Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in *Drosophila*

Patrick Morcillo, Christina Rosen, Mary K. Baylies, and Dale Dorsett¹

Molecular Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 USA

The mechanisms allowing remote enhancers to regulate promoters several kilobase pairs away are unknown but are blocked by the *Drosophila* suppressor of Hairy-wing protein (Suhw) that binds to gypsy retrovirus insertions between enhancers and promoters. Suhw bound to a gypsy insertion in the *cut* gene also appears to act interchromosomally to antagonize enhancer-promoter interactions on the homologous chromosome when activity of the *Chip* gene is reduced. This implicates *Chip* in enhancer-promoter communication. We cloned *Chip* and find that it encodes a homolog of the recently discovered mouse Nli/Ldb1/Clim-2 and *Xenopus* Xldb1 proteins that bind nuclear LIM domain proteins. Chip protein interacts with the LIM domains in the Apterous homeodomain protein, and *Chip* interacts genetically with *apterous*, showing that these interactions are important for Apterous function in vivo. Importantly, Chip also appears to have broad functions beyond interactions with LIM domain proteins. Chip is present in all nuclei examined and at numerous sites along the salivary gland polytene chromosomes. Embryos without Chip activity lack segments and show abnormal gap and pair-rule gene expression, although no LIM domain proteins are known to regulate segmentation. We conclude that Chip is a ubiquitous chromosomal factor required for normal expression of diverse genes at many stages of development. We suggest that Chip cooperates with different LIM domain proteins and other factors to structurally support remote enhancer-promoter interactions.

[Key Words: *apterous*; *even-skipped*; gap genes; homeodomain; LIM domain-binding protein; *suppressor of Hairy-wing*]

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Development of multicellular organisms requires precise temporal and spatial regulation of gene expression. Much of this regulation depends on proteins that bind transcription enhancers. For enhancers separated by a few hundred base pairs from their promoter, DNA looping may be sufficient to allow interactions between basal factors at the promoter and the enhancer-binding proteins. Many complex developmentally regulated genes, however, contain multiple enhancers, which can be many kilobase pairs from the promoter. Enhancers require more than DNA looping to interact with the promoter over such remote distances. For instance, either a UAS or a higher eukaryotic enhancer must be upstream and promoter-proximal to activate transcription in yeast cells, yet both will function downstream of the gene in higher eukaryotic cells (Struhl 1989). This suggests that, in contrast to yeast, higher eukaryotes have factors that facilitate remote enhancer-promoter interactions.

The effects of insertions of the gypsy retrovirus on enhancer activity in *Drosophila* lend support to the enhancer-facilitator hypothesis. Gypsy insertions block enhancer-promoter communication, without inactivating either the enhancer or promoter, when, and only when, they are between the enhancer and promoter (for review, see Dorsett 1996; Geyer 1997). The Suhw protein encoded by *suppressor of Hairy-wing* [*su(Hw)*] that binds to specific sequences in gypsy insertions is necessary and sufficient to block enhancers.

A common domain in Suhw is required for gypsy insertions to block enhancers in several different genes (Harrison et al. 1993; Kim et al. 1996), suggesting that Suhw blocks all enhancers by the same mechanism. Enhancer blocking is distance independent and reversible (Dorsett 1993), and blocked enhancers remain active because they can activate a second promoter in the other direction (Cai and Levine 1995; Scott and Geyer 1995). These observations rule out an epigenetic chromatin structure, or interference with enhancer-binding factors as blocking mechanisms. Although Suhw distorts DNA

¹Corresponding author.
E-MAIL d-dorsett@ski.mskcc.org; FAX (212) 717-3623.

(Shen et al. 1994), this is not sufficient to block enhancers in *Drosophila* (Kim et al. 1996) or an upstream activating sequence (UAS) in yeast (Kim et al. 1993). We postulate, therefore, that the Suhw enhancer-blocking domain interacts and interferes with higher eukaryotic proteins that facilitate interactions between the enhancer and promoter. We envision that enhancer-facilitators help form chromatin structures that bring enhancers and promoters closer together, and that they are different from enhancer-binding activators, coactivators, and basal factors in that they do not participate directly in the activation reaction. It can be inferred that the facilitating chromatin structure needs to be continuous, without interruptions, from the ability of Suhw to block the wing margin enhancer in *cut* from positions scattered throughout the 85-kb region between the enhancer and promoter (Jack et al. 1991), as well as the observation that blocking by gypsy insertions near the *cut* promoter or wing margin enhancer is qualitatively and quantitatively identical (Dorsett 1993).

To identify potential enhancer-facilitators, we conducted a screen for mutations that reduce activity of the remote wing margin enhancer in the *cut* locus (Morcillo et al. 1996a). The screen identified two known genes, *scalloped* (*sd*) and *mastermind* (*mam*), and a novel gene, *Chip*. The data suggest that *sd* and *mam* encode enhancer-binding factors and that *Chip* may encode an enhancer-facilitator. Both *sd* and *mam* mutants display stronger genetic interactions with wing margin enhancer deletions than with gypsy insertions in *cut*, consistent with roles as transcription activators (Fig. 1). Supporting this view, the *sd* protein (Sd) is homologous to the TEF-1 mammalian enhancer-binding factor (Campbell et al. 1992) and binds several sites in the wing margin enhancer DNA (Morcillo et al. 1996a), whereas Mam protein binds a limited number of sites in polytene chromosomes, including one near the *cut* locus (Bettler et al. 1996).

Chip is also needed for wing margin enhancer activity, but appears to play a unique role (Morcillo et al. 1996a). *Chip* mutations dominantly enhance the mutant phenotypes displayed by partially suppressed gypsy insertions in both *cut* and *Ultrabithorax* (*Ubx*) and are homozygous larval lethal, indicating that *Chip* regulates multiple genes. *Chip* is normally required for wing margin enhancer function because *Chip* mutations also enhance the cut wing phenotype of a *cut* mutation that lacks a gypsy insertion, and flies wild type for *cut* and heterozygous for *Chip* display cut wing phenotypes when either *sd* or *mam* are also heterozygous mutant. In contrast to *sd* and *mam* mutants, however, *Chip* mutants display stronger genetic interactions with gypsy insertions than with wing margin enhancer deletions. Thus, in a heterozygous *Chip* mutant, a heterozygous gypsy insertion in *cut* displays a cut wing phenotype, whereas a heterozygous enhancer deletion does not (Fig. 1). Dependence on the nature of the heterozygous lesion in the regulatory region strongly suggests that *Chip* directly regulates *cut*. More strikingly, it indicates that in a *Chip* heterozygote, a gypsy insertion is more deleterious to enhancer func-

