Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combinations of axis-patterning genes through a common intergenic control region

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The mechanisms that generate precise patterns of discrete cell types within developing fields are not well understood. One model for analyzing how cells interpret positional information in two dimensions is the regulation of proneural cluster formation within insect segments. Two adjacent proneural regulatory genes, *achaete* and *scute*, are expressed coincidently in cell clusters at reproducible anteroposterior (AP) and dorsoventral (DV) coordinates within the *Drosophila* embryo from which single neuroblasts later arise. Here, we show that the AP and DV position of these clusters is regulated through a common cis-acting region between the genes under the initial control of the products of the pair-rule and DV polarity genes and is later maintained by selected segment polarity genes. The combination of proneural gene activation/repression in AP stripes and repression within specific DV domains positions each cluster of *achaete/scute* expressing cells within segments; interactions between these cells then determine individual cell fates.

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During embryonic development in *Drosophila*, cell fates are determined according to their position in the embryo. Although a great deal has been learned about the genetic regulatory system governing axis formation and global positional information in the embryo, it is less well understood how individual or groups of cells decode this information and follow distinct fates. A combination of classical and molecular genetics has shown that the early zygotic patterning genes act progressively in a regulatory cascade to subdivide the embryo into its segmental units (for review, see Ingham 1988; Nüsslein-Volhard 1991). The gap genes interpret maternal anteroposterior (AP) gradients to specify the positional fate of large multisegment embryonic regions. The pair-rule and segment polarity genes refine this information and specify positional information to alternating segment-sized regions and to specific regions within each segment, respectively. Although the analysis of the regulatory interactions between the different classes of zygotic segmentation genes has yielded a formal understanding of how the basic segmental organization of the *Drosophila* embryo is laid down, very little is understood about how these genes specifically contribute to the pattern of differentiated cell types or tissues within a segment.

One of the first signs of intrasegmental pattern in the *Drosophila* embryo is the appearance of proneural gene expression of the *achaete–scute* complex loci (AS-C), *achaete* (ac), *scute* (sc), and *lethal of scute* (l'sc), in small clusters of cells during gastrulation (Cabrera et al. 1987; Martin-Bermudo et al. 1991; Skeath and Carroll 1992). The earliest phase of proneural gene expression is composed of a precise AP and dorsoventral (DV) pattern of cell clusters that is repeated within every segment. Genetic and cell ablation experiments suggest that the proneural clusters are groups of equipotential cells, all of which have a potential neural fate (Stern 1954; Taghert et al. 1984; Doe and Goodman 1985; Simpson 1990). This potential appears to be conferred to these cells by their expression of the proneural genes (Cabrera et al. 1987; Romani et al. 1989; Jimenez and Campos-Ortega 1990; Cubas et al. 1991; Martin-Bermudo et al. 1991; Skeath and Carroll 1991, 1992) and is eventually restricted to a single cell that becomes a neural precursor [a neuroblast (NB) or sensory mother cell]. The dynamics of proneural gene expression directly reflects this cell fate restriction, as proneural gene expression also becomes restricted from all cells of a cluster to just one, the future NB (Cabrero 1990; Cubas et al. 1991; Martin-Bermudo et al. 1991; Skeath and Carroll 1992). All of the first NBs to segregate into the embryonic central nervous system

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In this report we focus entirely on the initial clustered patterns of ac, sc, and l'sc expression (late stage 8/early stage 9) and on the genetic regulatory systems that establish the initial spatial limits of proneural gene expression.

The registration of proneural gene expression in the neuroectoderm is most easily discussed using a Cartesian coordinate system to refer to the positions of different proneural clusters within each hemisegment (summarized in Fig. 1e). Along the AP axis, each hemisegment is divided into four rows or quadrants—A, B, C, D—which successively divide each hemisegment, from the anterior to the posterior, into fourths. Along the DV axis, each hemisegment is divided into three columns: medial (m); intermediate (i); and lateral (l). Each hemisegment is then composed of 12 squares, each one uniquely identified by its AP and DV coordinates. Each square represents roughly four to six cells and outlines the boundary of a different proneural cluster.

