Identification of target genes of the homeotic gene *Antennapedia* by enhancer detection

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Localized expression of the homeotic gene *Antennapedia* (*Antp*) in *Drosophila melanogaster* is required for normal development of the thoracic segments. When the *Antp* gene is expressed ectopically in the larval primordium of the antenna, the antennal imaginal disc, the developmental fate of the disc is switched and the adult antenna is transformed to a mesothoracic leg. We screened ~550 different fly strains carrying single copies of an enhancer-detector transposon to identify regulatory elements and corresponding genes that are either activated or repressed in antennal discs in response to this transformation. Several regulatory elements that are either direct or indirect targets of *Antp* were found. One transposant that expresses the reporter gene (*lacZ*) in the antennal disc, but not in the leg disc, was studied in more detail. The enhancer detector in this strain is located near a similarly regulated gene at the *spalt* (*sal*) locus, which encodes a homeotic function involved in embryonic head and tail development. The expression of this newly discovered gene, *spalt major* (*salm*) is strongly repressed in gain-of-function mutants that express *Antp* in the antennal disc. Recessive loss-of-function mutations ($Antp^-$) have the opposite developmental effect; they cause the differentiation of antennal structures in the second leg disc. Accordingly, *salm* is derepressed in clones of homozygous $Antp^-$ cells. Therefore, we conclude that *Antp* negatively regulates *salm*. The time course of the interaction and reporter gene fusion experiments suggests (but does not prove) a direct interaction between *Antp* and *cis*-regulatory elements of *salm*. Our analysis of several enhancer-detector strains suggests that the basic patterning information in the antennal and leg imaginal discs is very similar.

[*Key Words*: *Drosophila*; homeotic genes; *Antennapedia* gene; enhancer detection; downstream genes; *spalt* locus]

Received September 18, 1991; revised version accepted October 11, 1991.

The unique identities of embryonic and adult body segments in the fruit fly *Drosophila melanogaster* are determined by the expression of specific homeotic genes within the segmental primordia (Lewis 1978; Kaufman et al. 1980). Each homeotic gene can intiate, either independently or in combination with other homeotic genes, one or more segment-specific developmental programs in the fly. Therefore, it has been proposed that the homeotic genes regulate the expression of a group of subordinate genes that establish segmental identity (Garcia-Bellido 1975). Some of these "downstream" or target genes probably control local developmental decisions within each segment, whereas others might encode structural, enzymatic, or other functions in differentiated cells. In support of this regulatory hypothesis, the homeotic gene products that determine the identity of abdominal, thoracic, and certain head segments all contain a highly conserved polypeptide region, the homeo domain, which has sequence-specific DNA-binding properties (for review, see Gehring and Hiromi 1986; Gehring 1987; Scott et al. 1989; Affolter et al. 1990; Hayashi and Scott 1990). Some *Drosophila* homeo domain-containing proteins have been shown to regulate the expression of reporter genes carrying homeo domain-binding sites in embryos, in cultured cells, and in vitro (e.g., Jaynes and O'Farrell 1988; Thali et al. 1988; Driever and Nüsslein-Vollhard 1989; Krasnow et al. 1989; Ohkuma et al. 1990). Furthermore, certain known vertebrate transcription factors contain a homeo domain (Herr et al. 1988; Guazzi et al. 1990). Therefore, it is assumed that homeo domain-containing homeotic proteins in *Drosophila* function by directly regulating genes at the transcriptional level.

Only a few putative target genes have been identified that are either directly or indirectly controlled by the homeotic genes of *Drosophila*; most of the best candidates are other homeotic genes that interact with one

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another to establish their specific expression patterns (e.g., Hafen et al. 1984; Struhl and White 1985). However, careful analysis of early embryonic gene expression patterns has, in some cases, revealed other potential target genes (Immerglück et al. 1990). Standard genetic approaches have not readily revealed genes that act downstream of the homeotic genes. One major reason for this shortcoming may be that most of the downstream genes are required for multiple roles in many different segments, so that their deletion either may not be associated with a homeotic or segment-specific phenotype or may be lethal at an early stage of development. To overcome these problems, Kennison and Tamkun (1988) took a classical genetic approach and screened for genes that interact with dominant homeotic mutations in a dosage-dependent fashion. They identified a number of interacting genes, although at least some of these genes are thought to act in parallel with and not downstream of the homeotic genes. Recently, Gould et al. (1990) constructed a genomic library containing sequences from the fraction of embryonic chromatin that is immunoprecipitated by antibodies against the protein encoded by the homeotic gene *Ultrabithorax* (*Ubx*). This library is enriched for *Ubx*-binding sites, and in two cases these sites are located near *Ubx*-regulated genes.

We have taken a combined molecular and genetic approach to look for genes controlled by the homeotic gene *Antennapedia* (*Antp*). This gene is required for normal development of the embryonic, larval, and adult thorax, particularly the mesothorax (Struhl 1981; Wakimoto and Kaufman 1981). Like other homeotic genes, its spatially localized expression pattern normally restricts its domain of action to specific body segments. Dominant gain-of-function mutations lead to the ectopic expression of *Antp* in the larval primordium of the antenna, the antennal imaginal disc and activate a thorax-specific developmental program in the disc so that the adult antenna is transformed into a mesothoracic leg (e.g., Hazelrigg and Kaufman 1983; Frischer et al. 1986; Jorgensen and Garber 1987; Schneuwly et al. 1987a,b; Gibson and Gehring 1988). Recessive loss-of-function mutations have the opposite effect: The absence of the *Antp* gene product in the mesothoracic disc results in transformation toward antennal structures (Struhl 1981), suggesting that *Antp* plays a pivotal role in determining the developmental differences between the antennal and mesothoracic leg imaginal discs.

A recently developed technique, called enhancer detection (or enhancer trapping), allows the expression patterns of large numbers of genes to be visualized and screened rapidly in vivo before the genes are cloned (O'Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989; Wilson et al. 1989; for review, see Wilson et al. 1990). In brief, single copies of a transposon carrying a fusion between the reporter gene *lacZ* and a weak promoter are introduced randomly into the *Drosophila* genome using a simple set of genetic crosses. The reporter construct responds to a wide range of regulatory elements, such as enhancers, in its vicinity (Bellen et al. 1989; Bier et al. 1989). Because, in many cases, these elements normally control *Drosophila* genes (Wilson et al. 1989), the enhancer detector frequently reflects part or all of the expression pattern of one or more neighboring genes. About 550 enhancer-detector strains have been screened for reporter gene expression in the imaginal discs of third-instar larvae (G. Gibson and W.J. Gehring, in prep.); in a few cases, the *lacZ* expression patterns in the leg and antennal discs differ significantly. In this paper we show that the staining pattern in the antennal discs of many of these strains is transformed towards the staining pattern in the leg disc in the presence of the *Antp* gene product. We characterize one of these strains in detail. It carries a disruptive insertion in a newly identified gene, *spalt major* (*salm*), which is located within the homeotic *spalt* (*sal*) locus. This locus is involved in embryonic head and tail development (Jürgens 1988). Dominant gain-of-function mutations leading to the ectopic expression of *Antp* in the antennal disc repress *salm* in the antennal disc, whereas recessive loss-of-function mutations have the opposite effect and derepress *salm* in the second leg disc. This indicates that *salm* is regulated negatively by *Antp*. Because down-regulation of *salm* requires roughly coincident expression of *Antp*, it is possible that *salm* is controlled directly by *Antp*. Our studies allow us to confirm and elaborate on previous hypotheses based on genetic experiments concerning the developmental function and action of *Antp* in *Drosophila*.

Results

Enhancer-detector screen for Antp*-regulated genes*

Late third-instar larvae of >500 enhancer-detector insertion strains (or transposants) from the screen of Bellen et al. (1989) were stained for β-galactosidase activity (G. Gibson and W.J. Gehring, in prep.). In 75 of these strains, the staining pattern includes *lacZ* expression in the antennal or leg imaginal discs. For nearly 80% (59 lines) of these 75 lines, the staining patterns in the two types of disc are not obviously different. However, for the other 16 strains, all of which also express *lacZ* in other larval tissues, the staining pattern in the leg discs (prothoracic, mesothoracic, and metathoracic) differs from that in the antennal disc. In these cases, enhancer-detector expression is affected presumably by genomic transcriptional control elements that are regulated differentially in the leg and antennal discs. Because the developmental differences between the antennal and mesothoracic leg discs are thought to be determined primarily by the *Antp* gene, these regulatory elements and any genes that they normally control may be regulated either directly or indirectly by *Antp*.