Figure 1. Model for regulation of *cut* by the remote wing margin enhancer and the *sd*, *mam*, and *Chip* genes. The model summarizes previous genetic and biochemical data (Morcillo et al. 1996a). The regulatory regions of *cut* genes (not to scale) are shown with the wing margin enhancer (wmE) to the left and the promoter (angled arrow) to the right. The *sd* protein (Sd) binds wing margin enhancer DNA and *mam* protein (Mam) is also postulated to be an enhancer-binding factor (see text). In females heterozygous for wild-type *cut* and an enhancer deletion allele (top pair), a heterozygous *sd* mutation (*sd*²/+) causes a partial cut wing phenotype (cut), while a heterozygous *Chip* mutation (*Chip*^{es.5}/+) displays wild-type wings (+). In contrast, when a female is heterozygous for wild-type *cut* and a gypsy insertion allele (bottom pair), which binds the enhancer-blocking *su(Hw)* protein (Suhw), a heterozygous *sd* mutation displays wild-type wings whereas a heterozygous *Chip* mutation causes a partial cut wing phenotype. These results, taken together, indicate that Suhw on one chromosome interferes with enhancer-promoter communication on both chromosomes and that *Chip* protein (Chip) facilitates enhancer-promoter communication.

tion than deletion of the enhancer. The simplest explanation is that Suhw bound to gypsy in one *cut* allele acts in a transvection-like manner to block the wing margin enhancer in the wild-type *cut* allele on the other chromosome (Fig. 1). This implies that *Chip* plays a crucial role in enhancer-promoter communication.

If *Chip* encodes an enhancer-facilitator as suggested by genetics, its products should be widely expressed, be localized in the nucleus, and affect expression of multiple genes. We cloned *Chip* to address these questions. On the basis of sequence and physical interactions, *Chip* is a homolog of vertebrate factors that interact with the LIM domains of nuclear proteins. Our results also indicate that beyond the specific interaction with LIM domain proteins, *Chip* is a ubiquitous chromosomal protein required for gene expression beginning early in embryogenesis.

Results

Cloning *Chip* by transposon tagging

Chip maps by recombination to 2-106.8 between *orange* and *speck* near the tip of chromosome 2R (Morcillo et al.

CHIP chromosomal protein regulates diverse genes

1996a). In a screen with γ rays, we isolated a deficiency, *Df(2R)Chip^{g230}*, that deletes *Chip*. The visible breakpoints of *Chip^{g230}* (60A3,7; 60B4,7) correlate well with the recombination mapping.

A lethal P-element insertion listed in FlyBase, *l(2)k04405* (Torok et al. 1993), was localized by in situ hybridization to 60B1-2, in the region deleted by *Chip^{g230}*. *l(2)k04405* fails to complement the larval lethality of *Chip* mutants. Like *Chip* mutations (Morcillo et al. 1996a), *l(2)k04405* also dominantly enhances the cut wing phenotype displayed by partially suppressed gypsy insertions in *cut* (not shown). To test whether the P insertion is the *Chip* mutation, we generated excisions with a P-transposase source (*CyO*, *HOP2*) by scoring for loss of the mini-white (*w*) marker in the P element. Of three independent excisions, two lost *Chip* lethality, confirming that the P insertion is a *Chip* mutation.

The *l(2)k04405* P-*lacW* insertion contains a bacterial plasmid origin and ampicillin resistance gene (Bier et al. 1989). The P insertion and 4.5-kb of flanking genomic sequences were rescued from genomic DNA by *SacII* digestion, ligation, and transformation of *Escherichia coli*. The genomic DNA flanking the P element was used to probe P1 phage inserts that contained DNA from the 60A to 60B cytological region (Hartl et al. 1994). This probe hybridized to a single 10-kb *EcoRI* fragment in the DS00543 phage insert.

Smaller fragments were subcloned from the 10-kb *EcoRI* fragment and used to probe Northern blots of RNA from wild-type and *Chip* mutant second and third instar larvae. An antisense RNA probe made from a 1-kb *EcoO109-BamHI* fragment (Fig. 2A) near the *l(2)k04405* P insertion detected a 2.2-kb polyadenylated RNA reduced ~50% in two heterozygous *Chip* mutants, *Chip^{g371}* and the *Chip^{g230}* deficiency. The 7-kb *BgIII-EcoRI* genomic fragment containing the probe fragment (Fig. 2A) rescues *Chip^{e5.5}* lethality when introduced into the genome by P element-mediated germ-line transformation, indicating that it contains the essential portions of the gene. We conclude that the 2.2-kb RNA is the *Chip* mRNA.

A cDNA library from imaginal discs was probed with the 1-kb *EcoO109-BamHI* genomic fragment (Fig. 2A) that detects the 2.2-kb RNA, and eight clones were isolated. Several had the same 5' end, and others had the same 3' end. The few clones containing both the common 5' and 3' ends were sequenced, revealing a long open reading frame (ORF) (Fig. 2A) and a poly(A) tail. The ORF encodes a protein of 577 amino acids (Fig. 2B). Comparison of the cDNA and genomic DNA sequences revealed a single small intron upstream of the ORF and that the *l(2)k04405* P-insertion site is a few base pairs upstream of the splice acceptor site (Fig. 2A).

To determine the molecular basis of the *Chip^{e5.5}*, *Chip^{g371}*, and *Chip^{g1}* alleles, the ORF was amplified by PCR from genomic DNA isolated from homozygous mutant second instar larvae. All three mutations are small deletions that shift the reading frame. The locations of these deletions are indicated in Figure 2A. Conceptual translation indicates that *Chip^{e5.5}* encodes a 153 amino

Figure 2. The *Chip* gene and predicted *Chip* protein sequence. (A) Structure of the *Chip* gene. Shown is the restriction map of the 7-kb *BgIII-EcoRI* genomic DNA fragment that rescues *Chip* homozygous mutant larval lethality. (Ba) *BamHI*; (Bg) *BgIII*; (E) *EcoRI*; (H) *HindIII*; (O) *EcoO109*; (P) *PstI*. Locations of the *l(2)k04405* P insertion, and the small deletions in *Chip^{e5.5}*, *Chip^{g371}*, and *Chip^{g1}* are indicated by arrows. The boxes below indicate the sequences present in the *Chip* cDNA clones, with the solid box indicating the ORF. The intron is indicated by a V. The *Chip* wild-type and mutant sequences have been deposited in GenBank (accession nos. AF010325-AF010328). (B) Sequence of *Chip* (D.m.) compared with the vertebrate homologs *Xldb1* (X.l.) and *Nli/Ldb1/Clim-2* (M.m.). Identities are silhouetted. The putative nuclear localization signal is underlined.

acid protein in which the first 141 are the same as wild type. *Chip^{g1}* encodes a 365-amino-acid protein, in which the first 300 are the same as wild type, and *Chip^{g371}* encodes a 182-amino-acid protein, in which the first 68 are wild type. These lesions confirm the identity of the *Chip* ORF.

