proteins required for the activity of insulator elements have been identified. For instance, the gypsy transposable element disrupts gene expression by integrating within the regulatory region of Drosophila genes. The mutagenic effects of gypsy are largely due to the enhancer-blocking activity of the su(Hw) protein bound to a cluster of reiterated sites in the transposon (Geyer et al. 1988; Peifer and Bender 1988; Holdridge and Dorsett 1991; Geyer and Corces 1992; Smith and Corces 1992). In addition to the su(Hw) protein, the gypsy insulator contains another protein, the product of the trithorax-group gene mod(mdg4), and its function may involve the assembly of higher-order chromosomal structures (Gerasimova et al. 1995; Gerasimova and Corces 1998). Similarly, activity of the insulators scs and scs’ may require a complex of proteins that include the product of the zeste-white 5 gene at scs (Gaszner et al. 1999) and BEAF32-A and -B at scs’ (Zhao et al. 1995; Hart et al. 1997). The enhancer-promoter in Drosophila also contains an insulating activity that requires the product of the trl gene, the GAGA-binding protein (Ohtsuki and Levine 1998). In vertebrates, a protein factor, CTCF, has been found to bind to the chicken β-globin insulator (Bell et al. 1999) and may be present at other vertebrate insulators as well.

Even though much has been learned about the structure and the activity of insulators in transgenic assays and their mutagenic activity when inserted in the regulatory region of genes, comparatively little is known about the normal function of insulators in the genome. For instance, while the mutagenic effects of SU(HW) binding sites in gypsy insertions are well known, the normal chromosomal binding sites for the su(Hw) protein have not been characterized, and their role in gene regulation is unclear. Similarly, no mutations have been recovered in scs or scs’ that would shed light on their function at their normal location. One major exception may be...
provided by a set of mutations at the Drosophila bithorax complex, which disrupt regulation at the locus. One of these mutations, Fab-7, corresponds to a deletion of DNA sequences located between the cis-regulatory regions iab-6 and iab-7, which control expression of the Abd-8 gene in parasegments 11 and 12, respectively. The Fab-7 element was shown to possess insulating activity in banding pattern, the Fab-7 gene in parasegments 11 and 12, respectively. The Abd-B et al. (1994). Artavanis-Tsakonas et al. (1985). These results suggest that the mutation is not evidence linking a genetically defined insulator element to specific defects in gene regulation or chromosome 1985). These results suggest that the mutation is not simply due to a loss of Notch regulatory elements such as enhancers or promoters. Rather, the Fab-7 gene functions in a conserved cell-cell signaling type is best explained by a chromosomal position effect originating in the 3C2–3C6 interval, which interferes with the proper expression of Notch (Keppy and Welshons 1977; Welshons and Welshons 1985, 1986). In this interpretation, the facstrain sequences would normally function to protect Notch against such position effects. In the studies reported here, we tested whether sequences from the Fab-7 region can function as a genetic insulator in transgenic assays.

**MATERIALS AND METHODS**

**Transformation vectors:** Plasmid pRW is a Drosophila transformation vector containing a white gene with ~2 kb of upstream regulatory sequences including two tissue-specific enhancer elements active in the eyes and testes, respectively (Vazquez and Schedl 1994; see Figure 4). A polynucleotide at position –315 separates the remote enhancer elements from the white promoter and provides unique restriction sites (XbaI and NotI) to test DNA fragments for enhancer-blocking activity. An upstream Xhol site allowed us to test fragments for insulating activity in a position-effect assay (white “maxigene”; Keppy and Schdel 1991). A reporter vector lacking the white upstream regulatory region was constructed by deleting the Xhol–XbaI fragment from pRW (pRWΔ). This vector was used to assay for position effects in the absence of the white enhancers (white “minigene”).

**DNA constructions:** Plasmid pN35, containing a 4.5-kb EcoRI genomic fragment from the Notch locus (~30 kb to ~25.5 kb), was digested with the appropriate restriction enzymes. Restriction fragments were separated on 1% agarose gels, purified, filled in with DNA Polymerase I (Klenow fragment), and cloned in one of the unique restriction sites of pRW or pRWΔ. To generate construct Bgl–XhoIΔHB, the BglIII–XhoI fragment was first subcloned in pKS– (Strategene, La Jolla, CA), digested with HindIII and BssHII, filled in with Klenow, and religated before being transferred to pRW. Construct Bgl–Xho [facet] contained a BglIII–XhoI genomic fragment from the mutant line fab7. Two oligonucleotides from the Notch 5′ region (5′-gcctatgatctcctgtggtcct-3′ and 5′-gctagctgtacgcgctc gggtc-3′) were used to PCR-amplify a 878-bp fragment [sequences 97 to 975 according to Ramos et al. (1989)]. An internal XbaI site was used to substitute the 3′ end of the
HindIII-BssHI fragment with its equivalent from the PCR-amplified fragment, generating the 60-bp-longer HindIII-BssH (+) fragment. One or two copies of this fragment were cloned in pWR to generate constructs PCR-1 and PCR-2, respectively. A 0.9-kb PvuII-PvuII fragment representing the minimal scs insulator element was used as a positive control (scs), while an NdI-Hpal DNA fragment from the AT-rich region of scs that lacks insulating activity was used as a negative control ("random"). Both fragments have been described (Vazquez and Sch edl 1994). Control constructs for the position-effect assay using the white maxigene, scs and random, correspond to constructs s-Ews and r-Ewr described in Kel l um and Sch edl (1991), while controls for the position-effect assay using the white minigene correspond to constructs s-ws’ and r-wr’.

All mT constructs were cloned in the 5‘-3’ orientation with respect to white except fragments HindIII-BssHI and BssH1-XhoI, which were tested in both orientations. For the latter two fragments, both orientations gave similar results and lines with both orientations were combined. The hsp70::laz construct (p70Z) was derived from plasmid p622c (H iromi and Geh r i ng 1987). It is a 4.2-kb fragment containing the hsp70 upstream regulatory region (sequences -194 to +271) including all promoter elements required for normal regulation, the hsp70 5‘ UTR, as well as the first seven hsp70 codons, fused in frame to the bacterial lacZ gene. Termination of transcription was provided by a 0.8-kb DNA fragment from the 3‘ region of hsp70. p70 was a truncated 1.3-kb fragment derived from the 5‘ end of p70Z, and it contains the same hsp70 promoter sequences (-194 to +271) and 0.9 kb of lacZ sequences.