The relative positions of proneural clusters have been precisely determined in single- and double-label experiments using antibodies specific for the ac [Fig. 1a,c, l'sc [Fig. 1b,c], and engrailed (en) [data not shown] proteins, as well as RNA in situ experiments using a probe specific for the sc transcript. During late stage 8, ac [and sc; data not shown] is expressed in four clusters per hemisegment—the m and l clusters of rows B and D (Fig. 1a,c,e; Skeath and Carroll 1992)—whereas l'sc is expressed in a circular pattern of eight adjoining proneural cell clusters—the m, i, and l clusters of rows A and C, and the m and l clusters of row D (Fig. 1b,c,e; Cabrera 1990; Martin-Bermudo et al. 1991). By mid-stage 9, one NB has delaminated from each proneural cluster (summarized in Fig. 1e; Martin-Bermudo et al. 1991; Skeath and Carroll 1992). Thus, the clustered pattern of proneural gene expression precisely prefigures the pattern of the first 10 NBs to segregate into the embryonic CNS (Fig. 1e). Along the AP axis, three of the four rows express different combinations of these genes and no two contiguous rows express the same combination: l'sc is expressed in rows A and C; ac and sc are expressed in row B; and all three are expressed in row D (Fig. 1c,e). At this stage, no proneural gene expression is found within the i clusters of rows B and D. During stages 9 and 10, a second and third phase of l'sc expression occurs and these regions, as well as many other regions of each hemisegment, accumulate l'sc protein (Cabrera 1990; Martin-Bermudo 1991). Because of the relative simplicity of the ac/sc expression pattern and the complications of interpretation that arise from the superimposition of the second and third phases of l'sc expression on the initial patterns of ac, sc, and l'sc expression, we focused on elucidating the genetic regulatory mechanisms that act within the early embryo to establish the initial limits of ac gene expression.

Other AS-C gene products affect the level but not the pattern of ac expression

During the development of the adult nervous system, both ac and sc are again expressed coincidently (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991).
It has been shown that during adult development the identical nature of these two patterns arises from cross-regulation between the ac and sc genes (Martinez and Modolell 1991; Skeath and Carroll 1991). To determine whether cross-regulation between the products of the proneural genes of the AS-C accounts for the generation of any aspect of the initial embryonic pattern of ac expression, we examined ac protein distribution in embryos hemizygous mutant for l'sc or sc. As simple point mutations have not been generated in ac, sc, or l'sc, we were forced to use synthetic deficiencies that removed l'sc or sc as well as ~20 kb of surrounding DNA (see Fig. 3a, below; Dambly-Chaudiere et al. 1988; Lindsley and Zimm 1992). The effects of these deletions on the ac expression pattern could arise either from the loss of l'sc or sc function or from the removal of ac regulatory sequences. Our conclusions, the evidence for which is detailed below, is that l'sc affects the level but not the spatial pattern of ac and that disruptions in the ac or sc patterns in certain synthetic deficiencies in the ac/sc region result from disruptions of a common cis-regulatory region between the genes.

In Df(1)sc^{4LgR} l'sc, this deletion removes DNA between +24.9 and +5.1, see Fig. 3a, below; Lindsley and Zimm 1992) embryos, the pattern of ac expression is not altered (Fig. 1d). However, in row D where ac and l'sc are normally coexpressed, the level of ac expression is frequently reduced (Fig. 1d, arrowheads), and very rarely ac expression is absent from the m cluster of row D (not shown). The NB that arises from this cluster is the NB that is most frequently absent in Df(1)sc^{4LgR} embryos (Martín-Bermudo et al. 1991). In addition, the time at which ac protein is detected within row D is delayed from late stage 7 in wild-type embryos until late stage 8/early stage 9 in Df(1)sc^{4LgR} embryos. Thus, l'sc appears to enhance the level of ac expression but does not control the spatial pattern of ac expression.

A shared regulatory region between the ac and sc genes
Critical insights into the cis-acting elements required for the spatial regulation of ac and sc come from the analysis of gene expression in embryos containing inversion breakpoints or deletions between ac and sc. These mu-
tants reveal that common intergenic control elements regulate ac and sc expression. For example, in embryos that lack the sc region of the AS-C [Df(1)sc8L4R [Lindsley and Zimm 1992]], a striking effect on the ac pattern is observed where ac is expressed only in row B [Fig. 2a, arrows] but almost completely absent from row D [Fig. 2a, arrowheads]. The sc8L4R deletion extends proximally for 22 kb from a point ~10 kb downstream of the ac transcription unit [Fig. 3a; coordinates +47.4 to +24.9, Lindsley and Zimm 1992]. Curiously, there is a reciprocal effect on sc expression in Df(1)y3PLsc8R [ac-] embryos: sc is expressed in row D [Fig. 2b, arrowheads] but not in row B [Fig. 2b, arrows]. The y3PLsc8R deletion removes the ac transcription unit and ~17 kb of 5' and 10 kb of 3' DNA [Fig. 3a, coordinates +73.0 to +47.4 [Lindsley and Zimm 1992]]. Two different interpretations can explain the reciprocal pattern alterations to ac and sc in these mutant embryos: (1) ac expression could be required to activate sc in row B and sc expression could be required to activate ac in row D; or (2) the Df(1)sc8L4R and Df(1)y3PLsc8R deletions could remove control elements required to activate ac in row D or sc in row B, respectively. Experiments described below establish that these deletions are removing control elements.

