Structure and Regulation of a Complex Locus: the cut Gene of Drosophila

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ABSTRACT

The cut locus encodes a homeobox protein that is localized in the nuclei of a variety of tissues and is required for proper morphogenesis of those tissues. Cut protein is required in embryonic and adult external sensory organs, where its absence results in conversion of the organs to chordotonal organs. Expression of cut also occurs in the Malpighian tubules, spiracles, central nervous system, and a number of other tissues. Gypsy transposon insertions upstream of the cut promoter block expression in subsets of these tissues. The effect of the gypsy insertions is polar, with those farthest from the promoter affecting the fewest tissues. The hypothesis that gypsy insertions block a series of tissue-specific enhancer elements that are distributed over a region of 80 kb upstream of the promoter predicts the location of the enhancers for cut expression in each of the tissues in which it is active in embryos. DNA fragments from this region drive expression of a reporter gene in each of the embryonic tissues in which endogenous cut gene is expressed. Each tissue has its own enhancer, and none of the enhancers require the activity of the endogenous cut gene to function.

The cut locus encodes a homeobox protein (Blochlinger et al. 1988) that is required for proper cell type specification and organogenesis of a number of tissues in Drosophila larvae and adults. The loss of cut function causes a complete transformation of most external sensory organs, which are mechanoreceptors and chemoreceptors with external cuticular components, to chordotonal organs, which are stretch receptors located inside the epidermis (Bodmer et al. 1987). Ectopic expression of cut causes the reverse transformation of chordotonal organs to external sensory organs (Blochlinger et al. 1991). In a number of other tissues the loss of cut function prevents proper differentiation, although it does not induce a transformation of one cell type to another. The Malpighian tubules of embryos that lack cut activity fail to differentiate morphologically or to express the white gene, which is normally transcribed in the tubules from the earliest time in their development, but the expression of several enhancer traps in the Malpighian tubules is unaltered by cut mutations (Liu and Jack 1992). The morphology of the posterior spiracles and maxillary complex is also altered by cut mutations (Wieschaus et al. 1984), and at least one enhancer trap normally expressed in the posterior spiracles is not expressed in cut mutants (Liu 1992). Finally, the lack of cut expression in the wing margin leads to the failure of the mechanoreceptors and noninnervated bristles of the wing margin to differentiate and to the death of most of the cells of the wing margin (Jack et al. 1991). The expression of cut in the margin occurs initially in a row of cells that lie on either side of the dorsal-ventral compartment boundary, which is located on the wing margin. These cells do not form sense organs but are, nevertheless, a component of the wing margin (Blair 1993; Couso et al. 1994) and have been called edge cells (Couso et al. 1994). The gypsy insertion mutations of cut block expression of the gene both in the edge cells and in the wing margin mechanoreceptors and noninnervated bristles (Jack et al. 1991).

Cut protein is expressed in each of the tissues that have been reported to be altered by cut mutations (Blochlinger et al. 1988; Jack et al. 1991). The localization and time of onset of expression of the protein suggest that cut is activated in response to the determination of the tissues and is involved in implementing the program of gene expression characteristic of the differentiated cells.

Cut is a complex locus, having both an array of mutations that block expression of the gene in various subsets of the tissues in which it is normally expressed and a group of null mutations that block expression in all tissues (Johnson and Judd 1979; Blochlinger et al. 1990; Liu et al. 1991). Johnson and Judd (1979) first proposed that the tissue-specific mutations alter regulatory elements of the gene, while null mutations alter the coding sequence. Evidence that has accumulated supports their proposal. Most mutations in the transcribed portion of the gene block expression in all tissues (Blochlinger et al. 1990; Liu et al. 1991). However, mutations distributed as far as 140 kb upstream of the start of transcription block expression in some tissues without affecting others (Blochlinger et al. 1990).
from fragments subcloned from those phage. Fragments A and B are, respectively, 7.8- and 3.5-kb EcoRI fragments from phage nR2.4. Fragments C and D are, respectively, a 5.4-kb EcoRI fragment and a 3.8-kb HindIII fragment from phage nR3.5. Fragments E and F are 6.5- and 3.3-kb EcoRI fragments from phage Mx3.5. The reporter constructs for restriction fragments A-F were generated by blunt ending the restriction fragments with T4 DNA polymerase and ligating them into the KpnI site of the HZ50 P-element transformation vector, which carries the lacZ gene fused to the hsp70 basal promoter (Hiromi and Gehring 1987).