**Germline transformations**: Plasmids were injected at a concentration of 400 mg/ml, together with 100 mg/ml of helper plasmid pUCH5-D2-3wc (“pTurbo”; T om l i son et al. 1988), into w1118 embryos prior to pole cell formation as described by Spr ad l ing and Rub in (1982). Single copy transgenic lines were established and analyzed as described (Vazquez and Sch edl 1994).

**Phenotypic analysis of transgenic lines**: Flies were raised at 20°-22°. All estimations of insulating or enhancer-blocking activity were based on at least two sets of eye color determinations by visual inspection under the dissecting microscope, using 2- to 4-day-old females heterozygous for the transgene, or as otherwise indicated. To minimize variability, comparisons were made between flies of the same age grown under similar conditions. To assay for testis pigmentation, testes were dissected from heterozygous or hemizygous 7- to 10-day-old males. Generally, there was a good correlation between the level of pigmentation in eyes and testes (Vazquez and Sch edl 1994), and data for testis pigmentation were omitted, except as otherwise indicated. For the enhancer-blocking assay, eye colors were placed in four categories: (1) yellow (~10% or less of wild-type expression), (2) orange (~25% of wild-type expression), (3) brown/ light red (~50% of wild-type expression), and (4) wild type (100% expression). In the absence of blocking, transgenic lines display eye colors ranging from brown to wild type (categories 3 and 4). With complete blocking, lines have yellow eyes similar to lines carrying an insulated white minigene (category 1). In our tables and figures, insulating activity in the enhancer-blocking assay was estimated as the percentage of lines with yellow or orange eyes (categories 1 and 2 divided by the total number of lines N), which corresponds to lines with <50% of the normal level of expression of white. Constructs were also compared by calculating an average level of expression for different sets of lines (on a scale from one to four).

To determine whether particular constructs (generally a set of test constructs and a control fragment) were similar or not, a two-tailed Student’s t-test was performed, and the associated P-values determined (null hypothesis test fragment is identical to control). This test calculates the probability that the difference between two sets of transgenic lines (test fragment and control) could be due to chance. A low P-value (0.05 or less) indicates that the two samples are very likely to be different.

In the position-effect assay using the white maxigene, eye colors were ranked on a scale of one to four, as described for the enhancer-blocking assay. Insulated transgenes generally give wild-type eyes (category 4), while noninsulated transgenes give a majority of lines with brown or light red eyes (category 3), in addition to wild type. Insulating activity was defined as the percentage of lines with wild-type eyes (category 4/ N). The average eye color was calculated for each construct, and a one-tailed Student’s t-test was used to determine the significance of the results as described above (a one-tailed test was used since the noninsulated constructs obligatorily have lighter eye pigmentation).

In the position-effect assay using the white minigene, eye colors were ranked on a scale from one to four as follows: 1, pale yellow; 2, yellow; 3, light orange; 4, dark orange or red. Insulated constructs generally give yellow eyes, while noninsulated constructs show a range of phenotypes from very pale yellow to dark orange, or sometimes darker. Insulating activity was defined as the percentage of lines with yellow eyes (category 2/ N). When the minigene is used to test for position effects, phenotypic variations in the noninsulated lines deviate from the insulated phenotype in both directions (lighter and darker eye colors). In this case, the differences in the average eye color are minimal, and a test aimed at comparing mean values as above is meaningless. Instead, lines were placed in two categories: (1) yellow eyes and (2) eyes different from yellow, and a two-tailed Student’s t-test was performed (null hypothesis test fragment is identical to control). For all these assays, a comparison of the activities on the basis of either the percentage of blocking or the calculated average eye-color values led to essentially similar conclusions.

**Chromatin mapping**: Nuclei were prepared from 0- to 12- and 0- to 24-hr embryos and from Drosophila Kc tissue culture cells and digested with DNase I or micrococcal nuclease as described in Ud a r d y et al. (1985) and Wor c e l et al. (1983). DNA from the chromatin digests was isolated, cleaved to completion with restriction enzymes, size-fractionated by electrophoresis on a 45-cm, 0.8% agarose gel and blotted to nitrocellulose filter. The nitrocellulose filters were probed with radiolabeled DNA fragments and analyzed by autoradiography.

**Photography**: Eyes of 2- to 4-day-old transformed females carrying one copy of the reporter construct were photographed using a Nikon HFX-I1A 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. Color prints were scanned using a Hewlett-Packard ScanJet IIcx scanner and DeskScan II software on a Power Macintosh 7100/ 66. Layout of the figures was done using Macromedia Freehand.

**RESULTS**

**Chromatin structure of the 3C6-3C7 interband region**: Cytological studies have placed the Notch transcription unit in polyteny band 3C7, while the promoter region and upstream sequences are located in the 3C6-3C7 interband (Rykowski et al. 1988; Figure 1). Much of the interband, including the fains deletion and the 5‘ end of Notch, is contained within a 4.6-kb EcoRI restriction fragment. As indicated in Figure 2, the beginning
Figure 1.—Cytology of polytene region 3C. The Notch transcription unit in wild-type polytene chromosomes maps to band 3C7 (solid box), while sequences immediately upstream of the transcription unit are located in the 3C5.6–3C7 interband (open box; bands 3C5 and 3C6 usually cannot be resolved). In the fa<sup>sm</sup> mutant, the Notch band is not visible, presumably because 3C7 fuses with 3C5.6. Small introns have been omitted. Adapted from Welshons and Keppy (1975), Rykowski et al. (1988), and Ramos et al. (1989).

To analyze the chromatin organization of the 3C6–3C7 interband region, we used three probes derived from the 4.6-kb EcoRI fragment. The first probe was an EcoRI-BglII fragment from the distal end of the 4.6-kb fragment (probe a; Figure 2A). In EcoRI restricted chromatin digests, the EcoRI-BglII probe displays the pattern of nucleosome cleavage reading from the distal EcoRI site toward the beginning of the Notch transcription unit. The second probe was a BglII-HindIII fragment from the middle of the 4.6-kb EcoRI fragment (probe b). In BglII restricted chromatin digests, this probe displays
the nuclease cleavage pattern reading from this distal BglII site, through the fa<sup>swb</sup> region and the Notch promoter, and into the main body of the Notch gene, up to a BglII site located 15 kb downstream. Finally, an XhoI-EcoRI fragment from the proximal end of the 4.6-kb EcoRI fragment (probe c) was used to display the nuclease cleavage products reading from near the beginning of the first Notch intron, through the transcription start sites and the fa<sup>swb</sup> region, up to the distal EcoRI site.