To distinguish between the two possibilities, we examined both ac and sc expression in Inversion(1)sc8 embryos [Fig. 2c,d]. The distal breakpoint of In(1)sc8 lies between the ac and sc genes at +47.4, ~12 kb upstream of sc and 10 kb downstream of ac [Fig. 3a, Lindsley and Zimm 1992], coincident with the distal breakpoint of Df(1)sc8L4R and the proximal breakpoint of the Df(1)y3PLsc8R deletions [Fig. 3a]. In(1)sc8, then, precisely separates the cis-acting sequences deleted in Df(1)sc8L4R away from the ac transcription unit and the cis-acting sequences deleted in Df(1)y3PLsc8R away from the sc transcription unit but preserves the coding regions of both genes. We reasoned that if ac activated sc in row B and sc activated ac in row D, then should ac be expressed in row B in In(1)sc8 embryos, sc expression would also be found there, and should sc be expressed in row D in these same embryos, ac would also be expressed there. On the other hand, if the control elements required to activate ac in row D are located, for the most part, proximal to the sc8 breakpoint [the region deleted in Df(1)sc8L4R embryos] and the control elements necessary to activate sc in row B are located distal to the sc8 breakpoint [the region deleted in Df(1)y3PLsc8R embryos], then in In(1)sc8 embryos, one would expect ac not to be expressed in row D and sc not to be expressed in row B. The results are consistent with the second model: ac is expressed only in row B [Fig. 2c, arrows], and sc is expressed only in row D of In(1)sc8 embryos [Fig. 2d, arrowheads]. This suggests that the initial embryonic patterns of ac and sc are not determined by cross-regulation but that

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**Intrasegmental pattern formation**

**Figure 2.** Dual control of ac and sc by a common intergenic regulatory region. (a) Ventrolateral view of an early stage 9 Df(1)sc8L4R [sc-] embryo labeled for the ac [arrows] and en [arrowheads] proteins; en expression marks row D. (b) Ventrolateral view of a stage 8 Df(1)y3PLsc8R [ac-] embryo probed for both sc [arrowheads] and wg RNA [wg expression marks row C]. (c) High magnification ventrolateral view of an early stage 9 In(1)sc8 embryo labeled for ac [arrows] and en [arrowheads]. (d) Lateral view of a late stage 8 In(1)sc8 embryo probed for sc RNA. (e) Lateral view of a stage 8 In(1)sc8 embryo labeled for ac protein. (f) lateral view of a stage 8 and [h] ventral view of a stage 9 In(1)sc8 embryo probed for sc RNA. (g) Ventral view of an early stage 9 In(1)ac embryo labeled for the ac protein. In a–h, arrows mark the position of row B; arrowheads mark the position of row D. Note that the expression pattern of ac in Df(1)sc8L4R and In(1)sc8 embryos is identical: ac is expressed in row B, but not in row D [a,c, arrowheads]. The reciprocal pattern alteration occurs to sc in both Df(1)y3PLsc8R and In(1)sc8 embryos: sc is expressed in row D [b,d, arrowheads] but not in row B [b,d, arrows]. In comparison to In(1)sc8 embryos, ac expression in In(1)sc8 embryos is reduced in row B [e, whereas sc expression is enhanced in row B [f,h, arrows]. Finally, although the level of ac expression is greatly diminished in In(1)ac embryos [g], its pattern is essentially normal. [a,b,d–f,h] Bar, 50 ~tm; [c,g] bar, 30 ~tm.
Figure 3. Localization of cis-acting elements regulating ac gene expression. (a) Physical map of the AS-C; (b--d) expression of ac and ac reporter constructs in late stage 8/early stage 9 embryos. (a) Physical map of the ac, sc, and l'sc loci (adapted from Campuzano et al. 1985). (A) Positions of breakpoints of chromosomal inversions. Horizontal arrows represent positions of ac, sc, and l'sc transcripts. Broken rectangular boxes indicate the position of cis-regulatory regions of ac and sc as suggested by results presented in Fig. 2. Bracketed lines below the map indicate the DNA fragments used in the reporter constructs. Construct 101R3.2 is a 3.2-kb EcoRI fragment that spans between positions 57.4 and 54.2, whereas 101H10 is a 10-kb HindIII fragment that spans roughly between positions 56.4 and 46.4. Broken bracketed lines below map indicate the extent of the DNA region deleted in the synthetic deficiencies of, from left to right, ac, sc, and l'sc. (b) Lateral view of ac RNA expression in a late stage 8 wild-type embryo. (c) Lateral view of lacZ RNA expression in a late stage 8 embryo that carries two copies of the 101H10 construct. (d) Lateral view of lacZ RNA expression in an early stage 9 embryo that carries two copies of the 101R3.2 construct. Note that lacZ expression in 101H10-bearing embryos and ac expression in wild-type embryos is found in two rows of cell clusters per segment [e, arrows]. Furthermore, as observed for ac expression [b], lacZ expression does not extend laterally past the neuroectoderm [c, black arrowheads] and is absent or reduced between the m and 1 columns [c, white arrowheads]. lacZ expression in 101R3.2 embryos is generally found in one broad stripe of cells per segment [d, arrows] that occasionally splits into two smaller stripes [d, small arrows]. Although lacZ expression declines lateral to the neuroectoderm in 101R3.2 embryos [arrowheads], no DV restriction of expression is observed within the neuroectoderm.