Reporters with fragments A1, A2, and A3 were constructed in the vector pcAspeR hsp43 B-gal (Pirrotta 1988; Thummel et al. 1988). The three fragments, which are 4.2-, 1.0- and 2.7-kb fragments of A, were generated by a BamHI digest of the cutA-HZ50 construct. Because the KpnI site in HZ50 is flanked by nearby BamHI sites, the digest of cutA-HZ50 divides the A fragment into three fragments. A1 and A3 have a small amount of HZ50 DNA outside the point at which the blunt ends of fragment A were ligated. Each fragment was then ligated into the unique BamHI site of the hsp43 polynucleotide.

Germline transformations were performed as described by Dorsett et al. (1989). The recipient strains were γ500 for the HZ50 vector and y w; Ki Δ2 for the hsp43 vector. Expression of each construct was tested in at least three independent insertion lines.

Drosophila stocks: Two mutations were used to examine the expression of the reporter constructs in a background lacking cut activity. The cut"45 mutation is described in Linesley and Zimm (1992). The mutation cut"67 is described in Bloomlinger et al. (1990).

Embryo and tissue staining: Antibody staining of embryos was as described previously (Liu et al. 1991) except that 0.04% NiCl2 was used in the dianzinobenzidine solution. The anti-Cut antibody clp2 (Bloomlinger et al. 1988), a gift of L. and Y.-N. Jan, was diluted 1:2000, and the polyclonal anti-β-galactosidase antibody (Cappel) was diluted 1:4000. Anti-β-galactosidase was preincubated with devitellinized Drosophila embryos to reduce background staining.

For double staining, embryos were incubated simultaneously with a rabbit polyclonal antibody clp2 or anti-β-galactosidase and the monoclonal antibody 22C10 (Zipursky et al. 1984), a gift of D. Ballinger. They were then incubated with the horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Boehringer-Mannheim), processed, and developed with dianzinobenzidine and NiCl2, as described previously, to produce the black color reaction. The embryos were then washed in PBT, incubated with the other horseradish peroxidase-conjugated secondary antibody, and developed in dianzinobenzidine without NiCl2 to produce the brown color reaction.

For X-gal staining, larval and pupal tissues were dissected in PBS and fixed in 3% formaldehyde in PBS for 10 min. They were washed in three changes of PBT for 20 min and incubated in staining solution (10 mM NaPO4, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3 mM K3[Fe(II)(CN)6], 3 mM K4[Fe(III)(CN)6]), 0.3% Triton X-100, 1/40 vol. 8% X-gal in dimethyl sulfoxide) for 2–24 hr. The tissues were then washed in two changes of PBT, rinsed in 70% EtOH to dissolve X-gal crystals, rinsed once more in PBS, and mounted in 90% glycerol, 10 mM Tris pH 7.2.

RESULTS

Expression of the cut gene has been demonstrated in a number of cell types, including the central nervous system, the embryonic and adult external sensory or-
gans, the spiracles, and the Malpighian tubules (Blochling et al. 1988). The effects of gypsy insertion mutations at cut and other loci suggest the working hypothesis that gypsy insertions block enhancer activity when the elements are interposed between the enhancers and their promoters, even in the large regulatory region of the cut locus. This hypothesis predicts the location of the enhancer elements for the individual cell types in which cut is expressed. Gypsy insertion mutations located \(~5-7\) kb upstream of the start of transcription, called lethal I mutations, block expression in all embryonic tissues and in adult sense organs, while insertions \(38\) kb upstream, called lethal III mutations, have no effect on expression in any embryonic tissues (Figure 1). Therefore, the enhancers for all of the embryonic enhancers and possibly for the adult external sensory organs are predicted to be in the \(32\)-kb interval between the insertion sites of the two types of cut muta-