The autoradiograms in Figure 3 show the pattern of DNase I and micrococcal nuclease (MN) cleavage in embryonic nuclei reading across the 3C6–3C7 interband region with probe c (Figure 3A) or probe a (Figure 3B). The chromatin organization of the DNA segment spanning the fa<sup>swb</sup> region is summarized in Figure 2B. Within the Notch transcription unit, there are several prominent DNase I hypersensitive (HS) regions, which are best visualized using the XhoI-EcoRI and BglII-HindIII fragments for end-labeling (data not shown). Two of these regions, A and B, are located in the first exon, close to the XhoI restriction site, while there are additional DNase I HS regions in the large first intron. In addition to these internal DNase I HS regions, a series of five prominent HS regions (sites 1–5) can be observed in the 5′ region of Notch (Figure 3A). The Notch promoter region contains two major DNase I HS sites, 1 and 2, which cover a DNA segment of ~150 bp. The promoter region also contains chromatin-specific MN cleavage products, including a broad region of nuclease sensitivity that appears to correspond to the 150-bp region between the distal and proximal promoters and a relatively strong MN site (site a) just upstream of the 5′-most initiation site (Figure 3A, lanes 2 and 3; Figure 3B, lanes 1 and 2). These chromatin-specific cleavage products are still present after extracting the embryonic nuclei with 0.35 m NaCl, but largely disappear when the nuclei are extracted with 0.75 m NaCl, a salt concentration that results in extensive nucleosome shuffling (Worcel et al. 1983). The start site for the major Notch transcript of Ramos et al. (1989) appears to be located within DNase I HS region 1, while the start site for the major Notch transcript of Kidd et al. (1986) appears to coincide with DNase I HS region 2.

Upstream of the promoter region are DNase I HS regions 3, 4, and 5, which together cover a DNA segment of nearly 200 bp. Region 3 spans the proximal fa<sup>swb</sup> breakpoint, while regions 4 and 5 are located entirely...
within the fas deletion. Region 5 appears to coincide with the proximal copy of the 47-bp repeat. Although there are chromatin-specific micrococcal nuclease fragments from regions 3 and 4 (sites a and b; compare lanes 2 and 7 in Figure 3A, lanes 1 and 6 in Figure 3B), the two most strongly labeled MN fragments in chromatin digests are located just upstream. The first maps near the proximal copy of the 47-bp repeat (site c), while the second maps near the distal copy (site d). Both fragments also correspond to cleavage sites for the enzyme on naked DNA. However, both sites appear to be enhanced in chromatin. Furthermore, the cleavage for site c coincides with DNasel HS region 5, suggesting that this micrococcal nuclease fragment is derived from a sequence that is exposed in chromatin. It should also be noted that there is a weak DNasel site in the distal copy of the 47-bp repeat, corresponding to MN site d (see Figure 3A, lane 1). These results suggest the presence of specific nucleoprotein structures in the promoter region of Notch and extending into the upstream face: strawberry sequences. In addition to the major DNAasel and MN sites, less prominent chromatin-specific sites appear to be distributed in the distal part of the region in a pattern that suggests the presence of an ordered nucleosomal array. These additional sites, however, are relatively weak and obscured by the presence of non-chromatin-specific cleavage sites, probably reflecting the generally open conformation of chromatin in this interband region.

**Identification of an enhancer-blocking activity in the 3C6-3C7 interband:** Two different transgenic assays, an enhancer-blocking and a position-effect assay, have been used to determine whether particular DNA fragments can function as genetic insulators in Drosophila. In the enhancer-blocking assay, candidate elements are tested for their ability to prevent enhancer-promoter interaction when interposed between an enhancer and a promoter (Kellum and Schedl 1992). In the position-effect assay, the DNA fragments are tested for their ability to insulate reporter genes against either positive or negative chromosomal position effects (Kellum and Schedl 1991). To determine whether the 3C6-3C7 interband region contains an insulator that can function independently of other elements from the Notch gene or elsewhere in the 3C5-7 chromosomal interval, we used an enhancer-blocking assay based on the white gene (Vazquez and Schedl 1994). In this assay, DNA fragments are inserted into the P-element vector pRW (see Figure 4) between the white upstream regulatory region that contains tissue-specific eye and testis enhancers and the promoter of the white gene. Flies transformed with vector pRW have high levels of expression of white, resulting in light red to bright red eyes and pigmented testes. An insulator element should reduce the level of expression of white when inserted between the enhancer and promoter, but should not inhibit expression when placed upstream of the enhancer (Holdridge and Schedl 1991; Geyer and Corces 1992; Kellum and Schedl 1992; Vazquez and Schedl 1994; Cai and Levine 1995; Chung et al. 1997). Figure 4 shows the phenotypes of two representative flies in which a 2.3-kb BglII-Xhol fragment from the 5′ region of Notch has been inserted upstream of the white enhancer (fly a) or between the white enhancer and promoter (fly b). This fragment completely suppressed enhancer activity when located between the enhancer and promoter, since transgenic flies showed a yellow eye color similar to the eye color of flies in which the white enhancer has been deleted. On the other hand, the same fragment had no effect when placed upstream of the enhancer, suggesting that it does not function through a silencing mechanism. Since the white upstream regulatory region contains both eye- and testis-specific enhancers, we also examined testis pigmentation in the transgenic lines. We found that the BglII-Xhol fragment severely suppressed testis pigmentation when inserted between the white regulatory region and the white promoter in a manner that paralleled that of the eye phenotype. Testis pigmentation, however, was found to be normal when the fragment was located upstream of the enhancer (data not shown). Therefore, the BglII-Xhol fragment behaves, in this assay, in a manner identical to other insulator elements. To further characterize the magnitude of the insulating activity, we directly compared the
As shown in Table 1 and Figure 5, the Notch fragment to the previously characterized insulator scs using the same assay (Vazquez and Schedl 1994). As shown in Table 1 and Figure 5, the BglII-Xhol fragment led to a significant reduction of expression (see materials and methods) in 94% of the lines, with eye colors usually ranging from yellow to light orange. This level of blocking was similar to that of the minimal 0.9-kb scs fragment.