the row D control elements of both ac and sc are found mostly proximal to the sc breakpoint, whereas the row B control elements of both ac and sc are mostly located distal to the breakpoint [Fig. 3a]. We should note that the In(1)scV2 effects are not the result of position effects on ac or sc created by juxtaposition of these genes to foreign DNA because deletion mutants and reporter constructs yield consistent results (see below).

The expression patterns of ac and sc in In(1)scV2 embryos also support the hypothesis that the elements necessary to activate ac and sc are shared. The scV2 breakpoint is found at +55.0 (Campuzano et al. 1985) and lies ~7 kb closer to the ac gene and 7 kb farther from the sc gene than the sc breakpoint [Fig. 3a; Lindsley and Zimm 1992]. Thus, in relation to In(1)scV2 embryos, more cis-acting DNA is removed from the ac gene and less from the sc gene in In(1)scV2 embryos. As was found in In(1)scV2 embryos, ac expression in row D is absent in In(1)scV2 embryos; however, ac expression in row B is also greatly reduced [Fig. 2e, arrows] or absent [not shown]. In contrast to ac, sc expression in In(1)scV2 embryos is restored in row B. During stage 8, sc RNA becomes detectable in row B [Fig. 2f, arrows] and, by stage 9, is expressed at moderate levels in row B [Fig. 2h, arrows]. The results obtained with In(1)scV2 and In(1)scV8 embryos suggest that at least a portion of the row B control elements of both ac and sc lie between the scV2 and sc breakpoint and that critical row D control elements for both genes lie proximal to the sc breakpoint (Fig. 3a).

The identification of regions 3' to ac that appear necessary to initiate the early clustered pattern of ac was not expected. To determine whether regions 5' to ac are important for the generation of the ac pattern, we examined ac expression in In(1)acV3 embryos. In(1)acV3 breaks distally at +59.2 and separates all 5' sequences from the ac-coding region, except for ~1 kb of DNA that lies im-

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The initial AP positions of proneural clusters are set up by pair-rule genes and are independent of segment polarity genes

Having established that the initial limits of ac expression are set up largely independently of the other members of the AS-C and having identified cis-acting regions required to initiate the clustered expression patterns of ac and sc, we then determined which trans-acting factors might act directly on these regulatory elements to generate the precise expression patterns of ac and sc. The AP and DV registration of these clusters and the time at which ac expression becomes detectable [early gastrulation] suggest that the segment polarity, the pair-rule, and the zygotic DV genes are the prime candidate genes to regulate ac expression directly. It has been proposed that the intersection of the expression patterns of these genes forms a grid within which the proneural genes are activated at specific coordinates [Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1990]. With this model in mind, we sought to identify the genes that control ac expression along each axis.

The similarity between ac and segment polarity gene expression is striking [Fig. 4a]. Although ac is expressed in two transverse rows of cell clusters per segment and many segment polarity genes are expressed in one stripe of cells around the embryo per segment, both are expressed in similar and sometimes identical domains along the AP axis. For example, the A and P borders of ac expression within row D coincide perfectly with the A and P borders of en expression, whereas ac expression within row B is found midway between stripes of en-expressing cells [Fig. 4a; Skeath and Carroll 1992]. ac and segment polarity gene expression, then, appear to respect similar boundaries. Because of this correspondence, it seemed likely that the establishment of the initial AP limits of ac expression would involve the segment polarity genes. We were surprised to find that this was not the case. In embryos singly mutant for all of the zygotic segment polarity genes [en, armadillo, wingless [wg], hedgehog, patched, gsb, nkd, and Cubitus interruptus D~] and in embryos doubly mutant for en and inverted, the initial embryonic pattern of ac was essentially identical to that observed in wild-type embryos [data not shown]. This suggested that the genes that establish the boundaries of segment polarity gene expression, the pair-rule genes, also regulate directly the initial AP boundaries of ac gene expression within segments.