**Figure 1.**—Map of the cut locus. The map shows the location of mutations along the cut locus DNA. Scale is in kilobases. The solid boxes on the upper line show the regions where mutations have been mapped onto the restriction map of cut. The stippled box is a region where some mutations have been located by recombination mapping. The horizontal arrow marks the start and direction of transcription. Above the boxes are listed the phenotypes or tissues in which cut expression is blocked by mutations in the region. The expanded segment of the gene shows the fragments that were tested for enhancer activity in reporter constructs. The vertical arrows show the most distal position of a lethal I mutation and the most proximal position of a lethal III mutation.

**Figure 2.**—Restriction map showing fragments that were used in enhancer constructs. L-39 is the lethal III gypsy insertion closest to the promoter and L-35 is the lethal I insertion farthest from the promoter. R, EcoRI; H, HindIII; B, BamHI. The restriction sites used for each fragment are described in MATERIAL AND METHODS.
tion. We examined restriction fragments covering most of the 32-kb interval (Figure 1) for enhancer activity by testing their ability in vivo to regulate an hsp70 promoter fused to the lacZ gene. Under these circumstances, at least one fragment drives expression in each of the tissues in which cut is expressed (Table 1).

Expression in the peripheral nervous system: Cut protein is expressed in the peripheral nervous system in all of the simple external sensory organs of embryos and adults and in the complex sense organs of the head in embryos (BLOCHLINGER et al. 1988, 1993; JACK et al. 1991). At least three and probably four different enhancers are apparently required for cut expression in the simple external sensory organs. We have previously shown that a fragment upstream of all the cut gypsy insertion mutations contains an enhancer that drives expression in the mechanoreceptors of the wing margin (JACK et al. 1991). Those mechanoreceptors are unique among external sensory organs in not requiring achaete or scute activity for development (GARCIA-BELLIDO and SANTAMARIA 1978). We have now identified one fragment from the lethal III-lethal I region that drives expression in most adult external sensory organs and another fragment from the region that drives expression in embryonic external sensory organs.

Fragment E drives reporter expression in all simple external sensory organs of adults with the exception of the mechanoreceptors and chemoreceptors on the wing margin. The reporter begins to be expressed in the sense organs of the wing blade and the notum during late third instar and early pupation, the same time that cut would normally be expressed in these cells (Figure 3A). However, no expression is evident in the wing margin chemoreceptors as late as 7 hr after pupariation (AP) (Figure 3A), when Cut protein is already present in these cells. In adults carrying the cutE-hsp70-lacZ construct, β-galactosidase is expressed in most external sense organs, including the bristles between the facets of the eye (Figure 3,B–D). However, no expression is observed in the external sense organs of embryos carrying the fragment E construct.

No fragment was found to drive a reporter in the wing margin chemoreceptors. Therefore, a separate enhancer may exist for these sense organs. No genetic evidence is currently available to suggest a location for this enhancer.

Fragment A, on the other hand, drives the reporter in the external sensory organs in the thoracic and abdominal segments of embryos. Expression driven by this fragment is weak and variable both between embryos and between segments in the same embryo. Dividing the 7.9-kb fragment into three smaller fragments resulted in the identification of a 2.7-kb fragment A3 that shows strong, uniform expression of the reporter in all