To test this hypothesis, the transposants were crossed to different fly strains that express the *Antp* protein product inappropriately in the antennal discs. Two of these strains carry dominant gain-of-function alleles of the *Antp* locus, namely $Antp^{Ns}$ and $Antp^{73b}$. The third, called H4, carries a P-element insertion containing an *Antp* cDNA, which includes the entire *Antp* protein-coding sequence, fused to the *hsp70* heat shock promoter

(Schneuwly et al. 1987b). Incubation of early and mid-stage third-instar larvae from this strain at 37°C leads to the transformation of adult antennal structures to leg structures (Schneuwly et al. 1987b; Gibson and Gehring 1988).

Initially, all 16 selected transposants were crossed to the $Antp^{73b}$ strain. In eight cases, ectopic expression of *Antp* in the antennal disc altered the staining pattern of the transposant significantly, so that it resembled more closely the staining pattern in the leg disc. Figure 1 shows the four clearest examples. Two of these strains (A184.3F1 and A405.1M2) are normally stained in antennal discs but lack at least some elements of this staining pattern in leg discs. The other two (C1.1S3 and A245.1F2) are stained in both types of discs, but in different patterns. For example, in strain C1.153, at least two roughly equally sized intersecting rings of staining can be detected in the leg disc (Fig. 1A). In the antennal disc, two concentric rings, one much smaller than the other, are observed (Fig. 1B). As judged from the fate map (Gehring 1966; Bryant 1978; Haynie and Bryant 1986), the larger one is located in the second antennal segment primordium and the smaller one in the primordium of the arista. If *Antp* is overexpressed in the antennal disc, an additional larger ring of staining, which intersects the larger concentric ring, appears in the antennal disc (Fig. 1D), so that the antennal staining pattern more closely resembles the leg disc pattern. In strain A245.1M2, leg discs (Fig. 1E) show a characteristic staining pattern in the shape of a horseshoe, whereas only a peripheral staining outside of the segmented region in the antennal disc can be detected (Fig. 1F). Staining in a horseshoe-shaped domain at the center of the antennal disc is only observed in the presence of ectopically expressed *Antp* (Fig. 1H). Only a single ring of staining is found in leg discs of strain A184.3F1 (Fig. 1I), whereas two concentric but incomplete rings are observed in the antennal disc (Fig. 1J). The additional outer ring in the antennal disc is lost in the presence of *Antp* (Fig. 1L). Finally, the leg imaginal discs of strain A405.1M2 are not stained (Fig. 1M). The ring of staining normally observed in the antennal disc in the primordium of the second antennal segment is strongly repressed in gain-of-function *Antp* genetic backgrounds (cf. Fig. 1N,P). In the other eight strains in which the enhancer detector is differentially regulated in antennal and leg discs, the differences in staining are relatively subtle (i.e., typically one or two more rings of staining in the leg disc). We were not able to determine with certainty whether ectopic *Antp* expression affects the antennal disc-staining pattern in these strains.

In further studies, we have examined a second set of enhancer-detector strains that stain in the third-instar larval leg or antennal imaginal discs. Of the 150 selected strains studied (kindly provided by M. Mlodzik, U. Gaul, and G. Rubin), we identified 9 strains with significantly different β-galactosidase expression patterns in the antennal and leg discs. In all cases the antennal disc expression pattern is transformed toward a leg-like expression pattern in an $Antp^{73b}$ genetic background. Two examples are shown in Figure 1. In strain rK781 the antennal disc is stained only weakly (Fig. 1R). If *Antp* is expressed in the antennal disc, a patch and a semicircular ring of staining appear; a similar staining pattern is visible in the normal leg disc (cf. Fig. 1Q,T). However, the patch of staining in the antennal disc does not seem as extensive as that in the leg disc. A peripheral domain of β-galactosidase expression is observed in a sector of the leg disc of strain rI781 (Fig. 1U), whereas the eye antennal disc is not stained (Fig. 1V). In $Antp^{73b}$ mutants (Fig. 1X), a similar domain of staining is also found in the antennal disc.

In summary, our enhancer-detector screen has identified a number of genomic transcriptional regulatory elements that are expressed in different patterns in the leg and antennal imaginal discs. In the most extreme cases, these elements and any genes that they might control are normally only active in one type of disc. Our results indicate that the homeotic gene *Antp* is responsible for this differential regulation.

Different dominant gain-of-function Antp *alleles have different effects on the enhancer-detector strains*

Jorgensen and Garber (1987) demonstrated that *Antp* transcripts are only detected in an anterior sector of the antennal disc in $Antp^{Ns}$ mutants, whereas in $Antp^{73b}$ mutants *Antp* is expressed more uniformly throughout the antennal disc. We examined the effect of the $Antp^{Ns}$ allele on *lacZ* expression in the antennal discs of selected enhancer-detector strains (see Fig. 1C,G,K,O,S,W). In contrast to the pattern transformations in an $Antp^{73b}$ mutant background, which appear to affect the entire antennal disc, only the staining pattern in the anterior part of the antennal disc is altered in an $Antp^{Ns}$ genetic background. For example, only the anterior region of mutant $Antp^{Ns}$ antennal discs from strain C1.1S3 exhibits the second outer ring of staining characteristic of leg discs in this strain (Fig. 1C). Likewise, in strains A184.3F1 and A405.1M2, *Antp*-induced repression of β-galactosidase staining is only observed in the anterior part of $Antp^{Ns}$ antennal discs (Fig. 1K,O). There are no enhancer-detector strains in which staining in the posterior part of the antennal disc is transformed by the $Antp^{Ns}$ allele. The domain in which pattern transformations are observed in an $Antp^{Ns}$ genetic background roughly corresponds to the domain in which *Antp* transcripts are observed in this mutant (Jorgensen and Garber 1987). However, we have not been able to produce sufficiently strong double labeling with antibodies to the *Antp* and β-galactosidase proteins to define the precise region of transformation.

Antenna-to-leg transformations of enhancer-detector staining patterns are induced by ectopic expression of Antp *only at specific stages of development*

When third-instar larvae carrying a heat shock–*Antp* (hs–*Antp*) fusion construct are incubated at 37°C during the early and middle stages of their development, the antennae of the resulting adult flies show transformations toward leg structures (Schneuwly et al. 1987b; Gib-