The 3C6-3C7 region insulates transgenes against position effects: To further establish the existence of an insulator element in the 5′ region of Notch, we tested the same BglIII-Xhol fragment in a position-effect assay. In this assay, putative insulators are tested for their ability to shield reporter transgenes from chromosomal position effects at the site of insertion. In the absence of insulators, a transgene will show a range of phenotypes due to variable levels of expression in different transgenic lines, while transgenes flanked by a variety of insulator elements will show the same phenotype in all lines (Kellum and Schedl 1991; Chung et al. 1993; Roseman et al. 1993). Two position-effect assays using the white gene have been used. In the first, a white maxigene, including the white enhancer, will give wild-type levels of expression (bright red eyes) when insulated, but will show a range of phenotypes (generally brown or light red to wild type) in the absence of an insulator. In the second assay, which uses a white minigene lacking the enhancer, insulated transgenes will give rise to flies with uniform yellow eyes, while noninsulated transgenes will show a range of phenotypes varying typically from pale yellow to bright orange. Both reporter constructs are equally effective in detecting position effects, as indicated by a similar proportion of lines that deviate from the basic (insulated) phenotype with either construct (~65–70%; Kellum and Schedl 1991).

To test whether the BglIII-Xhol fragment is able to insulate a reporter gene against position effects, this fragment was inserted in the Xhol site of prw, upstream of the enhancer in the white maxigene reporter construct. Eleven independent transgenic lines were isolated. All were found to have identical, wild-type eyes (Table 2 and Figure 6). This result was similar to that obtained when the maxigene was flanked by scs and scs′ (9 wild-type lines out of 9; construct scs). On the other hand, a noninsulated reporter construct (random) showed wild-type levels of pigmentation in only 2 out of 8 lines. Therefore, the BglIII-Xhol fragment is able to insulate the reporter transgene against position effects.

We also tested the BglIII-Xhol fragment in a position-effect assay using the white minigene. Five transgenic lines were obtained, all of which had yellow eyes similar to lines transformed with the white minigene insulated by scs (Table 3). On the other hand, a noninsulated minigene construct (random) gave a majority of lines with eye colors different from yellow. These results to-

Table 1

Enhancer blocking activity of the facet-strawberry region

<table>
<thead>
<tr>
<th>Line</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>N</th>
<th>Mean</th>
<th>P(+)</th>
<th>P(−)</th>
<th>%</th>
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<td>0.05</td>
<td>0.02</td>
<td>4</td>
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<tr>
<td>Bgl-Xho fa</td>
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<td>2</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>2.60</td>
<td>&lt;0.001</td>
<td>94</td>
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<tr>
<td>Bgl-Xho Δ</td>
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<td>2</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>2.60</td>
<td>&lt;0.001</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>

Eye colors were ranked on a scale from one to four (1, yellow; 2, orange; 3, brown/light red; 4, wild type). The number of lines in each category is listed, as well as the total number of lines (N). Mean: average eye color on a scale ranging from 1 (yellow) to 4 (wild type). P(+) and P(−) denote the P-values associated with the different constructs using the positive control (scs) or negative control (random) as the reference, based on a two-tailed Student’s t-test (null hypothesis: test fragment is identical to control). NA, not applicable (reference construct). %, percentage of lines showing blocking [defined as the number of lines with yellow or orange eyes (categories 1 and 2) divided by N].

Figure 5.—Enhancer-blocking activity of the wild-type and fa<sup>str</sup> Notch upstream region. DNA fragments were tested in the XbaI site of the enhancer blocking construct prw (see Figure 4). Blocking was defined as the percentage of lines showing <50% expression of white (yellow or orange eyes, Table 1). Fragments tested are a 0.9-kb PvuII scs fragment (scs), a 450-bp fragment without insulating activity (random), a Notch 5′ BglIII-Xhol wild-type fragment (Bgl-Xho), a BglIII-Xhol fragment with an internal HindIII-BspHII deletion (BXΔ), and a BglIII-Xhol fragment carrying the fa<sup>str</sup> deletion (facet).
The experiments described in the previous section construct, these transgenic assays corresponds to the putative cated 33 bp upstream of the distal \textit{facet-strawberry} enhancer and promoter of \textit{pRW}. Three transgenic lines characterized scs insulator element. The enhancer-blocking activity of the mutant \textit{Bgl} position effects. Furthermore, the \textit{Notch} upstream DNA fragment is as effective, in our assays, as the previously characterized scs insulator element.

\textbf{The \textit{facet-strawberry} deletion impairs insulator function}: The experiments described in the previous section suggest that the \textit{BglII-XhoI} fragment contains an element that can function as a genetic insulator. An important question is whether the insulating activity detected in these transgenic assays corresponds to the putative boundary element defined by the \textit{faswb} mutation. To address this question, we generated a set of constructs in which the \textit{faswb} sequences were deleted from the \textit{BglII-XhoI} fragment.

In the first set of experiments, we tested the deleted fragment in the enhancer-blocking assay (Figure 5 and Table 1). Two different constructs were used. The first construct carried a genomic \textit{BglII-XhoI} fragment isolated from a mutant \textit{faswb} line, inserted between the white enhancer and promoter of \textit{pRW}. Three transgenic lines were obtained for this construct. As indicated in Table 1, none of the lines showed a phenotype consistent with efficient blocking. Instead, these lines had orange or red eyes (as well as pigmented testes), suggesting that the enhancer-blocking activity of the mutant \textit{BglII-XhoI} fragment was severely compromised. The second construct, \textit{BglXhoΔH B}, is an “artificial” \textit{faswb} deletion that was generated by removing sequences between the \textit{HindIII} and \textit{BssHII} restriction sites in the \textit{BglII-XhoI} fragment (see Figure 8). The \textit{HindIII} restriction site is located 33 bp upstream of the distal \textit{faswb} breakpoint, while the \textit{BssHII} restriction site is located 4 bp upstream of the proximal breakpoint (Figure 2). As shown in Table 1 and Figure 5, the enhancer-blocking activity of the deleted \textit{BglII-XhoI} fragment was also substantially reduced, with 7 out of 10 lines showing higher than expected levels of pigmentation. The level of blocking of this construct (30%) was equivalent to that observed for the \textit{faswb} fragment and suggests that the loss of enhancer-blocking activity is due to the loss of sequences in the \textit{facet-strawberry} region. The effect of the mutation \textit{faswb} on enhancer blocking is illustrated in Figure 7, which shows two representative enhancer-blocking lines carrying the wild type (fly a) or mutated fragment (fly b).