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**TABLE 1**

Summary of reporter expression patterns driven by cut DNA fragments

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Embryonic expression patterns</th>
<th>Larval and imaginal expression patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (4.2 kb)</td>
<td>Posterior spiracles, probably sense organs</td>
<td>ND</td>
</tr>
<tr>
<td>A2 (1.0 kb)</td>
<td>Maxillary organs&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>A3 (2.7 kb)</td>
<td>External sensory organs</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Malpighian tubule tip cells</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Posterior spiracles, outer cells</td>
<td>ND</td>
</tr>
<tr>
<td>B (3.3 kb)</td>
<td>Malpighian tubules</td>
<td>Larval optic lobes and connectives between optic lobes and central ganglia</td>
</tr>
<tr>
<td>C (5.4 kb)</td>
<td>Posterior spiracles, inner cells</td>
<td>Tarsal organs near CNS</td>
</tr>
<tr>
<td></td>
<td>Antennal-maxillary organs</td>
<td>Hypophysis</td>
</tr>
<tr>
<td></td>
<td>Malpighian tubules</td>
<td>PNS fascicles near CNS</td>
</tr>
<tr>
<td>D (3.8 kb)</td>
<td>Posterior spiracles, inner cells</td>
<td>PNS fascicles near CNS</td>
</tr>
<tr>
<td></td>
<td>Antennal-maxillary organs</td>
<td>Two CNS cells per hemisegment</td>
</tr>
<tr>
<td></td>
<td>Hypophysis</td>
<td>Slight Malpighian tubule</td>
</tr>
<tr>
<td></td>
<td>Epiphysis</td>
<td>Slight Malpighian tubule</td>
</tr>
<tr>
<td></td>
<td>PNS fascicles near CNS</td>
<td>Anterior spiracles</td>
</tr>
<tr>
<td>E (6.5 kb)</td>
<td>Anterior spiracles</td>
<td>Tracheal branches</td>
</tr>
<tr>
<td></td>
<td>Tracheal branches</td>
<td>Ring under lateral epidermis</td>
</tr>
<tr>
<td></td>
<td>Ring under lateral epidermis</td>
<td>Weak Malpighian tubule</td>
</tr>
<tr>
<td>F (3.3 kb)</td>
<td>Many cells throughout CNS</td>
<td>Larval optic lobes and ventral ganglia</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only the larval CNS and pupal wings and legs were examined for this study.
<sup>b</sup> Only two insertion lines examined.
of the embryonic external sensory organs in the thoracic and abdominal segments but causes no expression in chordotonal organs (Figure 4) and a 4.2-kb fragment A1 that drives expression in the posterior spiracles, probably in the sense organs. A3 also drives reporter expression in the posterior spiracles, but the expression does not appear to be in the sense organs.

Cut protein is also expressed in the complex cephalic sense organs of embryos (BLOCHLINGER et al. 1988). These organs are innervated by multiple neurons and are formed by the coming together of the sensory cells of the gnathal segments to form large sensory structures. One of these, the antennomaxillary complex, is composed of three organs that lie at the opening of the atrium after head involution. The dorsal organ is the antennal component, and the terminal and ventral organs make up the maxillary complex (CAMPOS-ORTEGA and HARTENSTEIN 1985). The epiphysis and hypophysis are sense organs of the foregut that can be seen in stage 14 embryos in the clypeolabrum and lar-
bium, respectively. Expression of _cut_ in the cephalic sense organs appears to be regulated separately from the simple external sensory organs in the thoracic and abdominal segments. Although fragment A3 is capable of activating the reporter gene in all of the simple external sense organs, it activates expression in only two of the complex sense organs of the head: the maxillary organ and hypophysis (not shown).

Other fragments drive expression of the reporter construct in one or more of the sensory organs of the head. Fragments C and D drive expression in both the antennal and maxillary components of the antennomaxillary complex (see Figure 8D, fragment D). In addition, fragment C causes expression in the hypophysis (not shown), and fragment D causes expression in the hypophysis and epiphysis (see Figure 8D). Fragment B drives expression in both components of the antennomaxillary complex and in the epiphysis and hypophysis (not shown), fragment F causes some expression in the antenna (see Figure 6B), and A1 causes expression in the maxillary organ (not shown). Thus, multiple regulatory elements may be used to control _cut_ expression in these complex sense organs.

Expression of Cut protein is also observed in the nuclei of cells associated with the ventral end of the fascicles that carry the axons from the peripheral nervous system into the ganglia of the central nervous system. Fragment D drives reporter expression in this portion of the fascicles in both embryos and late third instar larvae (Figure 5).