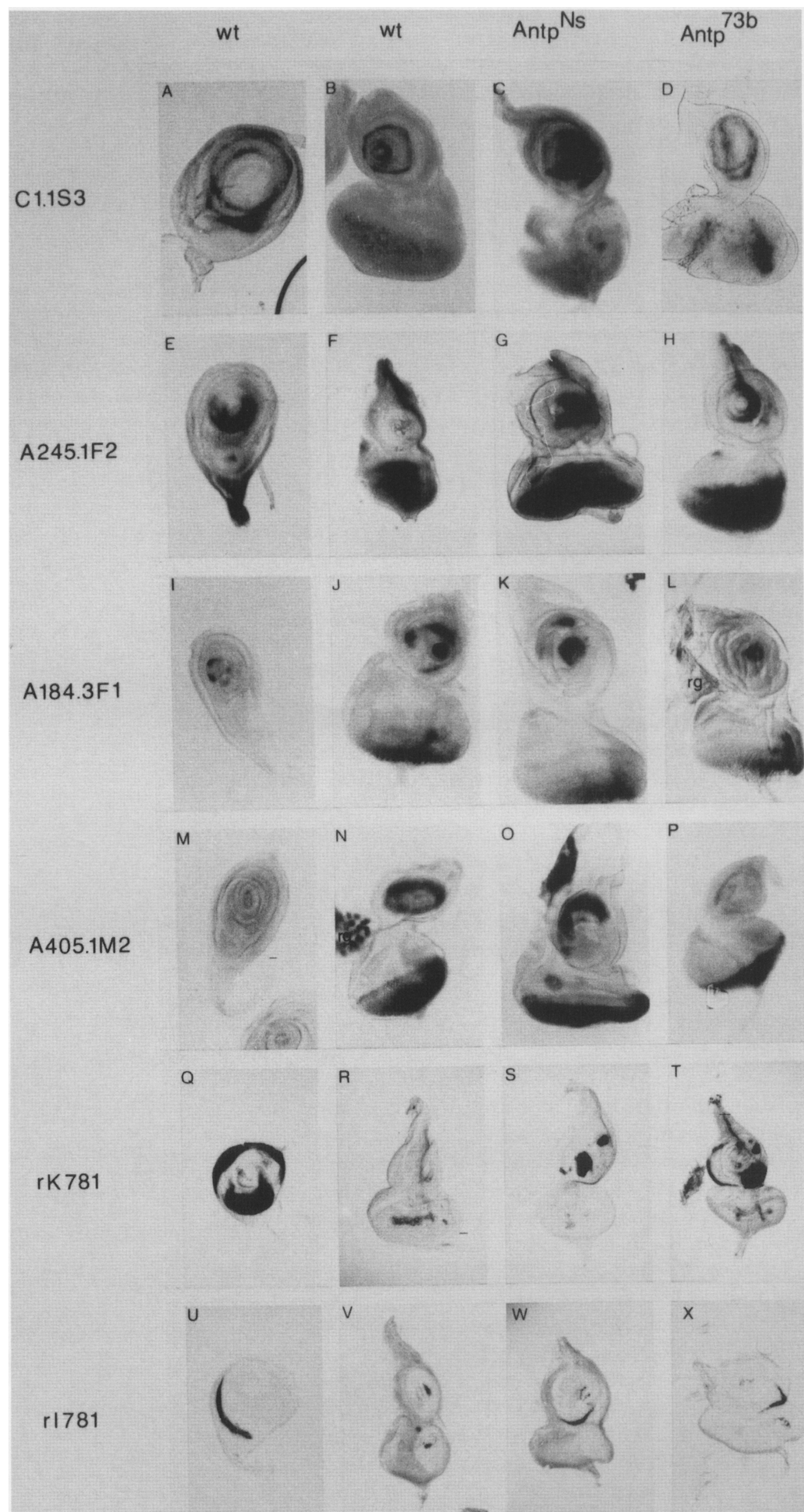

Figure 1. β-Galactosidase expression patterns of transposants carrying *Antp*-regulated enhancer detectors. Leg discs (*A,E, I,M,Q,U*) and eye antennal discs (*B,F,J, N,R,V*) of different transposant lines and eye antennal discs from transposant lines that had been crossed to $Antp^{Nc}$ (*C,G, K,O,S,W*) or $Antp^{73b}$ (*D,H,L,P,T,X*) flies were stained for β-galactosidase activity. The strains studied are C1.1S3 (*A–D*), A245.1F2 (*E–H*), A184.3F1 (*I–L*), A405.1M2 (*M–P*), rK781 (*Q–T*), and rI781 (*U–X*). The patterns of staining in the leg discs of all six strains differ from the normal staining patterns in antennal discs. In all cases, the antennal disc-staining pattern is transformed toward the corresponding leg disc pattern when *Antp* is expressed ectopically in the antennal disc (see text for details). The pattern transformations in the $Antp^{Ns}$ genetic background are restricted to an anterior region of the disc, whereas those in $Antp^{73b}$ larvae include all parts of the disc. The posterior edge of the antennal disc is at the *top* of each plate. In this orientation, the polar positional coordinates of the antennal disc appear to be rotated ~90° counterclockwise relative to the coordinates in the leg disc (cf. E and H, Q and T, etc.). In some eye antennal discs, patches of endogenous staining are observed (e.g., R,V); this staining is produced by variable numbers of macrophage-like cells that lie on the surface of the peripodial membrane of the disc. (rg) Ring gland.

son and Gehring 1988). A number of the *Antp*-regulated enhancer-detector strains were crossed to flies carrying this heat shock construct (H4 strain), and the progeny were heat shocked at different stages of larval development. We describe our results for two strains, A184.3F1 and A405.1M2.

A184.3F1 is normally stained in two incomplete concentric rings in the antennal discs during mid- and late third-instar larval stages (Fig. 1J); the outer ring, which is absent in leg discs and in transformed antennal discs (Fig. 1L), is located in the primordium of the second antennal segment (Schubiger 1968; Haynie and Bryant 1986). In the presence of the hs–*Antp* construct, repeated 45-min heat shocks at 4-hr intervals during the first half of third-instar larval development (between ~68 and 90 hr after fertilization) result in the complete absence of the outer ring of β-galactosidase staining in strain A184.3F1 during later larval development (data not shown). Repression is not observed when the heat shocks are applied at stages coincident with A184.3F1 staining. Therefore, it is essential that *Antp* is expressed during a time window prior to the A184.3F1 reporter to repress the outer ring of staining in antennal discs.

Gibson and Gehring (1988) have found that a single 1-hr heat shock induction of *Antp* transcription at 76–80 hr of development is sufficient to induce specific transformations in the second antennal segment of hs–*Antp* flies. Therefore, we reasoned that the *Antp*-induced repression of the outer ring of staining in strain A184.3F1 might be a reflection of the developmental process leading to transformation of the second antennal segment to the corresponding leg segment (trochanter, femur). Thus, A184.3F1 larvae carrying the hs–*Antp* construct were subjected to a single 1-hr heat shock at different developmental stages. A single heat shock at 76–77 hr was sufficient to repress completely *Antp*-regulated *lacZ* expression in A184.3F1 antennal discs, even though this expression is normally first observed only 24 hr later (cf. Figs. 1J and 2D). A heat shock at 72 or 84 hr of development had no significant effect on the A184.3F1 staining pattern. Some larvae from each experiment were allowed to develop to adulthood. Only those heat-shocked at 76–77 hr showed specific transformations of the second antennal segment. This is consistent with the notion that the altered staining pattern is caused by the transformation of the second antennal segment primordium to a leg segment primordium.

A second enhancer-detector strain, A405.1M2, is unique among the strains that we identified in our first screen, because β-galactosidase is first detected much earlier in the imaginal discs than in any other strains, namely at 74 hr of development. This expression is restricted to the primordium of the second antennal segment (Fig. 1N; Fig. 4N,O, below). However, other regions of the third-instar larva are also stained, including the region of the eye disc posterior to the morphogenetic furrow (Fig. 1N), most of the wing disc, and specific cells in the brain (data not shown). The period of antennal disc *lacZ* expression in this strain includes the entire time window during which heat shock-induced pulses of ectopically expressed *Antp* can transform the presumptive second antennal segment.

We tested the effect of a single 1-hr heat shock at 76 hr of development on A405.1M2 flies carrying the hs–*Antp* construct (A405.1M2/H4). Four hours after the heat shock (at 80 hr) antennal disc staining was repressed

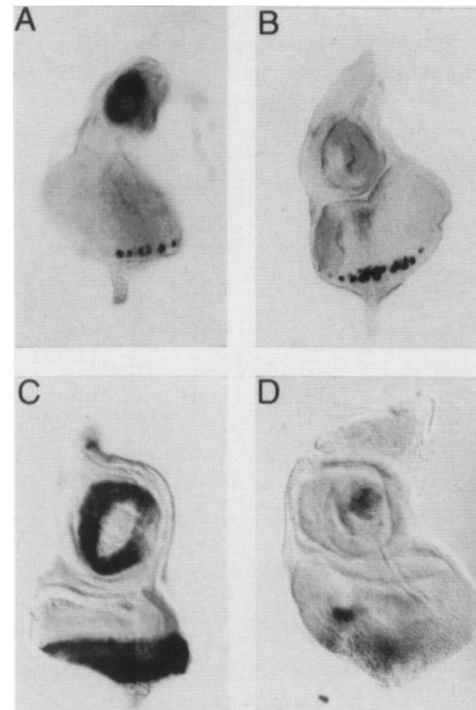

Figure 2. Effect of a single pulse of *Antp* expression on β-galactosidase staining in two enhancer-detector strains. Third-instar larvae of different enhancer-detector strains heterozygous for the hs–*Antp* construct (H4, Schneuwly et al. 1987b) were heat-shocked for 1 hr at 76–77 hr of development. Eye antennal discs were then dissected at later developmental stages and stained for β-galactosidase activity. (*A*) A heat-shocked 80-hr-old third-instar larva of strain A405.1M2. (*B*) A heat-shocked 80-hr-old larva of strain A405.1M2 carrying a single copy of the H4 hs–*Antp* fusion construct. Antennal disc staining is repressed. (*C*) A 100-hr-old A405.1M2/H4 larva heat-shocked at 76 hr. Staining in the antennal disc is no longer repressed by the earlier pulse of *Antp* expression. (*D*) A 100-hr-old third-instar larva of strain A184.3F1/H4, which was heat-shocked at 76 hr of development. The antennal disc-specific outer ring of staining shown in Fig. 1J is absent.