Chip is a functional homolog of vertebrate nuclear LIM domain-binding proteins

The protein encoded by the *Chip* ORF is homologous to the mouse *Nli/Ldb1/Clim-2* (Agulnick et al. 1996; Jurata

et al. 1996; Bach et al. 1997), mouse Clim-1 (Bach et al. 1997), and frog Xldb1 (Agulnick et al. 1996) vertebrate proteins that bind to the LIM domains of nuclear proteins. Chip residues 205–577 display 58% identity with the mouse Nli/Ldb1/Clim-2 protein (Fig. 2B). All of these proteins have a potential nuclear localization signal (Fig. 2B). There are no yeast homologs of Chip, suggesting that Chip and its relatives are specific to higher eukaryotes.

The major difference between Chip and the vertebrate homologs is that Chip has a proline-rich amino-terminal domain of ~200 amino acids (Fig. 2B). Thus, the truncated protein encoded by *Chip^{es.5}* does not contain the conserved domain, consistent with the observation that it behaves like a null allele (Morcillo et al. 1996a).

The Nli/Ldb1/Clim-2, Clim-1, and Xldb1 proteins were isolated because they physically interact with nuclear LIM domains (Agulnick et al. 1996; Jurata et al. 1996; Bach et al. 1997). There are mutants available for the *apterous* (*ap*) gene in *Drosophila*, which encodes a LIM-homeodomain (HD) protein involved in wing and muscle development (Bourgouin et al. 1992; Cohen et al. 1992). We tested whether *Chip* interacts genetically with *ap*. Flies heterozygous for either *Chip^{es.5}* or *ap^{56f}* have normal wings, but flies heterozygous for both display margin defects (Fig. 3A). *Chip^{es.5}/ap^{56f}* male progeny of *Chip^{es.5}* mothers and *ap^{56f}* fathers have 2.5 ± 0.1 ($n = 261$; error value is the standard error) margin gaps per fly, whereas progeny with the same genotype from *ap^{56f}* mothers and *Chip^{es.5}* fathers have 1.0 ± 0.2 ($n = 47$) gaps per fly. *Chip^{es.5}* also interacts with *ap⁴* (not shown). We believe it is unlikely that Ap directly regulates the *cut* wing margin enhancer because Ap is detected only in the dorsal half of the cells in which this enhancer is active (Blair 1993).

The interaction between *Chip* and *ap* and the homology between Chip and the vertebrate LIM domain-binding proteins raised the question of whether Chip interacts directly with the Ap LIM domains. This was tested in a yeast double-hybrid fusion experiment, in which full-length Chip was fused to the bacterial LexA DNA-binding protein, and the Ap LIM domains were fused to the transcription activation domain of the yeast GAL4 protein. The two fusion proteins were coexpressed in a yeast strain containing two reporter genes with LexA-binding sites in the promoter (Hollenberg et al. 1995). One reporter expresses the *lacZ* gene detected by X-gal staining, and the other expresses the *HIS3* gene, detected by selection on media lacking histidine. When the two fusion proteins are coexpressed, both reporters are activated (Fig. 3B). The reporters are not expressed when the GAL4 activation domain alone, or the GAL4 activation domain fused to Chip, is coexpressed with the LexA-Chip fusion. We conclude that Chip interacts with the Ap LIM domains.

CHIP is a widely expressed chromosomal factor

Ap is expressed in a tissue-specific pattern during development (Bourgouin et al. 1992; Cohen et al. 1992). If

Figure 3. Interactions between Chip and the Ap LIM protein. (A) Genetic interaction between *Chip* and *ap*. The wing from a *y* w*; Chip^{es.5}/ap^{56f}* male has gaps in the posterior margin. (B) Interaction between Chip and the Ap LIM domain in yeast. Yeast were transformed with a plasmid expressing the full length *Chip* ORF with the *lexA* DNA-binding protein fused to the amino terminus, and a second plasmid expressing the indicated protein containing the Gal4 activation domain (GAD, Gal4 activation domain alone; GAD-Chip, the Gal4 activation domain fused to the *Chip* ORF; GAD-APLIM, the Gal4 activation domain fused to the amino terminus of the Ap LIM domains). Interaction between Chip and the Ap LIM domains is indicated by the activation of the *lexA* operator–*HIS3* reporter gene as detected by growth on histidine omission plates (*his*⁻), and expression of the *lexA* operator–*lacZ* fusion gene as detected by X-Gal staining (X-Gal). The GAD-APLIM protein does not activate the reporters when coexpressed with the LexA DNA-binding domain alone (not shown).

Chip, as suggested by genetic studies, plays a more general role in gene expression than regulating Ap activity, it should be more widely expressed. A Northern blot of total RNA reveals that *Chip* mRNA is present at all developmental stages (Fig. 4). Strikingly, early embryos (0–30 min old, lane EE) have ~10-fold higher levels than later developmental stages. Because this is before zygotic gene expression, we conclude that *Chip* mRNA is maternally contributed during oogenesis.

To investigate expression of Chip protein, anti-Chip antibodies were raised in rabbits. The *Chip* cDNA was expressed in *E. coli*, producing a 70-kD protein used as antigen. Affinity-purified antibodies recognize a major band of 68 kD and minor bands of 82 and 22 kD in Western blots (not shown) of proteins extracted with 0.35 M NaCl from nuclei of cultured Schneider 2 *Drosophila* cells (Dorsett 1990). Embryos stained with these antibodies reveal that from the early cellular blastoderm stage (Fig. 5A) through gastrulation (Fig. 5C) to the end of

Figure 4. Expression of *Chip* transcripts during development. (top) An autoradiograph of a Northern blot showing the *Chip* 2.2-kb mRNA; (bottom) a photograph of the ethidium bromide-stained rRNA in the gel used for the Northern blot. Each lane contained 5 μ g of total RNA isolated from Oregon-R wild-type flies at the indicated developmental stage [EE, 0–30 min after egg laying (AEL); LE, 30 min to 16 hr AEL; L1–L3, first to third instar larvae; P1–P4, first to fourth day of pupation; M, 0- to 1-day old male adults; F, 0- to 1-day old adult females]. *Chip* transcript was quantitated by PhosphorImager and normalized to the amount of rRNA as determined by densitometry. After normalization, the *Chip* transcript level in early embryos (EE) is ~5-fold higher than in late embryos (LE) and ~10-fold higher than in larval and pupal stages.

embryogenesis (not shown), Chip protein is present in most, if not all, nuclei, including the pole cell nuclei. Staining is undetectable or very weak in syncytial blastoderm nuclei until just before cellularization (not shown) although Chip must be present at low levels, because lack of Chip activity affects expression of seg-

mentation genes at this stage (see below). Embryos produced from germ-line clones homozygous mutant for *Chip*^{es.5} (see below) do not stain at any stage (Fig. 5B), showing that the staining is Chip-specific. Chip is also present in the nuclei of larval tissues including imaginal discs (Fig. 5D–F), fat body (Fig. 5G), and salivary glands (Fig. 5H). These observations indicate that Chip is essentially a ubiquitous nuclear factor during embryogenesis and larval development.