**Expression in the central nervous system (CNS):** Cut protein is expressed in many cells throughout the central nervous system at all stages that we have examined. A number of the _cut_ restriction fragments that we tested were found to drive reporter expression in the CNS. Some fragments drive expression in large numbers of cells throughout the CNS, while others drive expression in relatively discrete subsets of CNS cells.

The most extensive expression in the CNS is driven by fragment F, which is closest to and overlaps the insertion sites of some of the _lethal_ 1 mutations. Reporter expression from this fragment is observed in many cells throughout the embryonic CNS (Figure 6A).

Expression driven by other fragments is more restricted. Reporter expression is observed from the fragment D construct in the embryonic CNS in two cells per hemisegment: one in the lateral part of the hemisegment and one near the midline (Figure 6B).

We also examined expression of the reporter constructs in the CNS at the end of the third larval instar. Expression from fragment E is observed in patches of cells that lie on either side of the midline of the ventral ganglion and correspond in number to the thoracic and abdominal segments (Figure 6C). Fragment B drives expression in two locations on the larval brain. One is a lattice of cells that form an arc around the periphery of each optic lobe. In addition, expression is observed in connectives between the two optic lobes and between each optic lobe and the adjoining ventral ganglion (Figure 6D). Fragment F drives expression somewhat more broadly in the larval CNS in cells of the optic lobes, the anterior half of the ventral ganglion, and in a pair of cells or groups of cells near the midline at the posterior tip of the ventral ganglion (Figure 6E).

The data concerning the effect of mutations on _cut_ expression are consistent with the location of multiple regulatory elements for the CNS in the region of the gene that was tested. However, the genetic data cannot confirm whether or not the reporter expression in the CNS is representative of the regulatory elements for _cut_ expression in these cells.

**Expression in the Malpighian tubules:** Cut protein is observed in all of the nuclei of the Malpighian tubules (BLOCHLINGER et al. 1988, 1990), including the tip cells, and loss of expression of the gene in the tubules blocks their development (BLOCHLINGER et al. 1990; LIU and JACK 1992; LIU et al. 1991). Two restriction fragments in the _lethal_ III-_lethal_ 1 interval are capable of driving strong reporter expression in the Malpighian tubules. By far the most complete reporter expression is driven by fragment B (Figure 7A and B). The _cutB-hsp70-lacZ_
construct expresses the reporter strongly in all cells of the Malpighian tubules from the time they begin to bud out from the hindgut (Figure 7A), and expression continues throughout development. This is identical to the pattern of expression of the cut gene in the Malpighian tubules. The A3 fragment produces an interesting pattern of Malpighian tubule expression. In flies bearing this construct, β-galactosidase from the lacZ reporter gene is localized strictly to the tip cells of each Malpighian tubule and in most cases also to the cells to which the tip cells are attached (Figure 7C). The tip cell is a morphologically distinct cell that is attached to the body of each tubule at the distal tip by a slender process. The tip cells control cell division in the tubules and ablation of the tip cell of an individual tubule halts its further development without affecting the other tubules (SKAER 1989). On closer observation of reporter expression driven by the larger 8-kb fragment A, which contains A3, we find that the reporter is also occasionally expressed in the tip cells of the Malpighian tubules but, as is the case in the peripheral nervous system, expression from the larger fragment is weaker and much more variable than expression driven by fragment A3. The observation of β-galactosidase in both the tip cell and the adjacent cell may not necessarily represent A3 expression in both cells. The two cells appear to be connected by a tube-shaped process of the tip cell, and distribution of the protein into the adjacent cell may occur by diffusion out of the tip cell.

The enhancer specific to the tip cell appears to be redundant since fragment B drives expression in all of the cells of the Malpighian tubules, including the tip cells. However, another possibility is that transcription driven by the cutB enhancer occurs in all cells except the tip cell and β-galactosidase enters the tip cell by diffusion. Then, cutA3 would be responsible for expression in the tip cells, and cutB would be responsible for all of the other cells of the tubules.