In embryos homozygous for null mutations for each pair-rule gene examined [fushi tarazu (ftz), odd-skipped (odd), paired (prd), even-skipped (eve), odd-paired (opa), sloppy-paired (slp), runt [data not shown], and hairy [data not shown]], the early clustered pattern of ac expression is altered [Figs. 4 and 5]. The effect of removing any one of these genes on ac expression, with the exception of runt, is to cause specific pattern alterations at a double segment periodicity. On the basis of their effect on ac expression, most pair-rule genes can be grouped broadly into two categories: those required to set up ac expression within row D [Fig. 4; ftz, odd, prd, eve], and those necessary to establish ac expression within row B [Fig. 5; opa, slp, prd]. It should be noted that in all pair-rule mutant embryos where it has been examined [ftz, prd, and odd], sc expression is altered in a manner identical to ac [not shown]. Therefore, the sc expression pattern ap-
Figure 4. Pair-rule genes positioning ac gene expression within row D. Ventral or lateral views of late stage 8 wild-type (a) and pair-rule mutant (b–h) embryos either singly labeled for the ac protein (b,c,e,g) or doubly labeled for the ac (brown) and en proteins (a,d,f,h, black/purple). The domain of en-expressing cells marks row D of each segment. (a) Lateral view of a wild-type embryo. At this stage, every segment contains two transverse rows of ac clusters: one found midway between stripes of en-expressing cells; the other coincident with the en stripes. In embryos mutant for particular pair-rule mutants, this reiterated pattern is disturbed (b–h). In eve$^{127}$ embryos (b), all ac clusters normally found in row D are deleted. In ftz embryos (c,d), every fourth transverse row of ac clusters disappears, corresponding to row D of every even-numbered segment (c,d, arrows). In odd embryos (e,f), the same row of ac clusters expands posteriorly and is three times its normal width (e,f, arrows). Every fourth transverse row of ac clusters is lost in prd embryos (g,h), and these clusters correspond to those found in row D of odd-numbered segments (g,h, arrows). In addition, the row B clusters found in the anterior half of each even-numbered segment expands posteriorly and fuses with the ac clusters in row D (g,h, arrowheads). (a,b,c,e,g) Bar, 50 μm; (d,f,h) bar, 30 μm.

ac appears to be regulated in trans in the same way as ac, which is also consistent with ac and sc being regulated by common cis-acting elements.

The combinatorial action of four pair-rule genes establishes ac expression within row D

In embryos mutant for ftz, every fourth row of ac clusters is removed [Fig. 4c,d, arrows]. The deleted clusters are those normally found in row D in every even-numbered segment. The same row of clusters expands posteriorly to roughly three times its normal width in embryos mutant for odd [Fig. 4e,f, arrows]. Within this region in wild-type embryos, the anterior edges of ac and ftz gene expression coincide precisely, whereas the posterior border of ac and the anterior border of odd expression abut [the registration of ac expression to pair-rule gene expression was deduced from the known expression patterns of ac and the indicated pair-rule genes (Carroll et al. 1988; Coulter et al. 1990)]. Thus, the borders of ac expression defining row D of the even-numbered segments may result from the combinatorial effect of ftz activation and odd repression.

A similar type of combinatorial mechanism involving different pair-rule genes can explain the borders of ac expression within row D of the odd-numbered segments. In prd mutant embryos, every fourth transverse row of ac clusters is deleted; these are the normal row D clusters of odd-numbered segments [Fig. 4g,h, arrows]. This same row of ac clusters is removed in embryos mutant for eve [Fig. 4b]. Because, in wild-type embryos, the anterior borders of ac and eve expression and the posterior borders of ac and prd expression coincide in row D of odd-numbered segments [Frasch and Levine 1987; Carroll et al. 1988; Baumgartner and Noll 1990], activation of ac expression within row D of odd-numbered segments may result from the overlapping domains of prd and eve gene expression. We note that prd and eve have additional effects beyond the odd-numbered D rows: prd$^{-}$ embryos also exhibit fusion of the B and D rows of even-numbered segments (see below), whereas in eve$^{-}$ embryos, the D rows of even-numbered segments are deleted, probably because of an indirect effect of eve on ftz expression (Carroll and Scott 1986; Frasch and Levine 1987).

In contrast to row D, we cannot propose a simple model to explain the establishment of ac expression in row B. In every segment, the loss of opa function removes ac expression from row B [Fig. 5a,b, arrows] and decreases the cluster size and level of ac expression in row D. In embryos singly mutant for either slp [Fig. 5c] or
prd [Fig. 4g,h], the B and D rows of ac expression fuse in the odd- and even-numbered segments, respectively. When the ac pattern is established, opa is expressed uniformly throughout the segmental primordia [M. Benedyk and S. DiNardo, pers. comm.], whereas the anterior edge of prd expression abuts the posterior edge of ac expression in row B in even-numbered segments [Baumgartner et al. 1991] and the posterior edge of slp expression abuts the anterior edge of ac expression in row D in odd-numbered segments [Grossniklaus et al. 1992]. The expression domains of ac and prd suggest that prd represses ac expression at the posterior border of row B in even-numbered segments, whereas the relative expression patterns of slp and ac suggest that slp could repress ac at the anterior border of row D in odd-numbered segments. It is also possible that slp acts alone, or in conjunction with other genes, to repress ac expression at the posterior border of row B in odd-numbered parasegments. The recent cloning and characterization of the slp locus may help to unravel the role slp plays in establishing the ac expression pattern [Grossniklaus et al. 1992]. No simple model can be made for the effect of opa on ac. It is possible that opa acts in row B as a general activator and in row D as an enhancer of ac expression, whereas other as yet unidentified genes would define the anterior borders of ac expression in row B. Regardless of the exact mechanism that establishes ac expression within row B, these results support the idea that the combinatorial action of distinct sets of pair-rule genes defines the periodicity of ac expression in the B and D rows of odd- and even-numbered segments.