Examination of the larval salivary glands stained with anti-Chip antibodies revealed that the polytene chromosomes stain in banded patterns (Fig. 5I). From examination of several nuclei, it is evident that Chip is present at many sites along the entire lengths of all chromosomes. The nucleoli and nonchromosomal portions of the nuclei do not display significant staining. Attempts to stain the polytene chromosomes after spreading were unsuccessful, indicating that the reagents used to spread the chromosomes either denature the Chip epitopes or remove Chip from the chromosomes. Although we cannot identify the specific loci with which Chip associates, the very large number of sites with which Chip associates is consistent with a general role in gene expression.

Chip is required for accurate expression of segmentation genes

Most homozygous *Chip* mutants die prior to the third instar larval stage (Morcillo et al. 1996a). If Chip is re-

Figure 5. Expression of Chip protein. Embryos and larval tissues were stained with affinity-purified anti-Chip antibodies. All panels are wild-type Oregon-R and the same magnification unless indicated otherwise. Embryos are lateral views with anterior to the left and dorsal to the top. All larval tissues are from third instar larvae. (A) Cellular blastoderm; (B) mutant cellular blastoderm from *Chip*^{es.5} germ-line clone; (C) gastrula; (D) wing imaginal disc; (E) eye-antennal imaginal disc; (F) leg imaginal discs; (G) larval fat body; (H) larval salivary gland; (I) salivary gland nucleus at 10-fold higher magnification than in panel H. The polytene chromosomes stain in a banded pattern.

Figure 6. *Chip^{e5.5}* germ-line clone embryonic cuticles. All panels are the same magnification. Anterior is to the left; dorsal is to the top. The wild-type cuticle is a lateral view and the mutant cuticles are slightly ventral aspects. (A) Wild-type Oregon-R embryo; (B) *Chip^{e5.5}* germ-line clone grown at 18°C. (C) *Chip^{e5.5}* germ-line clone grown at 25°C.

quired for the activity of many enhancers, one might expect that homozygous mutants would be embryonic lethal. We considered the possibility that maternally loaded *Chip* mRNA allows survival into the larval stages. To test this, germ-line clones homozygous mutant for *Chip^{e5.5}*, which is the only lethal mutation on its chromosome, were generated with the dominant female sterile yeast flip recombinase system (Chou and Perriemon 1996). The embryos from *Chip^{e5.5}* germ-line clones fail to hatch. The cuticles exhibit normal dorsal-ventral polarity, but severe segmentation defects. At 18°C, most have a single fused, irregularly shaped patch of ventral denticles (Fig. 6B). At 25°C, the phenotype is slightly less severe and most have a large fused denticle patch and one or two denticle bands resembling A7 and A8 of wild type (Fig. 6C). A few rare embryos at 25°C display a pair-rule-like phenotype with approximately half the normal number of segments (not shown).

We tested whether a wild-type paternal allele could influence the phenotype of *Chip^{e5.5}* germ-line clone embryos. Germ-line mosaic females were mated to males with *Chip^{e5.5}* on one chromosome and a P element containing a *fushi tarazu (ftz)-lacZ* fusion gene on the homolog with a wild-type *Chip* allele. The resulting embryos were stained with anti-β-galactosidase antibodies. We observed roughly equal numbers of staining and non-staining developing embryos with indistinguishable segmentation defects (not shown), indicating that a wild-type paternal *Chip* allele does not substantially alter development.

The unsegmented phenotype of *Chip^{e5.5}* germ-line clone embryos resembles that displayed by embryos homozygous for null alleles of the pair-rule gene *even-skipped (eve)* (Nüsslein-Volhard et al. 1985). To determine whether the phenotype reflects a lack of *eve* expression, we stained *Chip^{e5.5}* germ-line clone embryos raised at 18°C with anti-Eve antibodies. Eve is expressed,

Figure 7. Expression of Eve in *Chip^{e5.5}* germ-line clone embryos. Embryos stained with anti-Eve antibodies. All are lateral views with anterior to the left and dorsal to the top. (A) Early wild-type cellular blastoderm. (B) Early *Chip^{e5.5}* germ-line clone cellular blastoderm. Eve is overexpressed relative to wild-type. (C) Wild-type cellular blastoderm with seven stripes of Eve expression. (D) *Chip^{e5.5}* germ-line clone cellular blastoderm showing weak Eve expression.

although the pattern is abnormal. There are stripes (Fig. 7D), but the number is often less than the seven in wild type (Fig. 7C). Furthermore, the first stripe to appear (Fig. 7B) is often wider and stronger than in wild type (Fig. 7A), whereas later-appearing stripes (Fig. 7D) are narrower, weaker, and more uneven than wild type (Fig. 7C). All seven stripes are affected, although the effects on stripes 1 and 7 are less dramatic.

The Eve expression defects are insufficient to explain the unsegmented phenotype of *Chip^{e5.5}* germ-line clone embryos because hypomorphic *eve* mutations exhibit

Figure 8. Expression of gap gene proteins in wild-type and *Chip^{e5.5}* germ-line clone embryos. Cellular blastoderm embryos were stained with antibodies against the indicated gap proteins (Hb, Kr, Kni, and Gt). All are lateral views with anterior to the left and dorsal to the top. (A, C, E, G) Wild type. (B, D, F, H) Mutant embryos from *Chip^{e5.5}* germ-line clones. Kr and Kni expression is lower in the mutants, and the domain of Gt expression is expanded.

pair-rule phenotypes (Nüsslein-Volhard et al. 1985). Embryos homozygous mutant for two pair-rule genes can also have an unsegmented phenotype (Nüsslein-Volhard et al. 1985). Expression of a *ftz-lacZ* fusion gene, however, is also only partially defective in *Chip^{es.5}* germ-line embryos, displaying an abnormal stripe pattern that alternates with the Eve pattern (not shown). This suggests that pair-rule gene defects alone are insufficient to explain the cuticle phenotype.