Much weaker and more restricted expression is observed in the tubules of other constructs. Fragments C–E cause faint expression that is limited to about a third of the tubule cells in the middle for E or distal end for C and D (not shown).

**Expression in spiracles, tracheae, and other cells**

Cut protein is expressed in both the anterior and posterior spiracles in embryos. However, separate fragments drive expression in the anterior and posterior pairs. Four different fragments drive expression in the posterior spiracles. Fragments C and D cause strong lacZ expression in an identical group of cells in the internal portion of the posterior spiracles (Figure 8A and B). These two fragments overlap by ~1.2 kb in the region around +58 on the cut locus map (Figure 1), suggesting the possibility that the enhancer may reside in the region of overlap. Expression begins in the extended germ band stage in three groups of cells on each side (Figure 8A), and the groups later coalesce, forming an internal and probably nonneural component of the
Figure 7.—Expression of cut constructs in the Malpighian tubules. Embryos are stained with anti-β-galactosidase. (A and B) Reporter expression driven by cutB. The transformant line is cutB-HZ50(1.3). (A) A stage 11 embryo in which the tubule primordia have just begun to bud out from the hindgut. (B) A stage 15 embryo in which considerable elongation of the tubules has occurred. Three of the four tip cells are visible in the plain of focus (arrows). (C) An embryo with reporter expression in the tip cells (arrows) and the cells to which the tip cells are attached (arrow head). Expression is driven by the cutA3 fragment, and the tubules were dissected from a stage 12 embryo from transformant line cutA3-hs43(6.2b).

The cutE reporter is also expressed in another group of cells that forms a vertical column in the epidermis, just posterior to and associated with the segmental tracheal trunks from the mesothorax to abdominal segment 7 (Figure 8C, arrowhead, and D, arrow). The vertical column of cells forms in each segment about one row of cells posterior to the anterior segment furrow and just underneath the epidermis, slightly medial to the cells of the external sense organs. Close inspection of embryos stained with anti-Cut antibody reveals that Cut protein is expressed in the same cells.

The most anterior of the columns of reporter expressing cells, that in the mesothorax, contributes to the posterior portion of the anterior spiracle. This spiracle is primarily formed from the posterior third of the prothorax (Campos-Ortega and Hartenstein 1985), but a column of cut expressing cells along the anterior edge of the mesothorax forms an arc and joins cut expressing cells from the prothorax to form the spiracle. During stages 13 and 14 the reporter is also expressed in the cells of the posterior prothorax that form most of the anterior spiracle, but the expression diminishes and is not seen in the anterior part of spiracle in later stages.

Expression of the reporter constructs does not require activity of the cut gene: Some genes are known to sustain their own expression by autoactivation once they have been initially activated by other trans-acting regulators. Autoregulatory aspects of cut expression have been demonstrated by Bocchling et al. (1991). In that case, ectopic expression of Cut protein activated a mutant cut allele in cells in which cut expression is not detected in normal embryos.

However, the cut enhancers that are detected by the lacZ reporter do not show autoregulation. The presence of a wild-type cut gene does not appear to be necessary either to initiate or sustain reporter expression from any of the cut enhancer constructs. We examined activity of each reporter construct in mutant embryos carrying one of the inactive alleles cut^{107} or cut^{145}. Each of the constructs was expressed in the same tissues as in wild-type embryos, although the morphology of some of the tissues was altered by the mutations. Thus, cut expression driven by these enhancers is apparently activated and sustained by factors other than the Cut protein itself.