gsb and nkd segment polarity genes maintain the AP borders of ac ectodermal cell clusters

Although the initial pattern of ac expression (stage 8) in embryos singly mutant for all of the zygotic segment polarity genes is essentially identical to that observed in wild-type embryos, adjacent rows of ac clusters fuse in older embryos [mid-late stage 9] mutant for either gsb or nkd [Fig. 6]. In embryos mutant for gsb, ac expression in row B expands posteriorly into row C and eventually merges with ac expression in row D [Fig. 6c,e; arrows mark row C], whereas expression in row D appears to dissipate prematurely [Fig. 6, cf. a with c and e]. The directional expansion of ac expression in gsb mutant embryos is demonstrated by double-labeling with ac and en antibodies [Fig. 6e; en expression (purple) marks row D]. In wild-type embryos, the anterior limit of gsb expression abuts the posterior edge of ac expression in row B [J.B. Skeath and S.B. Carroll, data not shown; Baumgartner et al. 1987; A. Ungar and R. Holmgren, in prep.]. gsb activity appears necessary to maintain the posterior border of ac expression in row B by maintaining ac gene repression in row C. In embryos mutant for nkd, ac expression in rows B and D appears to expand: that of row B anteriorly into row A and that of row D posteriorly into row A [Fig. 6d,f; arrows mark row A]. The oriented expansion of ac expression into row A is most obvious in nkd mutant embryos double-labeled for ac and en [Fig. 6f]. Thus, along the AP axis, the concerted action of gsb and nkd appears to preserve the initial pair-rule-defined borders of ac expression by maintaining the repression of ac in rows C and A, respectively [Fig. 6b]. Interestingly, the removal of either of these genes has apparently no effect on l′se expression [Martin-Bermudo et al. 1991].

It should be noted that although only nkd and gsb control the initial pattern of ac gene expression, all zygotic segment polarity genes, with the possible exception of gsb, govern the spatial patterning of the later phases of ac gene expression in the PNS and CNS [J.B. Skeath and S.B. Carroll, unpubl.]. This raises the interesting possibility that two different sets of genes establish the AP pattern of ac gene expression at different times during embryogenesis.
The zygotic DV genes repress ac expression within specific DV domains

In a survey of representative zygotic (but by no means all) DV pattern mutants, we found that the ac domain frequently expanded, indicating that DV regulation of ac expression appears to occur largely through repression of ac expression within specific domains along the DV axis. These domains correspond to the region between the m clusters [regulated by twist (twi) and snail (sna)], the region dorsal to the l clusters [regulated by decapentaplegic (dpp) and tolloid (tld)], and the region between the m and l clusters [regulators unknown]. In embryos mutant for twi, the expression of which is largely restricted to the region between the two columns of m clusters (Thissel et al. 1988; Kosman et al. 1991), a partial fusion of these clusters occurs (Fig. 7b, arrowheads). The dorso-lateral extent of ac expression appears to be regulated by dpp and tld; the ventral limit of expression of each of these two genes extends roughly to the dorsal extent of ac expression within the l clusters (St. Johnston and Gelbart 1987; Shimell et al. 1991). Embryos mutant for each gene exhibit essentially identical alterations to the ac patterns where the l clusters expand dorsally (Fig. 7, cf. c and e with the wild-type embryo in a). The severity of the phenotype increases with age, as in stage 9 dpp [Fig. 7e] or tld [not shown] mutant embryos, the l extent of these clusters has expanded further dorsally than in stage 8 mutant embryos. In embryos mutant for zerknüllt (zen), the l clusters may expand dorsally to a small degree (Fig. 7d); however, this effect might also be attributable to the inability of cells to rearrange and condense during germ-band extension. Curiously, we found no mutants in which the m and l clusters fuse. These results argue that these DV genes directly or indirectly repress ac expression within specific domains of the DV axis.