completely (cf. Fig. 2A and B). However, within the next 20 hr, staining returned to normal levels (cf. Figs. 1N and 2C), suggesting that a single pulse of *Antp* expression is insufficient to repress β-galactosidase synthesis throughout the third-larval instar in antennal discs of strain A405.1M2. In support of this hypothesis, total repression of antennal disc staining in A405.1M2/H4 flies is only observed in late third-instar larvae, when six consecutive 45-min heat shocks are given at 4-hr intervals from 68 hr of development onward (see Fig. 4C, below). Such stringent conditions are sufficient to induce complete transformation of all adult antennal segments toward a leg fate and also lead to severe reduction of the adult eye (Gibson et al. 1990). Despite the grossly altered morphology of the resulting antennal discs, the discs still retain sufficient patterning information to develop into readily identifiable segmented legs. Because A405.1M2 larvae that do not carry the hs–*Antp* construct stain normally under the same heat shock regime, the repression ob-

served in hs–*Antp* larvae is *Antp* dependent. Furthermore, in other enhancer-detector strains (e.g., A245.1F2), domains of staining that are shared by the leg and antennal discs are not significantly repressed by repeated heat shock treatments in a hs–*Antp* background, suggesting that repression in strain A405.1M2 larvae is not a result of general transcriptional inhibition.

Consistent with the phenotypic effect observed in the eye of hs–*Antp* adults, β-galactosidase expression in the eye disc is also repressed in A405.1M2/H4 larvae exposed to successive heat shocks (Fig. 4C, below). However, other elements of the A405.1M2 staining pattern remain unaffected by extensive heat shock treatment at any time during third-instar larval development (data not shown). The brain and wing discs are still strongly stained, even though immunocytochemical staining with an antibody that specifically cross-reacts with the *Antp* protein indicates that *Antp* is expressed relatively uniformly in the imaginal discs and brains of heat-shocked hs–*Antp* larvae (Schneuwly et al. 1987b; G. Gibson, unpubl.).

Coregulation of the A405.1M2 enhancer detector and the neighboring salm *gene by* Antp

Most of the *Antp*-regulated enhancer-detector strains that we examined are only stained in the antennal or leg imaginal discs from mid- or late third-instar larval development onwards. One major exception, discussed above, is strain A405.1M2; *lacZ* expression in the antennal discs of this strain is first observed in the early third-instar larva at 74 hr after fertilization, only a few hours after the second molt. Because roughly coincident expression of *Antp* is required to repress this early staining, the regulatory element identified by the A405.1M2 enhancer detector may be controlled directly by *Antp*. We therefore chose to study strain A405.1M2 in more detail.

Previous genetic crosses indicated that the P[lArB] enhancer detector in strain A405.1M2 is located on the second chromosome balancer, CyO (see Bellen et al. 1989). The *Escherichia coli* origin of replication and the ampicillin-resistance gene in P[lArB] allowed a genomic DNA fragment lying on the 3' side of the A405.1M2 insertion to be cloned selectively by plasmid rescue (see Materials and methods; Steller and Pirotta 1986). This fragment was used as a probe for in situ hybridization to polytene chromosomes of wild-type flies to establish the cytological location of the transposon in A405.1M2. A single insertion was found at 33A on the second chromosome. This is the location of *sal*, a genetic locus that encodes a homeotic function involved in embryonic head and tail development (Jürgens 1988). The insertion in strain A405.1M2 disrupts *sal* function. The A405.1M2 insertion chromosome does not complement either of two *sal* alleles (sal^{2B57} or sal^{2A55}) described previously by Jürgens (1988). Previously, Frei et al. (1988) identified a candidate *sal* gene at the *sal* locus. P-element rescue experiments suggested that it is involved in embryonic *sal* function. The early embryonic expression pattern of this gene, which is partly consistent with the phenotype of *sal*, closely resembles the early staining pattern in strain A405.1M2 (Bellen et al. 1989). It consists of two precisely defined transverse bands of expression: one at the anterior end (58–70% egg length) and one at the posterior end (8–18% egg length) of the embryo. Another group of cells express β-galactosidase in the dorsal part of the presumptive head region at ~85% egg length. However, later embryonic expression of this gene does not resemble the staining pattern observed in A405.1M2 at similar stages, and no transcripts are detected in the third-larval instar (Frei et al. 1988). Subsequent experiments have shown that at least one other gene that lies much closer to the A405.1M2 enhancer-detector insertion shares the same early embryonic expression pattern (Fig. 3; R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.). We designate this newly identified gene as *salm* because it plays a primary role in *sal* function (R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.), whereas the previously identified gene (Frei et al. 1988) seems to have only an accessory function (Reuter et al. 1989; R. Schuh, unpubl.) and thus is called *spalt accessory* (*sala*).

R. Schuh and his colleagues have recently generated a specific polyclonal antibody against part of the *salm* protein product (R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.). We examined the *salm* protein expression pattern in imaginal discs of wild-type third-instar larvae (Fig. 4D). The protein is localized to the nucleus; unlike the *sala* gene, its pattern of expression is consistent with the β-galactosidase expression in imaginal discs of A405.1M2 third-instar larvae. There is also a strong correlation between A405.1M2 *lacZ* expression and *salm* expression in late embryogenesis (R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.). Therefore, the A405.1M2 enhancer detector responds primarily to regulatory sequences of the *salm* gene. Our results from the enhancer-detector screen suggest that *salm* expression in the antennal disc should be repressed in the presence of the *Antp* protein. Indeed, in the hs–*Antp* background, the level of protein in both the antennal and eye discs is reduced greatly by a stringent regime of repeated heat shocks during early third-instar larval development (Fig. 4F), whereas expression in the wing discs and brain is not affected. In an $Antp^{Ns}$ genetic background, *salm* protein expression is repressed completely in the same anterior sector of the disc that is affected in the A405.1M2 enhancer-detector strain (Fig. 4E). Consistently reduced expression of *salm* protein is observed also in the posterior region of the antennal disc. Presently, we have no obvious explanation for this difference in *salm* protein and A405.1M2 β-galactosidase expression in $Antp^{Ns}$ larvae. One possibility is that low expression levels of *Antp* protein in the posterior part of $Antp^{Ns}$ antennal discs modulate the level of *salm* protein by a mechanism to which the enhancer detector cannot respond, for example, by a regulatory element that does not affect the enhancer detector or by post-transcriptional control.