Discussion

Genetic complexity and cis-acting regulation of cut: From the beginning of our work on the cut locus, the
FIGURE 8.—Embryos showing reporter expression in some other tissues. Embryos are stained with anti-β-galactosidase. Expression in A and B is driven by the cutD fragment. (A) A stage 11 embryo from transformant line cutD-HZ50(5.1). Expression is in three groups of cells located within the area of the primordia of the posterior spiracles. (B) Dorsal view of a stage 16 embryo from transformant line cutD-HZ50(6.1). Reporter expression is strong in cells in the interior of the posterior spiracles and is also seen in sense organs of the head. (C and D) Embryonic reporter expression from cutE. The vertical columns of cells are part of a structure associated with the segmental tracheal branches (arrows in D). The circular groups of cells lie just underneath the epidermis. The transformant line is cutE-HZ50(1.4). The most anterior of the groups of cells shown in C contributes to the anterior spiracles.

molecular basis for the genetic complexity of the gene has been a major point of interest. The fact that different mutations within the gene cause mutant phenotypes that affect different tissues and that the mutations map to different locations in the gene suggested the hypothesis that the tissue-specific mutations altered the regulation of cut activity (Johnson and JuDD 1979). With the discovery that cut confers an external sensory organ identity on specific sense organs (Bodmer et al. 1987; Böchling et al. 1991) and that it is required in a variety of other tissues for proper differentiation (Böchlinger et al. 1990; Jack et al. 1991), the mechanism of tissue-specific regulation of the gene has become an important issue in understanding the molecular basis of the determination and differentiation of the tissues in which cut is expressed.

Analysis of the effects on embryonic cut expression of a large number of mutations (Böchlinger et al. 1990; Liu et al. 1991), in combination with the previous genetic and molecular mapping of mutations (Jack 1985; Johnson and JuDD 1979), demonstrated an association of the type and intragenic location of mutations with the tissues in which gene expression is altered (Jack et al. 1991). Most mutations that map downstream of the start of transcription are functionally null, preventing expression of the gene in all tissues. However, mutations upstream of the transcription start site block synthesis of the Cut protein in only a subset of the tissues in which it is normally expressed. Many of these mutations are insertions of the transposable element gypsy, which has been shown at the yellow (Geyer and Corces 1992) and hsp70 (Holdridge and Dorsett 1991) genes to inactivate enhancers whenever the element lies between the enhancer and its promoter. All that is required for this blocking of enhancer activity is the binding of a protein, the product of the suppressor of hairy wing [su(Hw)] gene, to sites within gypsy. Su(Hw) protein bound to sites between the enhancer and promoter is both necessary and sufficient to inactivate the enhancer, but binding sites just outside the enhancer–promoter interval have no effect on enhancer activity (Holdridge and Dorsett 1991; Geyer and Corces 1992; Smith and Corces 1992).

A number of observations indicate that although the enhancer–promoter distances are much greater at cut than they are at yellow or hsp70, the gypsy transposon causes mutations by the same mechanism. First, the farther the gypsy elements are from the start of transcription, the fewer tissues are affected by the mutations (Figure 1). Furthermore, all cut locus gypsy insertions cause a loss of cut expression in the wing margin that leads to the loss of tissue there, producing the cut wing phenotype (Jack et al. 1991; Böchlinger et al. 1993). An enhancer for the wing margin cells lies just beyond the most distal of the gypsy insertions (Jack et al. 1991), placing all of the insertions in the interval between it and the promoter.

The hypothesis that cut enhancers are inactivated by
gypsy insertions between them and their promoter predicts the location of other cut enhancers. The fact that gypsy lethal I insertions located ~6 kb upstream of the start of transcription block all embryonic cut expression, while lethal III insertions at around 38 kb upstream have no effect on embryonic expression (BODMER et al. 1987; BLOCHLINGER et al. 1990; LIU et al. 1991), predicts that the enhancers for all of the tissues in which cut is expressed in embryos lie in the 32-kb lethal III-lethal I interval (JACK et al. 1991). In fact, we have now identified DNA from that interval capable of activating the expression of a reporter in all of the embryonic tissues in which cut is normally expressed. DNA from the interval activates reporter expression in some larval and adult tissues, as well. In no case have we observed reporter expression in cells in which cut is not expressed. Figure 9 summarizes the location of the known cut locus enhancers. Together, the blocking of cut activity by gypsy elements in the enhancer–promoter intervals and the fidelity of the regulation of the heterologous promoter make a strong case that the enhancers identified by the activation of the reporter are the same ones that regulate the activity of the endogenous cut gene.