Even though the mutated fragment was substantially impaired in its ability to block the white enhancer, some residual insulating activity (\textasciitilde 30\%, compared to \textasciitilde 10\% for the negative control) was still present. Since the phenotype of the mutation \textit{faswb} has been proposed to arise from a position effect affecting \textit{Notch} expression, we asked whether the mutated fragment would be sufficiently impaired in its ability to insulate a reporter transgene against position effects by testing the \textit{faswb} \textit{BglIII-XhoI} fragment in the position-effect assay (i.e., when placed distal to the white enhancer; Table 2 and Figure 6). While all the lines (11/11) carrying the wild-type \textit{BglII-XhoI} fragment had wild-type eye pigmentation, 7 out of 8 transgenic lines carrying the mutated fragment had reduced eye color. This result is virtually identical to that obtained with noninsulated transgenes.

### Table 2

<table>
<thead>
<tr>
<th>Line</th>
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<th>Mean</th>
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<tr>
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<td>0.003</td>
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<td>15</td>
</tr>
<tr>
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<td>0</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>3.13</td>
<td>&lt;0.001</td>
<td>0.55</td>
<td>13</td>
</tr>
<tr>
<td>\textit{Bgl-Xho faswb Xho}</td>
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<td>0</td>
<td>7</td>
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<td>3.13</td>
<td>&lt;0.001</td>
<td>0.55</td>
<td>13</td>
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</tbody>
</table>

DNA fragments were tested in a white megagene position-effect assay. Eye-color phenotypes were ranked on a scale from one to four (1, yellow; 2, orange; 3, brown-light red; 4, wild type). P(+) and P(−) represent the \textit{P}-values associated with the constructs using scs [P(+)] or random [P(−)] as a control (null hypothesis: test fragment is identical to control). NA, not applicable (reference construct). % represents the percentage of lines showing wild-type expression levels. Other symbols are as in Table 1.
TABLE 3

Insulating activity of the facet-strawberry region (II)

<table>
<thead>
<tr>
<th>Line</th>
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<th>3</th>
<th>4</th>
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<th>Mean</th>
<th>SD</th>
<th>P(−)</th>
<th>%</th>
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<td>33</td>
</tr>
<tr>
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<td>2.00</td>
<td>0</td>
<td>0.004</td>
<td>100</td>
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</tbody>
</table>

DNA fragments were tested in a white minigene position-effect assay. Eye-color phenotypes were ranked on a scale from one to four (1, pale yellow; 2, yellow; 3, light orange; 4, dark orange to wild type). Mean, average eye color on a scale from one to four. SD, standard deviation. P(−): P-values associated with the constructs using the noninsulated control (random) as a reference (null hypothesis: tested fragment is identical to control). %, percentage of lines with yellow eyes (category 2). NA, not applicable.

(local). Therefore, we conclude that the mutated fragment is unable to insulate the white reporter gene against position effects. Taken together with the reduced enhancer-blocking activity of the $fa^{ab}$ deletion, these results show that the sequences deleted in the $fa^{ab}$ mutation are critical for the insulating activity detected in our transgenic assays.

**Localizing the insulating activity.** To further define the sequences that confer enhancer-blocking activity, we tested a number of subfragments from the 3C6–3C7 interband region in the enhancer-blocking assay (Figure 8, Table 4). As shown previously, the BglII-Xho fragment was able to block the white enhancer in 94% of the lines tested. In contrast, an overlapping EcoRI-HindIII fragment located in the distal part of the interband region showed blocking in less than one-third of the lines, localizing the insulating activity to the promoter-proximal part of the region. We then subdivided the 2.3-kb BglII-Xho fragment into a distal BglII-HindIII fragment, containing sequences just upstream of the $fa^{ab}$ region, and a proximal fragment, HindIII-Xho, spanning sequences including the $fa^{ab}$ region as well as the Notch promoter region. A fragment including an additional 240 bp of 3'-specific sequences, HindIII-HindIII, was also tested. As shown in Figure 8 and Table 4, the BglII-HindIII fragment showed a modest enhancer-blocking activity (33%), similar to the overlapping EcoRI-HindIII

![Figure 7. Reduced enhancer-blocking activity of $fa^{ab}$. (A) Structure of constructs Bgl-Xho (a) and Bgl-Xho [facet] (b; see also Figure 8). (B) Eyes of 3-day-old representative females carrying one copy of construct Bgl-Xho (fly a) or Bgl-Xho [facet] (fly b).](image-url)

**Figure 8.**—Summary of the enhancer-blocking experiments. A restriction map of the Notch 5' interband region is shown. The $fa^{ab}$ region as well as the first exon and beginning of the first intron of Notch are depicted as in Figure 2. The different fragments tested for enhancer-blocking activity are indicated below the map. The endpoints of the fragments are identified by their restriction sites. Numbers to the right indicate the level of enhancer-blocking activity as the percentage of transgenic lines for each construct showing <50% expression of white (yellow and orange eyes; see materials and methods).
Notch contains all known regulatory elements does not substantially impair the normal expression of Notch (Figure 2C; see discussion). This activity was not significantly different from that of the larger HindIII-XhoI fragment. Therefore, we conclude that the exclusion of the Notch transcriptional start sites and possibly of most, if not all, transcriptional regulatory elements does not substantially impair the insulating activity. We cannot totally exclude, however, that some transcriptional regulatory elements might still surround the sequences and is likely to contain most, if not all, minimal promoter sequences required for the normal expression of Notch (Figure 2C; see discussion).

An analysis of transgenic lines carrying the HindIII-BssHII or the BssHII-XhoI fragment indicates that the 0.9-kb upstream fragment was able to block or attenuate enhancer-promoter interactions in 50% of the lines, while the proximal, promoter-containing fragment did not show any significant enhancer-blocking activity (Figure 8 and Table 4). Since nuclease mapping experiments have revealed the presence of nuclease hypersensitive sites at or near the proximal fa\textsuperscript{min} breakpoint (DNAseI HS site 3 as well as the proximal MN site; see Figures 2 and 3A), it is possible that the BssHII site itself might be located in a region important for insulation. Therefore, we used the polymerase chain reaction to generate a DNA fragment, HindIII-BssHII(\textsuperscript{+}), that extends an additional 60 bp downstream from the BssHII site (see materials and methods). This fragment includes DNAseI HS sites 3–5, but ends \textasciitilde10 bp upstream of the 5’-most transcription start site (Figure 2C). Two sets of transgenic lines that carried either one or two copies of the PCR-generated fragment (PCR-1 and PCR-2; Table 4 and Figure 8) were obtained. Both constructs showed a substantially increased insulating activity compared to the shorter HindIII-BssHII fragment (>80 vs. 50%). This activity was not significantly different from that of the larger HindIII-XhoI fragment. Therefore, we conclude that the exclusion of the Notch transcriptional start sites and possibly of most, if not all, transcriptional regulatory elements does not substantially impair the insulating activity. We cannot totally exclude, however, that some transcriptional regulatory elements might still be present in the insulating fragment (see discussion).