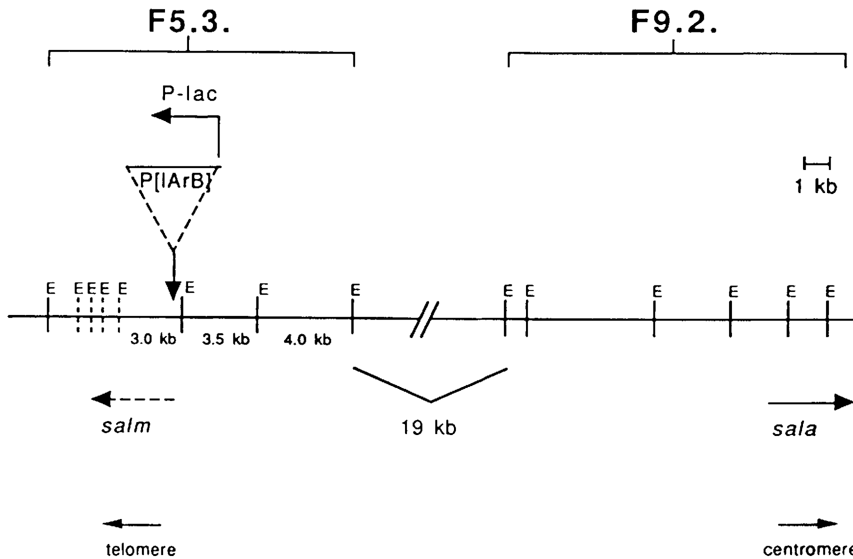

Figure 3. Genomic location of the P[lArB] transposon in strain A405.1M2. A 6-kb genomic fragment adjacent to the P[lArB] insertion in strain A405.1M2 was isolated by plasmid rescue using the restriction enzyme *Xho*I (see Materials and methods). This fragment hybridizes to chromosomal band 33A and is located within the *sal* locus. The diagram shows the approximate insertion position in relation to the restriction map described by Frei et al. (1988). P[lArB] is located within a 3.0-kb *Eco*RI fragment ~40 kb upstream of the *sala* gene described by Frei et al. (1988). The two bracketed regions in the map represent two λ clones (F5.3 and F9.2) isolated by Frei et al. (1988). The latter clone includes part of the *sala* gene described by these investigators. The former clone contains the *salm* transcription unit, which is not yet mapped completely. Details of this gene and the exact location of the insertion will be published elsewhere. P[lArB] is not drawn to scale; it is ~17 kb in length (Wilson et al. 1989). The 3.0-, 3.5-, and 4.0-kb *Eco*RI fragments that have been tested for enhancer activity are marked.

salm *expression is normally down-regulated by* Antp *in the second mesothoracic leg disc*

The pivotal role of *Antp* expression in distinguishing the developmental fate of the mesothoracic leg disc and the antennal imaginal disc has been demonstrated in two ways: (1) Ectopic expression of *Antp* in the antennal disc transforms it toward a second leg disc (see above), and (2) absence of *Antp* in mosaic clones within the anterior part of the mesothoracic leg disc transforms some but not all of the cells in these mosaic patches toward an antennal fate (Struhl 1981). Our enhancer-detector screen has identified transcriptional elements and corresponding genes, such as *salm*, that can be regulated by ectopic expression of *Antp* in the antennal imaginal disc. However, these experiments do not demonstrate directly that expression of *salm* or any other gene is normally controlled by *Antp* in the mesothoracic leg disc.

To address this question, we studied the expression of *salm* in $Antp^-$ loss-of-function mutants. Because such mutants are homozygous lethal, we generated homozygous $Antp^-$ clones in heterozygous animals by X-ray-induced mitotic recombination. The A405.1M2 enhancer detector was used as a marker for *salm*, as its associated staining is a faithful reflection of *salm* protein expression (see above). We examined initially mosaic clones in the adult fly so that clones could be marked independently by their antennal phenotype and by cuticular genetic markers. The A405.1M2 enhancer detector is expressed in the second segment of the adult antenna, a region corresponding to the area of expression in the antennal imaginal disc (see Fig. 4M,N,O). There is also some weak expression in the third antennal segment. No expression is observed in the adult leg and transformed antenna (data not shown). Therefore, regulation of *salm* in the adult antenna and leg correlates with its regulation in larvae. Clones homozygous for the null mutation $Antp^{rw10}$ (Wakimoto and Kaufman 1981) were induced in flies heterozygous for the A405.1M2 enhancer detector. Cells of these clones were marked genetically, because they did not carry the dominant bristle mutation *Kinked* (for details, see Material and methods). In addition, the "minute technique" of clonal analysis was used to produce relatively large clones (see Materials and methods; Morata and Ripoll 1975). By irradiating larvae between 24 and 48 hr of development, homozygous $Antp^{rw10}$ clones (carrying either the A405.1M2 detector or a wild-type second chromosome) were induced in a heterozygous *Kinked Minute* background.

Approximately 700 adult flies were screened for antennal structures in the mesothoracic legs. Nine such clones were identified in a total of eight different flies. Structures of the second antennal segment were only found in the trochanter and proximal femur while third antennal structures were restricted to the distal femur, tibia, and basitarsus. The eight flies in which these clones were observed were stained with X-gal; judging from the genetic marker, three of these flies also carried the A405.1M2 insertion. In only these three cases, the area of the clone that is transformed into antennal structures and a region surrounding it express β-galactosidase. For example, in Figure 4J, a clone is shown that includes tissue transformed to both second and third antennal segment structures. Staining is seen in the second anten-