Thus, the cis-acting regulatory sequences of the cut gene are spread over a region of ~85 kb upstream of the start of transcription, with the regulators for embryonic expression all located between ~6 and 38 kb upstream and an enhancer for the wing margin at ~85 kb upstream. The effect of gypsy insertions also suggests that an enhancer for the vibrissae lies at ~78 kb upstream of the promoter and an enhancer that is required for postembryonic viability lies in the region between 58 and 38 kb upstream.

An exception to the blocking of enhancers by gyypsy insertions is the kinked femur functional domain, which is similar genetically to regulatory regions of cut in that it is upstream of the promoter, and all of its mutations are rearrangements. Mutations in the protein coding region fail to complement kinked femur mutations. So, an intact coding region is required for wild-type kinked femur function, which is probably a cis-acting regulator of cut. However, although all of the known cut gyypsy insertions fall between the kinked femur region and the cut promoter, none block kinked femur activity.

Activation of cut by genes upstream in the regulatory hierarchy: The products of the genes of the Achaete–Scute Complex (AS-C) and the gene Krüppel are transcription factors (PANKRATZ et al. 1989; CABRERA and ALONSO 1991; VAN DOREN et al. 1992) that are required for the determination of, respectively, the external sensory organs (GARCIA-BELLIDO and SANTAMARIA 1978; GARCIA-BELLIDO 1979; GYPSSEN and DAMBLY-CHAUDIERE 1988) and the Malpighian tubules (HARBECKE and JANNING 1989; LIU et al. 1991; LIU and JACK 1992) and for the expression of cut in those tissues (JACK et al. 1991; LIU and JACK 1992). Therefore, cut could be activated directly by the AS-C genes or by Krüppel.

However, results described here suggest that direct regulation of cut by the AS-C genes and Krüppel is unlikely. Two lines of evidence address the question of regulation by the AS-C genes. The first is that the specific external sensory organs in which individual enhancers are expressed are different from the ones for which individual AS-C genes are required. The regulation of cut in most of the simple external sensory organs is divided between an embryonic and an adult enhancer. In contrast, no AS-C gene or group of genes is responsible specifically for the development of adult or embryonic external sensory organs. Rather, some adult external sense organs require achaete, and others re-
quire scute (Dubinin 1933; Garcia-Bellido 1979; Leys et al. 1989). And some embryonic external sense organs require either achaete or scute, while others require the gene ase (Dambly-Chaudière and Ghysen 1987). So, the observation that cut has one external sensory organ enhancer for all adult external sensory organs and another for all embryonic organs does not follow a pattern suggestive of direct regulation by the AS-C genes.

A second argument against direct activation of the enhancers stems from the fact that even though cut does not autoregulate the enhancers, both the AS-C genes and Krüppel are expressed transiently while external sensory organ and Malpighian tubule expression driven by the cut enhancers is sustained. Krüppel begins to be expressed very early, before the Malpighian tubules begin to bud out from the hindgut, and it ceases to be expressed before morphological differentiation occurs (Gaut et al. 1987). cut, on the other hand, begins to be expressed just before morphogenesis of the tubules begins and continues to be expressed at least through embryonic development (Blochlinger et al. 1988). Similarly, expression of achaete and scute is initiated and declines before the differentiation of the external sensory organs (Cubas et al. 1991; Skeath and Carroll 1991), while cut is expressed in the sense organs well after they differentiate (Blochlinger et al. 1988). If cut were activated directly by either the AS-C genes or by Krüppel, autoactivation would be the simplest mechanism to maintain cut expression after the activator gene ceases expression. However, the activity of the endogenous cut gene is not required to sustain the activity of the Malpighian tubule enhancer or the enhancers for the external sensory organs. So, if the AS-C genes or the Krüppel gene activate cut directly, a third activity would be required to sustain cut expression. A more likely possibility is that another gene both initiates and maintains cut activity.

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LITERATURE CITED


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