Pair-rule genes are regulated by gap genes. The observation that Eve and *ftz-lacZ* are expressed in abnormal alternating stripe patterns suggests that gap gene expression might also be partially defective in embryos lacking Chip activity. To confirm this, we stained *Chip^{es.5}* germ-line clone embryos incubated at 18°C with antibodies against the four gap proteins Krüppel (Kr), Knirps (Kni), Giant (Gt), and Hunchback (Hb). The Hb pattern is indistinguishable from wild type (Fig. 8A,B), and there are mild defects in the Kr and Kni patterns. At the cellular blastoderm stage, the Kr (Fig. 8C,D) and posterior Kni bands (Fig. 8E,F) are both slightly narrower, weaker, and more uneven in *Chip^{es.5}* germ-line clone embryos than in wild-type. Despite these mild abnormalities, the expression patterns of these three gap genes indicate that the maternally derived anterior-posterior Bicoid and Nanos protein gradients are present in embryos lacking active Chip.

Lack of active Chip affects Gt more severely than the other gap proteins. In wild-type precellular and early cellular blastoderm embryos, Gt is restricted to two broad bands (Fig. 8G), whereas in *Chip^{es.5}* germ-line clone embryos, Gt is expressed at low to moderate levels in the entire embryo, including the pole cells (Fig. 8H). Expression in domains to which Gt is restricted in wild type varies from moderate to high. In later stages, when Gt resolves into multiple bands of expression in wild-type, Gt expression in mutant embryos is similar to wild type (not shown).

Misexpression of Gt in embryos lacking active Chip can explain the decreased expression of Kr and Kni because Gt represses *Kr* and *kni* (Kraut and Levine 1991; Capovilla et al. 1992). Because Gt helps define the anterior border of Eve stripe 2 by repression (Small et al. 1991), it is conceivable that abnormal Gt expression also weakens Eve stripe 2. Misexpression of Gt, however, should not weaken other Eve stripes because some of these overlap wild-type Gt domains. We conclude that at least some of the effects on Eve expression in embryos lacking active Chip are independent of the effects on Gt expression.

The observed defects in gap gene and eve expression would be more consistent with partial segmentation than the observed unsegmented cuticle phenotype. We postulate, therefore, that the mutant cuticle phenotype reflects expression defects in multiple genes at different levels in the segmentation pathway. Importantly, however, the effects on the expression of gap genes, among the first zygotic genes expressed, shows that Chip is required for accurate gene expression very early in development. The fact that embryos lacking active Chip ex-

press segmentation genes also shows that Chip is not required for basal promoter activity.

Discussion

Chip is a unique general transcription factor

The *Chip* gene was identified in a genetic screen designed to identify factors that facilitate communication between remote enhancers and promoters (Morcillo et al. 1996a). We postulate that enhancer-facilitator proteins are not directly involved in the transcription activation reaction, but help form chromatin structures that bring the enhancer and promoter closer together. We infer that Chip is a potential enhancer-facilitator because in *Chip* mutants, the Suhw protein that binds gypsy insertions and blocks enhancer-promoter communication can act interchromosomally to block the remote wing margin enhancer in the *cut* gene (Morcillo et al. 1996a).

The observations reported here support the view that Chip, as expected for an enhancer-facilitator, plays a general role in gene expression. *Chip* mRNA is present at all stages of development, and Chip is a virtually ubiquitous nuclear protein. In contrast, other transcription factors that support *cut* wing margin enhancer activity, such as Sd (Campbell et al. 1992; Williams et al. 1993) and Mam (Bettler et al. 1996; Schmid et al. 1996), are expressed in specific temporal and spatial patterns. Chip protein also associates with numerous sites along the entire lengths of the salivary gland polytene chromosomes as expected for a general factor, and in contrast to a more gene- and tissue-specific factor like Mam, which associates with a limited number of sites (Bettler et al. 1996). Furthermore, reductions in Chip activity affect expression of diverse sets of genes at various stages of development, including gap genes, which are among the first genes expressed.

Chip is unlikely to be a basal transcription factor because lack of Chip activity does not cause a loss of promoter activity for any of the segmentation genes examined. However, mutations in the TATA-binding protein (TBP)-associated factors (TAF_{II}s) associated with the basal transcription apparatus drastically reduce expression of some segmentation genes and have no effects on others (Sauer et al. 1996). Thus, we cannot entirely rule out the possibility that Chip is a basal factor that affects a promoter's response to specific enhancers, although this would not explain why reduced Chip activity allows interchromosomal enhancer blocking by Suhw.

The possibility that Chip creates an open chromatin structure, thereby improving the access of transcription factors to DNA, is also unlikely. In contrast to other factors proposed to have this function, such as the *mod-(mdg4)* [*E(var)3-93D*] proteins (Dorn et al. 1993) or GAGA factor (Farkas et al. 1994), mutations reducing Chip activity do not modify the position effect variegation that occurs when chromosomal rearrangements place a euchromatic gene next to heterochromatin (Morcillo et al. 1996a).

The predicted amino acid sequence further supports

that view that Chip plays a unique role in enhancer function because it is not homologous to any cloned transcription factors such as enhancer-binding proteins, basal factors, or coactivators, and does not contain any known protein motifs. Moreover, Chip homologs were detected in higher eukaryotes but not in yeast. Part of the motivation for the hypothesis that higher eukaryotes have factors that facilitate enhancer-promoter interactions is the observation that a yeast UAS can behave like an enhancer in higher eukaryotic cells but acts only promoter-proximal in yeast (Struhl 1989).

Chip-LIM protein interactions in development

The vertebrate homologs of Chip provide clues to some of Chip's molecular activities. The mouse Nli/Ldb1/Clim-2, *Xenopus* Xldb1, and mouse Clim-1 proteins were isolated because they bind to LIM domains of nuclear proteins (Jurata et al. 1996; Agulnick et al. 1997; Bach et al. 1997). The LIM domain is a class of Zn²⁺-chelating protein motifs involved in a variety of protein-protein interactions (for review, see Sanchez-Garcia and Rabbitts 1994; Dawid et al. 1995). LIM domains are found in both nuclear and cytoplasmic proteins and there are several subclasses of LIM domains. Many nuclear LIM domain proteins also contain the HD DNA-binding motif, whereas others (LMO proteins) consist primarily of LIM domains. Nli/Ldb1/Clim-2, Clim-1, and Xldb1 bind LIM domains of the subclass found in LIM-HD and nuclear LMO proteins (Agulnick et al. 1996; Jurata et al. 1996; Bach et al. 1997).