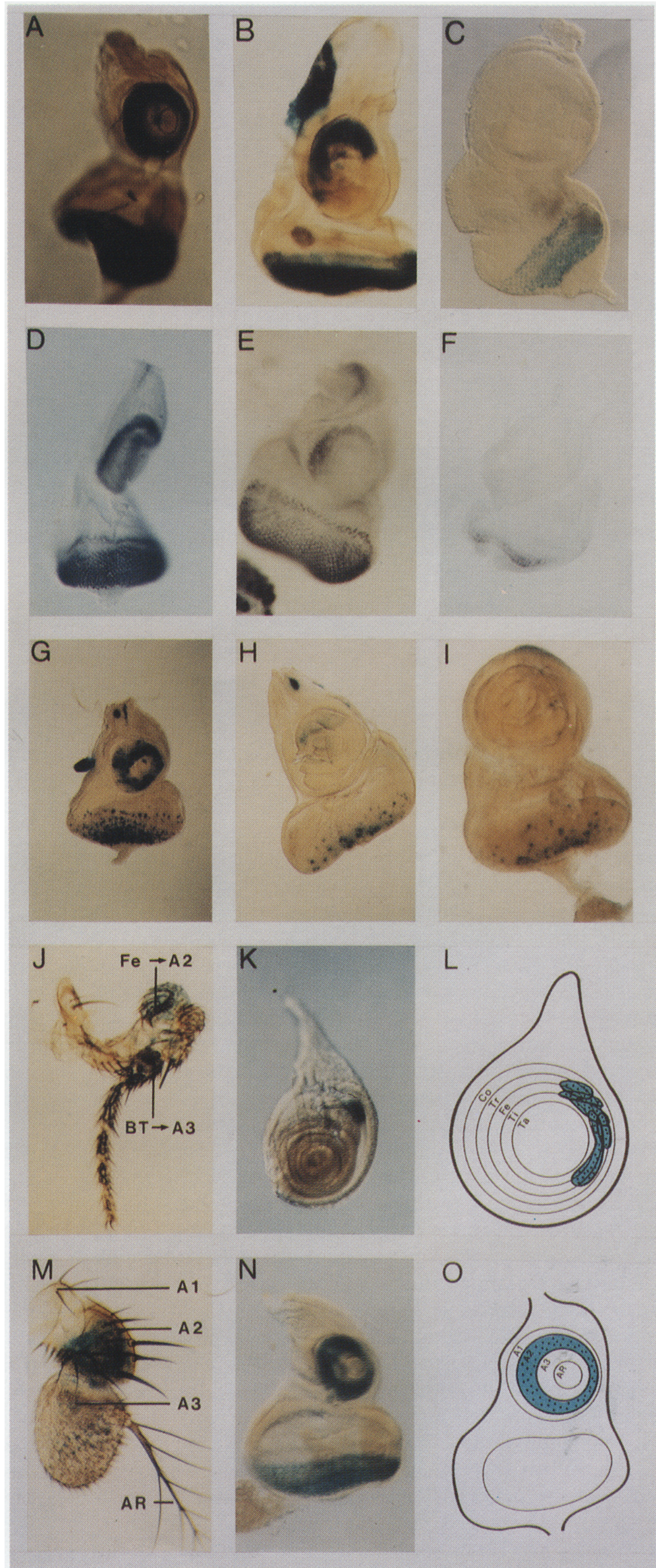

Figure 4. Regulation of the A405.1M2 enhancer detector and *salm* in dominant gain-of-function and recessive loss-of-function *Antp* mutants. (*A–C*) Repression of β-galactosidase staining in heterozygous A405.1M2 larvae (*salm* enhancer detector) by ectopic expression of *Antp* in the eye antennal disc. A405.1M2 staining in a wild-type genetic background (*A*); in $Antp^{Ns}$ showing repression in an anterior sector (*B*); in H4 (hsp–*Antp*) larvae after a series of six 45-min heat shocks each applied in 4-hr intervals from 68 hr of development onward (*C*). Complete repression of A405.1M2 expression is observed in the antennal disc. (*D–F*) Repression of *salm* protein expression in larvae by ectopic expression of *Antp* in the eye antennal disc. Discs were dissected from a wild-type larva (*D*), $Antp^{Ns}$ larvae (*E*), and H4 larvae (*F*) after the heat shock regime described in *C*. (*G–I*) Repression of β-galactosidase staining in larvae homozygous for a P-element insertion containing a 3.5-kb *Eco*RI fragment upstream from the *salm* gene fused to a *lacZ* reporter gene (3.5-kb HZ50PL; see Fig. 3) by ectopic expression of *Antp* in the eye antennal disc. (*G*) Wild-type genetic background; (*H*) $Antp^{Ns}$: partial repression; (*I*) In H4 after the heat shock regime described in *C*. (*J–L*) Analysis of homozygous $Antp^-$ clones (recessive loss-of-function) in flies heterozygous for the A405.1M2 insertion. Flies showing transformations were stained for β-galactosidase activity. (*J*) Partial transformation of the adult femur to structures of the second antennal segment and of the basitarsus to third antennal segment structures. Both transformed regions express β-galactosidase, either strongly or weakly. (*K*) Expression of the A405.1M2 enhancer detector in a second leg imaginal disc in an area where an $Antp^-$ clone is located. (*L*) β-Galactosidase-staining patterns associated with putative homozygous $Antp^-$ clones are superimposed on the fate map of the leg imaginal disc (Schubiger 1968). (*M–O*) Normal expression of the A405.1M2 enhancer detector in larvae and adults. (*M*) Adult antenna showing strong staining of the second antennal segment (A2) and weak staining of the third antennal segment (A3). (A1) First antennal segment; (AR) Arista. (*N*) Larval expression of β-galactosidase is observed in a ring corresponding to the primordium of the second antennal segment (*O*) (Gehring 1966; Haynie 1975).

nal segment tissues but is not entirely restricted to this region, and it extends into neighboring *Kinked*$^+$ (*Antp*$^-$) leg tissue that is not transformed into antennal structures. Weak staining is also observed in the third antennal segment structures. This expression may correspond to the weak staining seen in the third segment of A405.1M2 adult antennae.

We were also interested in determining whether derepression of A405.1M2 enhancer-detector expression was observed in *Antp*$^-$ mosaic clones in mesothoracic leg imaginal discs at the larval stage. No convenient markers were available to delimit the extent of clones at this stage. However, we stained ~900 irradiated A405.1M2 larvae heterozygous for *Antp*^{rw10} and simply screened for patches of inappropriate staining within the leg imaginal discs. Five leg discs showed a region of β-galactosidase expression (Fig. 4K), whereas no such staining was ever observed in nonmosaic control animals. Therefore, we conclude that in homozygous *Antp*^{rw10} clones, *salm* is derepressed in certain regions of the leg disc. Two other findings support the hypothesis that these patches are the result of derepression of *salm* in *Antp*$^-$ clones rather than an artifact of the staining procedure. First, they were all found in the anterior part of the mesothoracic leg discs, corresponding to the region that shows adult transformations. Second, the staining is observed in the primordia of the trochanter and proximal femur (see Fig. 4K,L), the regions that are transformed into second antennal segment structures in *Antp*$^-$ clones.

In conclusion, our results demonstrate that *Antp* expression in the mesothoracic leg disc is normally required to prevent expression of the *salm* gene, particularly in the regions that will develop into the anterior parts of the trochanter and femur.

Identification of a cis*-regulatory element whose activity is affected by* Antp

To analyze the interaction between *Antp* and *salm*, we attempted to identify the *cis*-regulatory region of *salm* whose activity is regulated by *Antp*. Three genomic *Eco*RI fragments located in the vicinity of the A405.1M2 transposon insertion site (Fig. 3) were subcloned into the P-element plasmid vector HZ50PL (Hiromi and Gehring 1987) upstream of a *lacZ* reporter gene fused to a basal promoter. These constructs were introduced into flies by P-element-mediated transformation (Rubin and Spradling 1982), and several transformant lines were analyzed for each construct. A 4-kb *Eco*RI fragment located several kilobases upstream of the *salm* gene (Fig. 3) directs *lacZ* expression in specific larval neuronal cells (data not shown); however, this staining pattern does not resemble any element of the larval expression pattern of the *salm* gene product. In contrast, the adjacent 3.5-kb fragment, which lies closer to the *salm* gene, specifically drives *lacZ* transcription in larval cells that normally produce the *salm* protein (Fig. 4G). Staining in the antennal and wing imaginal discs and in the brain matches the staining observed in strain A405.1M2. In addition, expression in the antennal disc is repressed specifically in *Antp*^{Ns} and H4 genetic backgrounds (Fig. 4H,I), as expected from our experiments with A405.1M2.

Therefore, the 3.5-kb *Eco*RI fragment contains a *cis*-regulatory element from the *sal* locus that drives transcription in the antennal disc and is repressed by *Antp*. This genomic fragment also directs *lacZ* expression in late embryos in a pattern corresponding to the A405.1M2 strain; however, no staining is observed in early transformant embryos that carry the 3.5-kb HZ50PL construct, indicating that separate regulatory elements are required for early embryonic expression of *salm*.

Discussion

We have used the enhancer-detector method to identify a number of genomic regulatory elements and corresponding genes in *Drosophila* that are influenced by the homeotic *Antp* gene product. We have concentrated our studies on enhancer-detector strains in which the β-galactosidase reporter gene is expressed in distinctly different patterns in the antennal and leg imaginal discs of the third-instar larva. In these strains, ectopic expression of *Antp* in the antennal disc leads to a transformation of the antennal-specific *lacZ* staining pattern toward a leg-specific pattern. These changes are correlated with the antenna-to-leg transformations induced in the adult by ectopic *Antp* expression. The general change of cell fate in the antennal disc in response to *Antp* presumably is reflected by these pattern alterations. Even in cases where staining is repressed, this is unlikely to be caused by the death of cells that normally express *lacZ*, because the extensive regions of staining in the antennal discs of strains such as A405.1M2 are clearly not deleted in transformed discs (e.g., see Fig. 10). The derepression of A405.1M2 expression in mesothoracic *Antp*$^-$ mosaic clones suggests further that the effect of *Antp* in this strain is related to changes in cell identity. In the case of A405.1M2, the regulatory element identified by enhancer detection behaves like a *cis*-regulatory element and, in wild-type flies, appears to control a neighboring *Drosophila* gene *salm*. Previous studies suggest that many of the other enhancer-detector lines identified in this study will allow the isolation of genes (e.g., Wilson et al. 1989) that are regulated similarly by *Antp*.

The antennal/leg imaginal disc system is particularly well suited for an investigation of the genetic and molecular pathways by which the homeotic genes establish segmental identity. Although the two types of discs produce morphologically distinct structures, the decision to take one of these two fates is determined primarily by a single homeotic gene, *Antp*, during the early third-instar larval stage (e.g., Struhl 1981; Wakimoto and Kaufman 1981; Schneuwly et al. 1987b). Unlike cells in many other larval tissues, cells of both the antennal and leg discs presumably contain the interacting regulatory factors required for *Antp* to initiate a precise leg-specific developmental program. This is well illustrated by the A405.1M2 insertion near the *salm* gene. When *Antp* protein is overexpressed throughout the third-instar larva by

heat shock induction, repression of the *salm* gene is only observed in the eye-antennal imaginal disc and not in other tissues like the brain and wing disc. The regions affected are those that are phenotypically altered in the adult.

The inference from genetic studies and evolutionary considerations that the antennal and leg discs are closely related is also supported by our screen. In most enhancer-detector strains that express *lacZ* in these discs, the staining patterns are identical in the two discs (G. Gibson and W.J. Gehring, in prep.). Some of the regulatory elements identified in these fly lines may be controlled by *Antp* in the leg disc; however, if they are, another regulatory gene must be able to exert a similar locally restricted control in the antennal disc where *Antp* is not normally expressed. One advantage of our approach is that it focuses on a limited set of *Antp*-regulated genes whose altered expression correlates with a discrete homeotic transformation.