The Notch 5’ region does not act through promoter competition: As discussed in the previous section, the promoter-containing fragment (BssHII-XhoI) did not

### Table 4

Mapping of the insulating activity

<table>
<thead>
<tr>
<th>Eye color</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>N</th>
<th>Mean</th>
<th>P(BX)</th>
<th>P(rand)</th>
<th>%</th>
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<td>0.015</td>
<td>75</td>
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</table>

DNA fragments (see Figure 8) were tested for enhancer-blocking activity in the XbaI or NotI sites of pRW. Blocking activity (%) was determined as in Table 1. P(BX) and P(rand) represent the P-values associated with the different constructs using constructs BglII-XhoI and “random” as reference, respectively (null hypothesis: test fragment is identical to control). Other symbols are as in Table 1.
show any significant enhancer-blocking activity in our assay, but instead behaved in a manner similar to that observed with control (noninsulating) DNA fragments (Figure 8 and Table 4), suggesting that the Notch promoter does not compete with white. Since promoters have been shown to compete for regulatory elements (Chung et al. 1997; Dillon et al. 1997; Ohtsuki and Levine 1998), we asked whether the white enhancer-blocking assay would be sensitive enough to detect such effects. We therefore tested two DNA fragments containing the hsp70 promoter in the enhancer-blocking assay (Table 4). The first construct, p70Z, is a 4.2-kb DNA fragment containing the hsp70 promoter region (sequences −194 to +271 including all promoter elements, 5′ UTR, and first seven codons) fused in frame to the bacterial lacZ gene and followed by hsp70 gene transcription termination signals. This hsp70-lacZ construct has been used as a reporter gene for the characterization of transcriptional regulatory elements (Hiromi and Gehring 1987). The hsp70 promoter is therefore likely to interact with a variety of regulatory elements, including the white enhancer. The second construct, p70, is a 1.3-kb DNA fragment derived from p70Z that contains the same hsp70 upstream regulatory sequences, along with a 0.9-kb truncated fragment of the lacZ gene. This fragment is therefore comparable in size and structure to the BssHII-XhoI Notch fragment.

While the Notch promoter fragment had no discernible effect in the enhancer-blocking assay, the hsp70 promoter constructs showed a weak but significant reduction in the level of expression of the white gene (Table 4). Gene dosage experiments indicate that the hsp70 promoter fragment (p70) causes a reduction of ∼50% in the level of expression of white, while the larger p70Z construct showed an even more substantial reduction. It must be noted, however, that none of the lines obtained showed complete suppression of white, as indicated by the absence of flies with yellow eyes or unpigmented testis (Table 4 and data not shown). The greater reduction in white expression observed in p70Z lines is probably due to the increased distance between the white enhancer and white promoter (∼5 kb in p70Z vs. 1.5 kb in p70). A similar distance effect has been observed among competing promoters at the human β-globin locus (Dillon et al. 1997). The reduced expression of white observed with p70 and p70Z, together with the observation that the lacZ gene in p70Z lines was expressed in a pattern similar to white (as indicated by the presence of β-galactosidase staining in eyes and testis; data not shown), indicates that the hsp70 promoter can respond to the white enhancers and is able to compete with the white promoter, leading to a noticeable decrease in the level of expression of white. Such a decrease may even be detected when a relatively small fragment containing a truncated transcription unit is used (p70). Therefore, the lack of effect of the BssHII-XhoI Notch fragment cannot be explained by a lack of sensitivity of our assay, but instead indicates that this fragment does not compete with the white promoter to any significant level. These results indicate that the Notch promoter elements, per se, do not affect white expression in the enhancer-blocking assay and therefore cannot account for the insulating activity of the Notch upstream DNA region. DNA sequences in the proximal BssHII-XhoI fragment, however, must contribute to the overall insulating activity, since the deletion of this fragment leads to a substantial decrease in activity (50% activity for HindIII-BssHII, compared to 90–100% for the larger HindIII-XhoI and HindIII-HindIII fragments). Such sequences, however, seem to be located mainly in a 60-bp DNA region just 3′ of the BssHII site, as indicated by the higher enhancer-blocking activity of the PCR-generated HindIII-BssHII(+) fragment. In addition, these sequences seem to function only in conjunction with the upstream faab sequences and therefore are likely to contain part of the functional elements required for the activity of the faab insulator (compare, for instance, constructs Bgl-XhoIΔHB and Bgl-Hind in Figure 8).

**DISCUSSION**

In the studies reported here, we tested DNA fragments from the 3C6–3C7 interband region, corresponding to the distal end of the Notch locus, for insulating activity in two different transgenic assays. This interband contains the Notch promoter, as well as additional 5′ sequences including an 880-bp DNA region deleted in the Notch mutation faab. In the first assay, we found that a BglIII-XhoI DNA fragment spanning the faab region was able to confer position-independent expression to two different white reporter constructs, insulating them against both positive and negative position effects. In the second assay, we found that the same BglIII-XhoI DNA fragment was able to block enhancer-promoter interactions when inserted between the enhancer and promoter of the white gene, but did not reduce white expression when located upstream of the enhancer. Therefore, the Notch 5′ region contains an activity similar to previously described insulators (Kellum and Schedl 1991, 1992).

That the sequences defined by the faab mutation are critical for insulating activity is demonstrated by the analysis of two deletion derivatives of the BglIII-XhoI DNA fragment. One derivative, Bgl-XhoΔ, was generated using convenient restriction sites, and it removes most of the sequences deleted in faab. The other, Bgl-Xho[facet], is a BglIII-XhoI fragment isolated from a faab genomic clone. In both cases, the deletion of the faab sequences reduced the enhancer-blocking activity of the BglIII-XhoI restriction fragment by ∼70%. Similarly, when the mutant fragment was tested in a position-effect assay, it was unable to insulate a white reporter construct against chromosomal influences. Instead of the wild-type eye
phenotype observed in lines carrying a white maxigene flank by the BglII-Xhol fragment, lines that carried the mutant fragment generally had reduced expression of white. This result is particularly compelling, since the reduction in the level of expression of white in these lines is similar to the Notch loss-of-function phenotype observed in the fa\textsuperscript{aem} mutant.