LIM domains appear to have negative effects on the activities of LIM-HD proteins. Thus, mutation or truncation of the LIM domains increases the DNA-binding activity of the Islet-1 (ISL-1) and Mec-3 LIM-HD proteins (Sanchez-Garcia et al. 1993; Xue et al. 1993). Mutation of the LIM domains also allows injected Xlim-1, a LIM-HD protein, to induce secondary axes in *Xenopus* development (Taira et al. 1994). Similar to mutation of the LIM domains, coinjection of the Xldb1 Chip homolog with Xlim-1 leads to axis duplication (Agulnick et al. 1996). This suggests that binding of Xldb1 overcomes the negative effects of the LIM domains.

Although the coinjection experiment indicates that Xldb1 can interact with Xlim-1 in vivo and affect development, it does not show that this interaction is required for normal development, or that Xldb1 is an essential protein. The homozygous larval lethality of mutant *Chip* alleles (Morcillo et al. 1996a) and the embryonic lethality of *Chip*^{e5.5} germ-line clones, therefore, is the first demonstration that a member of this class of LIM domain-binding proteins is an essential developmental factor. The interaction between Chip and the Ap LIM domains in a yeast double-hybrid fusion experiment shows that Chip is a functional homolog of Nli/Ldb1/Clim-2, Clim-1, and Xldb1. More importantly, the synergistic wing margin phenotype displayed by flies heterozygous for both *apterous* and *Chip* mutations indicates that the Chip-Ap interaction is normally required in vivo.

Do LIM domain and LIM domain-binding proteins have broad roles in gene expression and development?

Like Chip, Xldb1 is nuclear (Agulnick et al. 1996) and Nli/Ldb1/Clim-2 is expressed in a variety of tissues (Agulnick et al. 1996; Jurata et al. 1996; Bach et al. 1997). In contrast, LIM domain proteins such as Ap and Xlim-1 are expressed in tissue-specific patterns and have specific developmental tasks (for review, see Dawid et al. 1995). Curiously, Chip plays a key role in segmentation, yet no LIM domain proteins are known to be involved in segmentation. Dlmo, a LMO protein, is present during *Drosophila* embryogenesis (Zhu et al. 1995), but there are no *Dlmo* mutants and its functions are unknown. The direct targets of Chip regulation during embryogenesis are unknown, but *gt* and *eve* are two candidates on the basis of the evidence presented here; however, they are not known to be regulated by LIM domain proteins. Moreover, Chip regulates expression of *cut* and *Ubx* during imaginal disc development (Morcillo et al. 1996a), and these genes are also not known to be regulated by LIM domain proteins. It is possible, therefore, that LIM domain proteins play broader roles in development than appreciated previously, and that there are several unknown LIM domain proteins required for segmentation and imaginal disc development. Consistent with this idea, additional *Drosophila* LIM-HD proteins have recently been discovered. The Arrowhead protein is involved in establishment of some imaginal precursor cells (Curtiss and Heilig 1995, 1997a,b), the Islet protein governs axon pathfinding and neurotransmitter identity (Thor and Thomas 1997), and a *Drosophila* Lim-3 homolog is involved in embryonic nervous system development (A. Tomlinson, S. Thor, and J. Thomas, pers. comm.).

Another possible explanation for the broad functions of Chip is that it may interact with other proteins without LIM domains. The two mouse Chip homologs, Nli/Ldb1/Clim-2 and Clim-1 interact directly with P-Otx, a HD protein that lacks LIM domains (Bach et al. 1997). There are several non-LIM HD proteins involved in *Drosophila* segmentation and regulation of *Ubx*, and it is feasible that Chip interacts with one or more of these proteins.

How do Chip and its homologs regulate enhancer activity?

LIM-HD proteins are likely to function like other homeodomain proteins. Because HD proteins are commonly thought of as activators and repressors that bind to enhancers, the interaction between Chip and the Ap LIM-HD protein appears at first to suggest that Chip is a coactivator. Recent evidence, however, indicates that the vertebrate Chip homologs may regulate interactions between different activator proteins. Both mouse Chip homologs potentiate synergistic activation of transcription by the P-LIM/LHX3 and P-Otx HD proteins in transfection experiments with reporter gene constructs (Bach et al. 1997). Nli/Ldb1/Clim-2 also blocks syner-

gistic activation by the Lmx1 LIM-HD and E47 basic helix-loop-helix (bHLH) proteins (Jurata and Gill 1997). Nli/Ldb1/Clim-2 forms homodimers and higher order complexes with Lmx1 that contain multiple Nli/Ldb1/Clim-2 molecules (Jurata and Gill 1997), suggesting that it may also bridge together different activator proteins. Similarly, Nli/Ldb1/Clim-2 is a component of an erythroid DNA-binding complex containing the Lmo2 LIM-only protein, the Tal1 and E47 bHLH proteins, and the Gata-1 zinc finger protein (Wadman et al. 1997). Thus Chip and its homologs may function at enhancers to bring together diverse transcription factors and form higher order activation complexes, and in some cases, to block formation of such complexes.

Although Chip and its homologs are likely to function at enhancers to regulate interactions between diverse activator molecules, this alone cannot explain the genetic observation that, in contrast to an enhancer-binding factor, Chip is antagonistic to Suhw enhancer-blocking activity (Morcillo et al. 1996a). Enhancers blocked by Suhw can still activate another promoter in the other direction (Cai and Levine 1995; Scott and Geyer 1995), indicating that Suhw does not reduce the binding or activation potential of enhancer-binding factors. Therefore, the specific genetic antagonism between Chip and Suhw indicates that Chip plays an additional role in enhancer-promoter communication. Because Chip and its homologs interact with diverse HD proteins, it is also of interest that HD proteins may have functions other than activation and repression. Homeodomain proteins bind DNA with low sequence specificity *in vitro* (Desplan et al. 1988; Hoey and Levine 1988; Walter and Biggin 1996). Strikingly, and in contrast to other transcription factors, all the HD proteins examined *in vivo* bind nearly uniformly throughout their genetically defined target genes, instead of to specific enhancers. In UV cross-linking experiments, Eve and Ftz bind throughout at least 10 kb of eve, as well as several kilobase pairs of *Ubx* and *ftz* (Walter et al. 1994; Walter and Biggin 1996). Similarly, *in vivo* formaldehyde cross-linking experiments reveal that the Abd-B homeodomain protein binds throughout several kilobase pairs of the *empty spiracles* gene (Orlando et al. 1997). Thus, it has been proposed that HD proteins may help organize the structure of regulatory regions through interactions between themselves and other proteins (Walter et al. 1994). Similar to the way they may function at enhancers, the Chip family of proteins may also mediate interactions between HD proteins to help form structures that support interactions between distant regulatory elements and their promoters.