Establishment of positional information in imaginal discs

Both the leg and the antennal imaginal discs have a rounded shape and the disc epithelium folds to form a series of concentric rings (Bryant 1978). In the course of metamorphosis, the outermost ring remains attached to the thorax or head capsule, respectively, whereas the inner rings expand and are pushed out like a telescope, with each ring giving rise to a leg or antennal segment. Fate mapping of third-instar larval imaginal discs (Gehring 1966; Schubiger 1968; Haynie 1975; Bryant 1978) indicates that the central portion of each leg and antennal disc gives rise to the most distal structures while the peripheral portion of the disc gives rise to proximal structures. Segmental boundaries on the fate map are represented by the concentric rings of the discs. The leg discs contain one ring each for coxa, trochanter, femur, and tibia and two rings for the tarsal segments, whereas the antennal disc has only four rings, representing the three antennal segments and the most distal primordium of the arista. Data obtained previously (Gehring 1970; Bryant 1978) and the results of our mosaic analysis suggest the following transformations of leg structures into antennal structures: coxa into first antennal segment, trochanter and proximal femur into second antennal segment, tibia and basitarsus into third antennal segment, and tarsal segments 1–4 into arista. The rings of staining observed in many of the enhancer-detector strains examined reflect this organization within the antennal and leg discs (see Fig. 1) and are confined to the primordia of specific segments in the leg or antenna (e.g., strain A405.1M2). When segmentally restricted leg-specific staining is induced in the antennal disc by ectopic expression of *Antp*, this staining generally is confined to the corresponding segmental primordium in the antennal disc. Furthermore, strains stained in a specific sector of the leg disc show a similar sector of staining in transformed antennal discs (e.g., Fig. 1H,T,X), and these sectors are approximately in the same relative positions. Even complex patterns normally observed in the leg disc can be reproduced in transformed antennal discs (cf. Fig. 1A and D). Our results are therefore consistent with a model in which patterning information within the antennal and leg discs is set up by an identical set of genes largely independent of homeotic gene expression (cf. the polar coordinate model of French et al. 1976). These genes then interact with the homeotic genes to establish the identity of the disc in much the same way as the pair-rule and segment polarity genes are thought to interact with the homeotic genes to define cell fates in the larval epidermis (e.g., see Akam 1987).

Further experiments are necessary to establish whether any of the *Antp*-regulated genes identified play a role in leg or antennal development. Analysis of homozygous-viable mutations of the newly identified genes and genetic mosaics containing cells homozygous for nonviable mutant alleles will allow us to determine the precise developmental function of these genes.

The role of Antp *in establishing leg identity*

In this paper we have concentrated largely on the characterization of an enhancer-detector insertion in the *sal* locus (A405.1M2) that reflects the expression pattern of the *salm* gene. The *salm* gene is expressed in the primordium of the second antennal segment in the antennal disc but not in the leg disc; the antennal disc expression is entirely repressed by high levels of ectopically expressed *Antp*. This antennal-specific regulation is also found in the adult, although weak expression also is detected in parts of the third antennal segment. The gene is of particular interest because its early period of antennal disc expression overlaps with the time window during which the *Antp* gene must be transcribed to induce antenna-to-leg transformations in the second antennal segment. The *salm* gene is therefore a potential candidate to be regulated directly by the homeo domain-containing *Antp* protein.

Our results already suggest that *salm* is regulated differently by *Antp* than some other enhancer detectors and their neighboring genes. Typical enhancer-detector strains, such as A184.3F1, first express *lacZ* in the antennal or leg disc during mid- to late third-instar larval development. However, transformation of the antennal disc staining pattern toward a leg-specific staining pattern is not observed if *Antp* is ectopically expressed at these stages. Only ectopic expression in the early third-instar larva, the developmental stage during which *Antp* can induce antenna-to-leg transformations, leads to alterations in the antennal disc staining patterns. In contrast, *salm* is only repressed by *Antp* during and shortly after the time period when *Antp* is ectopically expressed in the antennal disc. Continued down-regulation of *salm* in the larval primordium of the second antennal segment requires the presence of *Antp* at times when *Antp* no longer induces transformations in this segment. This correlation between expression of *salm* and absence of *Antp*, even if the tissue does not display antennal iden-

tity, is also observed in our analysis of mosaic clones in the adult.

Because there is a distal-to-proximal temporal response to heat shock-induced *Antp* protein during development of the antennal imaginal disc, we have suggested previously that the requirement for *Antp* protein might be transient rather than maintained (Gibson and Gehring 1988). Instead of directly controlling terminally expressed genes in the leg and antennal developmental pathways, *Antp* could function via other intermediate regulatory genes whose expression it modulates directly. Our results are consistent with this model. Genes represented by enhancer detectors that are first expressed in the late third-larval instar are unlikely to be regulated directly by *Antp* but are useful downstream markers for the state of determination of imaginal disc cells. *salm*, on the other hand, appears to be a much stronger candidate for direct regulation, particularly because its expression correlates more with the absence of *Antp* than with the fate of cells in which it is synthesized. Interestingly, it has recently been found that *salm* encodes a protein with *Krüppel*-like zinc-finger DNA-binding motifs (R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.), suggesting that this gene may have a regulatory function. It is tempting to speculate that it may be one of the class of regulatory genes that are controlled by the homeotic genes and transmit information concerning segmental identity to limited domains within the imaginal discs.

We have also demonstrated by mosaic analysis that the expression of β-galactosidase in A405.1M2 and presumably of the *salm* gene itself is normally repressed by *Antp* in the mesothoracic leg disc. Struhl (1981) observed that some cells of the mesothoracic leg disc that lack *Antp* form patches of antennal-like cells in the adult. He suggested that *Antp* represses the expression of "head-forming genes" in the leg disc. Although we do not yet know whether *salm* plays a role in antennal development, it may well represent the first example of such a head-forming gene.

In preparation to test whether *salm* is regulated directly by *Antp*, we have identified a 3.5-kb genomic fragment flanking the transcription unit of the *salm* gene that drives appropriate expression of a *lacZ* reporter gene in the antennal disc and is repressed in mesothoracic leg and antennal imaginal disc cells expressing *Antp*. Currently we are delimiting the minimum extent of the *Antp*-regulated *cis*-regulatory element within this fragment. The *cis*-regulatory element will then be screened for *Antp* protein-binding sites. The 3.5-kb *cis*-regulatory element does not appear to contain the regulatory sequences that control all facets of the *salm* expression pattern. The early embryonic pattern (Bellen et al. 1989) that is shared by both the *salm* and the *sala* genes is not dictated by any genomic sequences tested so far. The sequences that drive spatially restricted *sal* transcription in the cellular blastoderm are therefore probably distinct from *Antp*-regulated *cis*-regulatory element; this is consistent with the observation that such expression is not obviously affected by ectopic overexpression of the *Antp* protein in embryos (data not shown).

We are hopeful that future studies will lead ultimately to an understanding of the molecular mechanisms by which *Antp* can reprogram the fate of the primordium for a complete morphological structure, the antenna, and transform it into a leg. One important observation can be made. Ectopically expressed *Antp* appears to exert its effects directly or indirectly by both repressing and activating the expression of genes in the antennal disc. In some cases, the regulatory elements identified in our screen are normally active in only one of the two types of disc we have examined (e.g., strains A405.1M2, rK781, and rI781). Therefore, it seems likely that future studies of these and other enhancer-detector strains will reveal both head-specific and thorax-specific *Antp*-regulated genes that play a role in determining the structural differences between the antenna and the leg of the adult fly.

Materials and methods

Fly culture and strains

Flies were raised at 25°C on standard medium containing cornmeal, sugar, yeast, and agar. The cytology of the *Antp* alleles $Antp^{Ns}$ and $Antp^{73b}$ and the phenotype of flies carrying these alleles are described in Schneuwly and Gehring (1985). The H4 strain was constructed by Schneuwly et al. (1987b). The generation of the enhancer-detector insertion strains and the nomenclature of these strains are described in Bellen et al. (1989).