While the sequences deleted in fa\textsuperscript{aem} are clearly important for insulation in the white transgene assays, they are not on their own sufficient to completely reproduce the strong enhancer-blocking activity of the BglII-Xhol fragment. We found that a HindIII-BssHII DNA fragment that contains all but 4 bp of fa\textsuperscript{aem} sequences has only an intermediate level of activity (50% blocking). On the other hand, a PCR-generated DNA fragment encompassing the fa\textsuperscript{aem} region and including an additional 60 bp immediately 3' of the proximal fa\textsuperscript{aem} breakpoint showed 80% of the activity present in the BglII-Xhol fragment. The sequences around the proximal fa\textsuperscript{aem} breakpoint are characterized by a set of nuclease hypersensitive sites, a feature that has also been observed in other insulator elements. Such sites may represent targets for boundary-associated proteins implicated in the insulating activity (Zhao et al. 1995; Hart et al. 1997; Gaszner et al. 1999). Therefore, the reduced activity of the HindIII-BssHII fragment might be due to the absence of one critical set of such binding sites overlapping with or located just 3' of the BssHII site.

**Does the Notch promoter contribute to the insulating activity?** On the basis of our analysis of different DNA fragments from the 5' region of the Notch locus, it is clear that most of the insulating activity (∼80%) is located within a region that includes the fa\textsuperscript{aem} sequences and the 60-bp region immediately downstream. Additional sequences may also contribute, to a lesser extent, to enhancer-blocking activity. Since the Notch promoter region is located just 3' of the fa\textsuperscript{aem} proximal breakpoint, the strong blocking activity of the BglII-Xhol fragment (or the HindIII-HindIII and HindIII-Xhol fragments; see Figure 8) could be due to the combined effects of an insulator (corresponding approximately to the fa\textsuperscript{aem} sequences) and promoter competition. By providing an alternative target for the white enhancer, the Notch promoter could cause a decrease in the level of expression of white in our enhancer-blocking assay, a result that could be interpreted as evidence for an insulator. Such a mechanism is not uncommon. For instance, at the human β-globin gene locus, different promoters compete for regulation by a unique locus control region in a distance-dependent manner (Wijgerde et al. 1995; Dillon et al. 1997). Similarly, the divergently transcribed yolk protein genes in Drosophila share, and possibly compete for, a unique enhancer element (Scott and Geyer 1995). Consistent with this idea, we found that an hsp70 promoter (fused to the bacterial lacZ gene) was able to reduce the level of expression of white when inserted between the white enhancer and white promoter. The effect was dependent on the size of the insert, suggesting that the distance between the enhancer and the competing promoters might be important. That the hsp70 promoter was indeed competing for the white enhancer was indicated by the fact that expression of the hsp70::lacZ transgene was detected in the same tissues as white (eyes and testes; our unpublished data). The effects of promoter competition, however, were weaker than those observed with a typical insulator. Even with the largest construct p70Z inserted between the white enhancer and promoter, expression of white was never completely suppressed. These results, however, indicate that an unrelated promoter is able to compete with the white promoter in our enhancer-blocking assay, leading to a decrease in the level of expression of white that may mimic the effects of a weak insulator.

We were unable, however, to obtain evidence that the Notch promoter is capable of competing with the white promoter. Drosophila core promoter elements generally consist of one or more of three conserved elements: a TATA element located at around −30, an initiator element (Inr) located near the transcription start site, and a downstream element (Dpe) located at +30 (Arkhipova 1995; Burke and Kadonaga 1996, 1997; Smale 1997). Class I promoters are characterized by a well-conserved TATA element in addition to the Inr element, while class II promoters lack a TATA element but instead generally contain a well-conserved Dpe element. Even though the promoter and regulatory sequences of Notch have not been completely characterized, all known transcriptional start sites of Notch are located within the BssHII-Xhol fragment (Figure 2). Kidd et al. (1986) reported two DNA sequences upstream of their major start site that bear some resemblance to the TATA and the mammalian CAT elements. The CAT element maps near the BssHII site, while the putative TATA element maps ∼30 bp downstream. The degree of homology between the putative TATA element and the consensus, however, is rather poor. On the other hand, Ramos et al. (1989) concluded that there were no good matches to the TATA consensus sequence in the Notch 5' promoter region, suggesting that the Notch promoter may be a class II promoter. Such TATA-less promoters are not uncommon in Drosophila, where they may be present in up to 50% of all genes (Arkhipova 1995). We found three sequences with some homology to the Inr element near some of the reported Notch initiation sites (Figure 2C). We also found sequences with homology to the minimal Dpe sequence motif Ga/tCG (Burke and Kadonaga 1996, 1997). We were unable, however, to find convincing evidence for putative TATA elements in that region, based on sequence similarity. Whether the Notch promoters are class I, or more likely class II promoters, all minimal promoter elements are likely to be located in
the near vicinity of the transcriptional start sites within the BssHII-XhoI fragment.

When the BssHII-XhoI fragment containing the putative promoter element was placed (in either orientation) between the white enhancer and the mini-white reporter gene, transgenic lines showed levels of expression of white identical to the control lines (Table 4), suggesting that the Notch promoter region did not significantly compete with the white promoter or interfere with enhancer-promoter communication. That the Notch promoter region has no detectable affects on white expression is also supported by the analysis of the deletion derivatives of the BglII-XhoI fragment (Bgl-XhoI and Bgl-XhoII). Both fragments are deleted for the fa^ab^ sequences, but retain the Notch promoter region as well as sequences in the BglII-HindIII fragment 5' of the deletion. In the enhancer-blocking assay, these fragments showed insulating activity identical to that of the upstream BglII-HindIII fragment alone (Figure 8 and Table 4). In agreement with these results, when the large BssHII-XhoI fragment, which includes the facet-strawberry sequences as well as the Notch promoter region, was placed in the 5'-3' orientation upstream of the white enhancer (i.e., with the promoter region immediately adjacent to the white enhancer), no reduction in the level of expression of white was detected, as we would expect if the Notch promoter were competing for the white enhancer (Figure 4 and Table 2). Instead, all lines showed a uniform, wild-type level of expression consistent with insulation. It is interesting to note that an activity required for promoter competition associated with the eye promoter required the TATA element (Ohtsuki and Levine 1998). This may explain why the Notch promoter, which lacks a good consensus to the TATA element, is less efficient than the class I hsp70 promoter in competing for the white enhancer.