Materials and methods

Screen for new Chip alleles

Approximately 220,000 progeny were screened after γ ray mutagenesis for enhancers of the *ct^{L-32}; su(Hw)^{e2}* cut wing phenotype as described previously (Morcillo et al. 1996a) except the second chromosome was marked with a P[w⁺] element at 57B. Three new *Chip* alleles: *Chip^{g96.1}*, *Chip^{g230}*, and *Chip^{g371}* were

identified. *Chip^{e5.5}* was mapped to 2-106.8 by use of P elements as markers as described elsewhere (Morcillo et al. 1996a,b).

Reversion of the l(2)k04405 P-element insertion

l(2)k04405 males were crossed to females with the *CyO*, *HOP2* transposase chromosome (provided by W. Gelbart, Harvard University, Boston, MA). F1 *CyO*, *HOP2/l(2)k04405* males were crossed to *yellow^{*} (y^{*}) w^{*}; CyO*, *Df(2R)Kr4*, *Kr^{B80}*, *Dp(1;2)y⁺* females. Three independent *y^{*}w^{*}; CyO*, *Df(2R)Kr4*, *Kr^{B80}*, *Dp(1;2)y⁺/w⁻* F2 males were crossed to *Chip^{g230}/CyO*, *Df(2R)Kr4*, *Kr^{B80}*, *Dp(1;2)y⁺*, and *Chip^{e5.5}/CyO*, *Df(2R)Kr4*, *Kr^{B80}*, *Dp(1;2)y⁺* females to test for complementation of *Chip* lethality.

Rescue of the l(2)k04405 P-element insertion from genomic DNA

The *P-lacW* (Bier et al. 1989) element in *l(2)k04405* (Torok et al. 1993) was designed to be rescued by transformation of *E. coli* with genomic DNA. Three micrograms of genomic DNA from homozygous *l(2)k04405* second instar larvae were digested with *Sac*II, religated, and used to transform *DH5 α* *E. coli*. The rescued plasmid contains ~4.5 kb of genomic DNA.

Isolation of Chip genomic DNA

P1 phage DNA was prepared as described by Hartl et al. (1994). Southern blots of *Eco*RI-digested P1 phage DNA were prepared as described in Sambrook et al. (1987). A 4.5-kb *Hind*III fragment of the rescued *l(2)k04405* plasmid containing the flanking genomic DNA was gel-purified, labeled with ³²P by random priming (Sambrook et al. 1987), and used as probe. A 10-kb fragment of the DS00543 P1 phage insert hybridizing to the probe was gel-purified and cloned into the *Eco*RI site of pBlue-script (SK-) (Stratagene). Several kilobase pairs around the P-insertion site were sequenced with Sequenase v. 2 (U.S. Biochemical) according to the manufacturer's protocols.

RNA preparation and Northern blots

RNA isolation and Northern blot hybridization were performed as described previously (Dorsett et al. 1989). Single-stranded ³²P-labeled RNA probes were prepared with the 1-kb *Eco*O109–*Bam*HI fragment (Fig. 2A) as template. Northern blots were quantitated with a PhosphorImager. As an internal standard, rRNA was quantitated by densitometric scanning of a photographic negative.

P-element rescue of Chip

A 7.4-kb *Bgl*II–*Eco*RI fragment (Fig. 2A) was cloned into the *Bam*HI and *Eco*RI sites of pCasper (Pirrotta 1988). The resulting plasmid was used for P element-mediated germ-line transformation (Rubin and Spradling 1982). Transformant flies were crossed to *Chip^{e5.5}* flies to test for complementation of *Chip* lethality.

Chip cDNA cloning

A third instar imaginal disc cDNA library in λ gt10 (provided by J. Kim, University of Wisconsin, Madison) was screened by use of standard protocols (Sambrook et al. 1987) with probes prepared by random-priming of the 1-kb *Eco*O109–*Bam*HI genomic DNA fragment (Fig. 2A). Eight hybridizing plaques were plaque-purified and DNA prepared. The *Eco*RI inserts were cloned into

pBluescript (SK-), restriction mapped, and full-length clones were sequenced.

Sequencing of *Chip* mutant alleles

The *Chip* ORF was amplified from genomic DNA isolated as described elsewhere (Levis et al. 1982) from homozygous mutant *Chip*^{es.5}, *Chip*^{g1}, and *Chip*^{g371} second instar larvae by PCR [primer 1 (5'-ATTAGAATTCATGAATCGTAGGGGTTTGA-3') and primer 2 (5'-ATTAAGATCTGTGTGTAGAGTA-GACGAC-3')] (Saiki et al. 1988). Amplified DNA was digested with *Bgl*II and *Eco*RI, cloned into the *Bam*HI and *Eco*RI sites of pBluescript (SK-), and sequenced.

Genetic interaction between *Chip* and *ap*

y^{w}; CyO, Df(2R)Kr4, Kr^{B80}, Dp(1;2)y⁺/Chip^{es.5}* flies were crossed to *y^{w*}; CyO, Df(2R)Kr4, Kr^{B80}, Dp(1;2)y⁺/ap^{56f}* flies at 25°C. The mutant wing margin phenotype of F1 males displaying a *y* phenotype was quantitated by scoring for nicks as described previously (Morcillo et al. 1996a).

Yeast double-hybrid fusion experiments

Yeast double-hybrid fusion experiments were conducted as described by Bartel and Fields (1995). The *Chip* ORF was amplified by PCR (primer 1 and primer 2), digested with *Bgl*II and *Eco*RI, and cloned into the *Bam*HI and *Eco*RI sites of pBTM116 (Bartel and Fields 1995), fusing it to the carboxyl terminus of the bacterial *lexA* reading frame. The segment of *ap* encoding the two LIM domains (Cohen et al. 1992) was amplified by PCR [primer 3 (5'-ATTAGGATCCAGCAAAATAACGCGCAACG-3') and primer 4 (5'-ATTAGGATCCCGTTAGGTGGCGCTCAT-3')] from a cDNA clone (provided by D. Segal, University of Tel Aviv, Israel), digested with *Bam*HI, and cloned into the *Bam*HI site of pGAD10 (provided by S. Fields, University of Washington, Seattle), thereby fusing the Gal4 activation domain to the amino terminus of the LIM domains. To provide a control plasmid, the *Chip* ORF was amplified by PCR [primer 2 and primer 5 (5'-ATTAAGATCTATGAATCGTAGGGGTTTGAA-3')], digested with *Bgl*II, and cloned into the *Bam*HI site of pGAD10. L40 yeast (*MATa, his3Δ200, trp1-901, leu2-3,112, ade2, LYS::(lexAop)₄-HIS3, URA3::(lexAop)₈-lacZ*) (Hollenberg et al. 1995) were transformed by use of the LiCl method (Sherman et al. 1986) with the indicated plasmids by selection on leucine and tryptophan double omission plates containing 2% glucose (Sherman et al. 1986) at 30°C. To evaluate *lacZ* reporter expression, colonies were stained with X-Gal. Activation of the *HIS3* reporter was evaluated by growth on leucine, tryptophan, and histidine triple omission plates at 25°C.