Heat shock treatment of larvae

Embryos from the H4 strain (Schneuwly et al. 1987b) and other control strains were collected for 1 hr on yeasted grape juice plates and allowed to develop at 25°C. They were transferred within 1 hr of hatching (taken as 22 hr of development) to yeasted cornmeal tubes and grown further at 25°C. Heat shocks were given by immersing the tubes into a 37°C water bath. In most cases, a series of six 45-min heat shocks were given at 68, 72, 76, 80, 84, and 90 hr of development (68 hr roughly corresponds to the end of the second larval stage). The heat-shocked larvae were dissected, and the discs were analyzed typically at ~115–120 hr of development. Appropriate control experiments were performed with non-H4 strains to show that heat shock did not alter the expression of β-galactosidase or *salm* in the absence of the hs–*Antp* construct.

Staining for β-galactosidase activity

Embryos were stained as described by Bellen et al. (1989). For third-instar larvae, heads were removed in buffered saline (BSS) or phosphate-buffered saline (PBS) and everted with forceps. The excess fat and glands were trimmed off, leaving the brain and attached imaginal discs relatively free from the rest of the larva. These tissues were transferred to a microtiter plate and were fixed for 15–20 min in 0.75% glutaraldehyde in PBS. They were washed once in PBS and stained in X-gal staining solution (Bellen et al. 1989) from 5 hr to overnight at 37°C. The stained discs were dissected further in a drop of BSS or PBS on a siliconized glass slide and covered with a cover slip. Excess liquid was removed from under the coverslip, and a liquid chamber was produced by sealing around the coverslip edges with nail polish. The discs were photographed within 3 days, as the stain-

ing becomes more diffuse with time. Adults were stained using the same procedure, but the fixation time was 30 min rather than 15 min. Typically, legs and antennae were removed from the rest of the body before fixation. The time of staining was performed overnight for up to 24 hr.

Molecular characterization of the A405.1M2 transposant

Isolation of genomic DNA from strain A405.1M2 and the cloning of insertion-associated genomic DNA by plasmid rescue experiments were performed as described by Wilson et al. (1989). A clone containing a 6-kb genomic region was isolated from *Xho*I-digested genomic DNA. To confirm that rescued clones contained genomic sequences adjacent to the P[lArB] insertion, we performed genomic Southern analysis of DNA from transposant and nontransformed flies (for details, see Wilson et al. 1989).

Fragments isolated from genomic-rescued clones and other genomic clones were labeled by random priming after the protocol included in the random priming kit from Amersham. Three micrograms of genomic DNA was digested with the same enzyme used for the plasmid-rescue experiment and fractionated on an 0.8% agarose gel by electrophoresis. Southern blots were prepared as described by Southern (1975) and were prehybridized, hybridized, and washed at high stringency, according to McGinnis et al. (1984). For precise mapping of the insertion site of P[lArB] in the genome of the transposant A405.1M2, *Eco*RI-digested phage DNA of the chromosomal walk at the 32F/33A cytological region (Frei et al. 1988) was fractionated by agarose gel electrophoresis and analyzed by Southern blotting as described above. The isolation and characterization of *salm* cDNA clones will be described elsewhere (R. Kühnlein, A. Weber, G. Frommer, J.T. Wagner-Bernholz, H. Jäckle, W.J. Gehring, and R. Schuh, in prep.).

Cytogenetic mapping

Biotin labeling of DNA probes and in situ hybridization to larval salivary gland chromosomes were performed as described by Langer-Safer et al. (1982). Because the A405.1M2 insertion is located on the CyO chromosome, the insertion was mapped by using genomic DNA obtained from a plasmid rescue experiment (see above) to probe cytological preparations from wild-type larvae.

Immunolocalization of salm *protein in imaginal discs*

Imaginal discs were dissected as described above in the β-galactosidase staining procedure. They were fixed in fixation solution (0.002 EGTA, 0.5% NP-40, 3.7% formaldehyde in PBS) for 30 min and were then washed three times for 5 min in PBS. The discs were permeabilized by incubating for 30 min in 0.05% Triton X-100, 0.05% NP-40, 0.05% DOC, and 2 mg/ml of BSA in PBS. Nonspecific binding was blocked by subsequent incubation for 30 min in blocking solution (0.1% Triton X-100 and 2 mg/ml of BSA in PBS). The *salm* antibody was applied to the discs in a 1 : 5 dilution in blocking solution and incubated at 4°C overnight. The preparation was then washed three times for 10 min in 0.1% Triton X-100, 2 mg/ml of BSA, and 5% milk powder in PBS. Subsequent steps were performed with the ABC kit of Vectastain. The second antibody, preabsorbed on fixed discs, was diluted 1 : 100 and applied to the discs in blocking solution. The preparation was incubated for 1 hr and was then washed three times for 15 min in blocking solution. The ABC complex was preincubated for 30 min at room temperature and added to the discs for 30 min. After one 15-min wash in blocking solution and two 15-min washes in PBS, bound HRP was detected with DAB solution (0.5 mg/ml 3,3-diaminobenzidine (DAB), 0.01% H_2O_2, and 0.03% $NiCl_2$].The staining reaction was stopped after 10 min by washing in PBS. The discs were dissected more completely and mounted in Canada Balsam (Aldrich).

Clonal analysis

Larvae and adults were obtained from a cross of males and females of the genotype

$$\frac{\mathrm{Cy[A405.1M2]}}{+};\frac{Antp^{rw10}}{Ki\ \mathrm{M(3)S31}}$$

and were of two genotypes prior to irradiation

$$\frac{+;\ Antp^{rw10}}{+\ Ki\ \mathrm{M(3)S31}}\ \mathrm{or}\ \frac{\mathrm{Cy[A405.1M2]}}{+};\frac{+}{+};\frac{Antp^{rw10}}{Ki\ \mathrm{M(3)S31}}$$

Mitotic recombination was induced by irradiation with 1000 rad. Embryos were collected from 20 females for 24 hr in split bottles and were grown at 25°C for another 24 hr before irradiation. After irradiation, cultures were left to age at 25°C until the third-larval or adult stages. Larvae and adults were stained and analyzed as described above. The legs that showed antennal tissue were removed and embedded in Faures' medium for detailed microscopic analysis.

Construction of lacZ *fusion genes*

Phage F5.3 of the genomic walk covering the *sal* locus (Frei et al. 1988) was digested with *Eco*RI and fractionated by agarose gel electrophoresis. The 3.0-, 3.5-, and 4.0-kb *Eco*RI fragments (Fig. 3) were gel isolated and cloned separately into the *Eco*RI site of a kanamycin-resistant vector, pHSS7 (U. Walldorf, unpubl.). The subcloned regions could then be excised with *Not*I, producing a fragment containing the original phage *Eco*RI fragment flanked on both sides by polylinker sequences. These fragments were then cloned into the *Not*I site of the HZ50PL vector (Hiromi and Gehring 1987) in such a way that their orientation with respect to the *lacZ* reporter gene was the same as their normal orientation with respect to the *salm* gene.

Germ-line transformation of P-element constructs

The P-element-mediated transformation procedure and the establishment of balanced or homozygous transformant stocks have been described previously (Hiromi and Gehring 1987). Helper plasmid Δ2-3 and the construct DNA were injected at concentrations of 0.1 μg/μl and 0.5 μg/μl, respectively.

Initially, three lines were obtained that carried the 3.5-HZ50PL insertion in the *sal* locus (33A). By using the jump-start technique (Cooley et al. 1988; Robertson et al. 1988), additional fly strains could be constructed that had the P element inserted on the third chromosome (mapped to 66C and 98F).

Acknowledgments

We thank S. Flister for helping with the screen, M. Mlodzik, U. Gaul, and G. Rubin for providing some of the enhancer-detector flies, and R. Nöthiger for providing the *Ki* M(3)S31 strain. We thank E. Hafen for the help in using the X-ray machine. Many thanks go to M. Affolter, R. Leemans, and A. Schier for critical reading of the manuscript, and to U. Kloter for technical help. Also, we thank E. Wenger-Marquardt for her help during the preparation of the manuscript. This work was supported by

grants from the Swiss National Science Foundation and the Kantons Basel-Stadt and Basel-Landschaft. C.W. was a recipient of a Royal Society exchange fellowship.

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