Discussion

The role of global patterning genes in regulating intrasegmental patterns

The analysis of the regulation of the early zygotic genes involved in embryonic pattern formation has yielded a
Formal genetic understanding of how the basic segmental organization of the Drosophila embryo is created (for review, see Ingham 1988; Nüsslein-Volhard 1991; Ingham and Martinez-Arias 1992), however, little is understood about the mechanisms that generate the precise patterns of discrete cell types within segments. For example, segment polarity genes, the expression of which are the most restricted of the early pattern-forming genes, are expressed in one stripe per segment that consists by stage 8 of ~100 cells. Although segment polarity genes specify positional cell identity (i.e., anterior or posterior position) within segments, they do not assign cells discrete fates: Cells found within the expression domain of any one such gene differentiate into a number of discrete cell types (e.g., NB, myoblast), which may be part of much larger tissue systems or structures (e.g., the nervous system, musculature, imaginal discs). Even though it has been widely assumed for some time that zygotic segment polarity genes control the initial patterns of cell- or tissue-specific regulation (which is limited to the D register of every segment; DiNardo and O’Farrell 1987) in the same way that they control ac expression. Furthermore, rule and DV genes appear to carve out domains of ac expression as proneural clusters. Within the embryonic thoracic segments, the points of intersection between the expression domains of a segment polarity gene, wg, and a DV gene, dpp, appear to activate Brista/Distal-less in small groups of cells, as they are set aside as the precursors of the larval leg imaginal discs (Cohen et al., 1992). The position of the salivary gland precursors is specified through activation by the homeotic gene Sex combs reduced along the AP axis and by repression by dpp and dorsal along the DV axis (Panzer et al. 1992).

The levels of proneural gene regulation reflect a stepwise refinement of cell fate

The genetic regulation of proneural gene expression, specifically that of ac, is a model for the progressive refinement of positional identity that leads to organized arrays of specific cell types (NBs). The establishment of the segmentally repetitive pattern of ac-expressing proneural clusters is the first step in this process. We have shown that along the AP axis the combinatorial action of specific pair-rule genes generates the periodic pattern of ac expression [Fig. 8]. Within the D register of even-numbered segments, the ftz gene, through activation, and the odd gene, through repression, appear to define the precise anterior and posterior borders of ac expression, respectively. Within the D register of odd-numbered segments the coexpression ofprd and eve proteins apparently activates ac expression. It is important to note that within row D, the prd, eve, ftz, and odd genes control en gene expression (which is limited to the D register of every segment, DiNardo and O’Farrell 1987) in the same way that they control ac expression. Furthermore,
Figure 8. Two-dimensional control of gene expression. A schematic indicating the expression domains of ftz and odd pair-rule genes and a theoretical DV regulator within a ventrolateral region of an even-numbered parasegment [bracketed boxes indicate the domain of each protein]. The specific position of proneural gene expression in a cluster of four (to six) cells within the D register [see text] results from a combination of activation by ftz (in the D, A, and B registers), and repression by odd (in the A, B, and C register) and the DV regulators (in the dorsolateral region). The action of each protein appears to be mediated by at least one cis-acting region between the ac and sc loci that influences the expression of both genes.

whereas the pair-rule genes also appear to be the prime determinants of the AP periodicity of l'sc [Martin-Bermudo et al. 1991], l'sc is expressed largely out of phase [in the A and C register] with respect to ac/sc [Fig. 1]. Clearly, a more detailed knowledge of the cis-acting elements involved in en, ac, and l'sc regulation will be necessary to sort out how pair-rule proteins position the expression of each of these target genes.

The fact that the pair-rule, and not the segment polarity genes, define the precise AP determinants of the AP periodicity of l'sc [Martin-Bermudo et al. 1991], l'sc is expressed largely out of phase [in the A and C register] with respect to ac/sc [Fig. 1]. Clearly, a more detailed knowledge of the cis-acting elements involved in en, ac, and l'sc regulation will be necessary to sort out how pair-rule proteins position the expression of each of these target genes.

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Distinct cis-acting regulatory elements are involved in the control of the pattern and level of ac and sc expression

Our initial dissection of the cis-acting regions of the ac gene suggests that the elements required to initiate the correct spatial expression patterns of ac and sc lie between the two genes and are roughly separable into at least two distinct regions: one critical for the initiation of expression in row B, located 3' to the ac gene and 5' to the sc breakpoint, and one critical for the initiation of expression in row D, located 3' to the sc breakpoint and 5' to the ac gene (Figs. 2 and 3). Furthermore, our initial results from reporter constructs containing large regions of DNA from between the two genes show that this region can drive weak lacZ expression in a pattern that resembles the endogenous ac (and sc) pattern. It is possible that these regions may be further subdivided into enhancers operating in either even- or odd-numbered parasegments responsive to specific combinations of pair-rule genes. It is also likely that each register of proneural gene expression is driven by redundant elements spread over the large intergenic regulatory region.
Although the intergenic region may be sufficient to initiate the proper spatial pattern of ac [sc] gene expression, the proper level of ac expression may require additional control elements located 5' to ac responsive to the input of the ac, sc, and l'sc proteins. We have shown that the level of ac gene expression (in row D) is reduced in l'sc mutant embryos, and Van Doren et al. (1991) have identified a number of consensus-binding sites for the ac, sc, and l'sc proteins that lie within 1 kb 5' of the ac promoter. Taken together, these observations suggest that 3' regulatory regions would largely control the spatial patterning of ac gene expression, whereas 5' elements would augment its level of expression. Further analysis of these 3' spatial control elements will require higher levels of reporter gene expression than that created here. It may be necessary to surround the reporter gene with both 5' and 3' regulatory regions of ac in future constructs to achieve the levels of reporter gene expression necessary for a more detailed dissection of these spatial control elements.