Expression of *Chip* in *E. coli* and antibody preparation

Chip protein was expressed in *E. coli* as a fusion with glutathione *S*-transferase (GST). Part of the *Chip* cDNA open reading was amplified by PCR from a cDNA clone [primer 5 and primer 6 (5'-ATTAGAATTCAACGCTTCTGGTTTCGTCACA-3')]. The 1.4-kb PCR product was digested with *Bgl*II and *Eco*RI and cloned into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia Biotech). The *Bam*HI-*Eco*RI fragment of the resulting clone was replaced by the 0.7-kb *Bam*HI-*Eco*RI fragment of the *Chip* cDNA clone to produce the expression plasmid.

Seven liters of DH5α *E. coli* with the *Chip* expression plasmid were grown to saturation in Luria broth at 30°C and protein expression induced by 0.5 mM IPTG for 3 hr. All subsequent steps were conducted at 4°C. The *E. coli* pellets were suspended

in 11 ml/liter of culture in EB [20 mM HEPES at pH 7.5, 0.1 M NaCl, 1 mM 2-mercaptoethanol, 0.1% NP-40, 50 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of leupeptin and pepstatin, 2 μg/ml of aprotinin, 10% glycerol]. The cells were lysed by sonication, and the lysate was clarified by centrifugation at 13,000g for 15 min. The pellet was suspended in EB, sonicated, clarified by centrifugation, and the two supernatants were pooled. The supernatant was incubated with 4 ml glutathione-agarose beads (Sigma, equilibrated with EB) overnight with gentle agitation. The beads were washed twice with 50 ml of EB for 10 min, twice with TB (20 mM Tris-HCl at pH 8.4, 0.15 M NaCl, 1 mM 2-mercaptoethanol, and 10% glycerol), and twice with TB containing 0.1 M CaCl₂. The beads were suspended in 6 ml of TB containing 0.1 M CaCl₂ and *Chip* protein eluted by thrombin cleavage. Fresh thrombin (1.5 U/ml) was added every hour during the 3-hr incubation at 25°C with gentle agitation. PMSF (1 mM) and EDTA (2 mM) were added prior to dialysis of the supernatant against 100 volumes of 25 mM HEPES at pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM PMSF, and then against the same buffer lacking PMSF. The dialysate was concentrated with a Centricon filter. The final extract (0.6 ml) contained 1.44 mg/ml of protein, ~40% *Chip*, as estimated by SDS-PAGE. The major contaminant appeared to be *E. coli* GroEL.

E. coli *Chip* was used as antigen in rabbits at the Pocono Rabbit Farm and Laboratory (Canandensis, PA) according to their standard purified protein antigen protocol. The anti-*Chip* antibodies were affinity-purified from crude serum on a Western blot of an SDS-PAGE separation of bacterial *Chip* extract as described elsewhere (Harlow and Lane 1988). The purified antibody was dialyzed overnight at 4°C against buffer containing 50 mM Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 10% glycerol. Glycerol was added to bring the final concentration to 50% and the purified antibody stored at -20°C.

Antibody staining

Embryos were dechorionated with bleach and fixed in a 1:1 solution of heptane and 4% paraformaldehyde in PBS (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, and 2.7 mM KCl₂, at pH 7.0) for 20 min with gentle agitation. The embryos were washed with heptane, devitellinized with a 1:1 mix of heptane and methanol, and dehydrated with methanol. Larval tissues were dissected in cold PBS (pH 7.2) containing 0.3% Triton X-100, fixed in PBS (pH 7.2) containing 4% paraformaldehyde, and dehydrated in methanol. Embryos and larval tissues were rehydrated in PBST (pH 7.4 PBS containing 0.3% Triton X-100), blocked with PBST containing 0.5% bovine serum albumin (PBSTB) for 30 min, and incubated with primary antibodies overnight at 4°C in PBSTB with gentle agitation. After several washes in PBST and blocking with PBSTB for 30 min, the tissues were incubated with a 1:200 dilution of the secondary antibodies (Vector Laboratories) for 45 min. The colorimetric reaction was conducted according to instructions provided with the Vectastain kit (Vector Laboratories). The anti-Eve (provided by Y. Hiromi, Princeton University, NJ) and anti-β-galactosidase antibodies were used as described previously (Baylies et al. 1995) and affinity-purified anti-*Chip* antibody was diluted 1:100. For the anti-gap protein antibodies (provided by J. Reintz, Mount Sinai Medical School, New York, NY) the following dilutions were used: Hb, 1:2000; Kr, 1:2000; Kni, 1:1000, and Gt, 1:2000. Embryos and larval tissues were mounted in araldite and photographed with a 20× objective and bright-field or Nomarski optics. Salivary gland nuclei were photographed with bright field optics with a 100× oil immersion objective and a 2× Barlow lens.

Generation of germ-line clones and cuticle preparation

Female $y^1 w^{1118} P\{ry^{+7.2} = hsFLP1; P\{w^{+mW.hs} \Rightarrow w^{hs} > \} G13 FRT 42B Chip^{e5.5}/P\{w^{+mW.hs} \Rightarrow w^{hs} > \} G13 P\{w^{+mC} = ovoD1-18\} 2R1 P\{w^{+mC} = ovoD1-18\} 2R2$ third instar larvae were heat shocked to generate germ-line clones as described by others (Chou and Perrimon 1996). Eclosing females were mated to males heterozygous for *Chip*^{e5.5} and embryos collected on apple juice agar plates. Cuticles were prepared by dechorionating and devitellinizing embryos as described above. Devitellinized embryos were rehydrated twice with PBST, and clarified by addition of a 1:1 solution of Hoyer's medium (Ashburner 1989) and 85% lactic acid and incubation at 70°C overnight. Mutant cuticles were photographed by use of Nomarski optics with a 20× objective and wild-type cuticles were photographed with dark field optics.

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