**Association between promoters and insulator elements:** Our results suggest that the insulator element defined by the facet-strawberry mutation is located immediately upstream of, or perhaps partially overlaps with, the Notch promoter. A close association between insulator elements and promoters such as the one observed here is not uncommon. For instance, Ohtsuki and Levine (1998) found that the eye promoter contains two closely associated but separable activities that interfere with enhancer-promoter communication: one activity, which requires the GAGA-binding factor (the product of the tri gene), functions as an insulator, while the second, which requires a functional TATA box element, acts through promoter competition. Similarly, the scs' element of the hsp70 heat-shock locus appears to coincide with the promoter region of the aurora gene (Glover et al. 1995), and putative transcripts have also been reported near scs (see comment by Avramova and Tikhonov 1999). Finally, the su(Hw) protein may play a dual role as an insulator and as a transcriptional regulatory element in the gypsy transposable element (Smith and Corces 1995). On the other hand, putative boundary elements at the bithorax complex, such as Fab-7, are located between regulatory elements and far away from Abd-A and Abd-B promoters (reviewed in Mihaly et al. 1998). A similar situation is observed at the chicken and human β-globin gene locus, where insulator elements are associated with locus control elements located far away from the globin genes (reviewed in Bell and Felsenfeld 1999). Whether these differences have functional significance or whether they reflect differences in gene density and organization in different species is unclear. It is clear, however, that sequence elements required for promoter function, such as the GAGA motif or SU(HW) binding sites, may have a function in both transcriptional regulation and insulation. Other motifs, such as the TATA element, by competing for enhancer activation, may have effects that somewhat resemble insulator elements in an enhancer-blocking assay, even though possibly acting through different mechanisms. In the Notch 5' region, we were able to separate, to a large extent, the insulating activity located in the facet-strawberry region from the putative Notch promoter region. These experiments, however, suffer from the caveat that the Notch promoters have not been functionally defined and are solely predicted on the basis of the mapping of mRNA start sites and sequence analysis. Therefore, we cannot exclude that there is some overlap in the sequences required for the insulating activity detected in our assays and Notch promoter function or regulation.

**What is the role of the facet-strawberry element?** The fa^ab^ mutation was identified as an unusual Notch allele, which is caused by a small deletion at the distal end of the locus, just upstream of the Notch transcription start sites. The fa^ab^ mutation compromises Notch gene activity in the eye, causing a rough, glossy phenotype. The defects observed in fa^ab^ are consistent with an altered level of expression of Notch, which could be due to the loss of transcriptional regulatory elements. Extensive genetic analysis, however, suggests that the defects in Notch activity in fa^ab^ are probably due to the deletion of conventional Notch regulatory elements, such as enhancers or promoters. Such loss-of-function mutations are generally not reversible. For instance, studies by Green (1961, 1962) on the rates of back mutation (reversion) at a number of scs, white, and yellow alleles suggest that most alleles that could be reverted by X rays at frequencies of 10^{-5} or higher correspond to the insertion of transposable elements (and are probably due to the excision of the transposon), while most other alleles could not be reverted at detectable rates. On the other hand, the fa^ab^ phenotype could be partially or completely suppressed, or in some cases enhanced, by a number of preexisting chromosomal rearrangements with breakpoints in the 3C1–3C6 region. These results led to the conclusion...
that the defects in Notch expression in this mutant may result from a position effect acting on Notch and originating in the 3C region distal to Notch (Keppy and Welshons 1977; Welshons and Welshons 1985, 1986; Ramos et al. 1989). Normally, the facet-strawberry sequences would insulate Notch from such interference. In the mutant, the insulating activity would be impaired, leading to an altered expression of Notch. In this model, a variety of chromosomal deletions or rearrangements that remove the source of the interference would be expected to restore normal levels of Notch expression, as observed. Since the fa<sup>rob</sup> mutation is most consistent with a partial loss-of-function phenotype, such position effect is likely to reduce the level of expression of Notch.

One possibility is that this reduction is due to downregulation of Notch by a remote silencer. Alternatively, the 3C1–3C5 interval may contain one or more promoters that could compete with the Notch promoter for activation by Notch enhancer elements, thereby reducing the level of expression of Notch. In both cases, an insulator element located just upstream of the Notch promoter region would be expected to prevent such negative regulatory effects.

The results of our transgene assays are consistent with the hypothesis of Welshons and co-workers that the mutation fa<sup>rob</sup> exposes Notch to a regulatory position effect by inactivating (deleting) an insulator element at the 5’ end of the Notch locus. However, the exact mechanism that affects expression of Notch in the fa<sup>rob</sup> mutant is not known and alternative explanations for the effects of fa<sup>rob</sup> are possible. For instance, it has been suggested that fa<sup>rob</sup> might delete the 3’ region, including signals for transcriptional termination, of a gene located in 3C6 (Kidd et al. 1986). Therefore, the effects of fa<sup>rob</sup> might be due to transcriptional interference due to readthrough from an upstream transcription unit over the Notch promoter. In this view, the suppression of fa<sup>rob</sup> observed in a variety of chromosome rearrangements could be explained if such rearrangements abolished transcription of the interfering gene. Even though transcripts originating in the 3C6 region have been detected (Kidd et al. 1986), there is no evidence that they are linked to the facet-strawberry phenotype. In addition, the fa<sup>rob</sup> phenotype can be suppressed or enhanced to different levels by a variety of chromosomal deficiencies and other chromosomal rearrangements in the 3C1–3C6 region. For instance, a small inversion located in the 3C2–3 interval far away from Notch enhances the fa<sup>rob</sup> phenotype. These results seem to argue against a simple promoter fusion or run-off mechanism (Welshons and Welshons 1986). The available genetic evidence is not sufficient to distinguish between these different mechanisms, and a molecular characterization of the transcripts and regulatory elements in the 3C1–3C6 interval may be ultimately required to identify the exact nature of the position effect acting on Notch and the mechanism of action of fa<sup>rob</sup>.

We thank Simon Kidd for kindly providing wild-type and facet-strawberry genomic DNA clones; Gretchen Calhoun for help with F-element-mediated transformations; Mirko Kuit for help with the preparation of the figures; and members of the Schedl lab for their advice and support. J.V. especially thanks Frank Grosvenor and John Sedat for their hospitality during the preparation of this manuscript. This work was supported in part by the Swiss Science Foundation (J.V.) and the National Institutes of Health (P.S.).

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