Once the global pattern of ac-positive proneural clusters is generated, local interactions between cells determine individual cell fates. Initially, within each cluster, a competitive process occurs, involving interactions between the proneural and neurogenic genes, which singles out one cell from the cluster as the future NB (de Celis et al. 1991a,b; Heitzler and Simpson 1991). An increased level of proneural protein expression in this cell relative to the remaining cells of the cluster reflects this decision (Cubas et al. 1991; Martin-Bermudo et al. 1991; Skeath and Carroll 1991, 1992). The future NB, then, starts a competitive process that determines the remaining cells of the cluster reflects this decision.

Materials and methods

Fly strains

Fly stocks were obtained from the laboratories of Walter Gehring, Michael Hoffmann, Steve DiNardo, Eric Wieschaus, and Alain Ghysen and from the Bowling Green, Indiana, and Tübingen stock centers. Fly stocks harboring the following DNA rearrangements in the AS-C were used: Df(1)pry,sc, Df(1)sc,sc-LAR, Df(1)sc,scLAR, In(1)sc,sc, In(1)ac, and In(1)scV2. Fly stocks carrying the following mutations to segment polarity genes were used: en^C1, armadillo^W22, wg^2^C2, hedgehog^1^C22, patched^1^N108, gsb^1^X26, nkd^1^B29, Cubitus interruptus^Dominant (Ci^D), and en^B5^ [en and invected double mutant]. Fly strains carrying the following pair–rule mutations were used: ftx^W20, odd^H1066, prd^H44, eve^1^27, opto^H31, spl^S284, runt^Df(1)B57, and hairy^7^H46. Fly stocks harboring the following DV mutations were used: dpp^61, snal^605, tld^h4, twi^H7, and zen^W26. The identity of the In(1)sc^a, In(1)ac^a, In(1)sc^v2, Df(1)pry,sc, sc-LAR, and Ci^D fly stocks was verified by the cuticular phenotypes of adult flies. The identity of all pair–rule mutant stocks and the armadillo^W22, wg^2^C2, hedgehog^1^C22, patched^1^N108, and nkd^1^B29 fly stocks was confirmed by determining the expression pattern of en in each stock. In all stocks the expected alteration to en expression occurred in ~25% of the properly staged embryos. The identity of the en^a, gsb^1^X26, and Df(1)sc,scLAR fly strains was determined by staining embryos from each stock for en, gsb, or l'sc protein, respectively, and observing that ~25% of the appropriately staged embryos of each stock were devoid of protein expression. The identity of the en^C1 fly stock was verified by the cuticular phenotype of each embryo from this stock.

ac reporter constructs

Putative regulatory regions of ac were cloned into phsplacCaSpeR (H. Nelson and A. Laughon, in prep.), which has a disabled heat shock promoter fused to the lacZ gene. Transformed lines were established by standard methods using the white gene as a selectable marker. Reporter constructs were co-injected with a helper F-element, pEF 25.7WC [Koress and Rubin 1984], into procellular embryos from the yw line [Lindsay and Zimm 1992]. The 3.2-kb ac construct shown in Figure 3 (101R3.2) was constructed by religation of the 1.0- and 2.2-kb EcoRI fragments (between 57.4 and 54.2) from l'sc101 [Campuzano et al. 1985] into pBlueScript[KS] (Stratagene) to generate p101R3.2. Construct 101H10 was subcloned as a HindIII fragment from l'sc101 [a kind gift from Juan Modolell, Centro de Biologia Molecular, Madrid, Spain] into pBlueScript[KS] (Stratagene) to generate p101H10. Both fragments were then isolated as KpnI–NotI fragments and were cloned into the unique KpnI cloning sites of phsplac CaSpeR in the sense orientation with respect to lac.

Four lines of the 101H10 construct and three lines of the 101R3.2 construct were obtained. All lines assayed resulted from independent integration events. Three of the four 101H10 lines exhibited the identical expression pattern (shown in Fig. 3), with the only line showing no detectable lacZ expression. All three of the 101R3.2 lines exhibited the expression pattern shown in Figure 3, although two lines expressed lacZ at lower levels.

Immunohistochemistry and in situ hybridization

Embryos were fixed in 4 ml of fix buffer [100 mM PIPES at pH 6.9, 2 mM EGTA, 1 mM MgSO4], 1 ml of 37% formaldehyde [Sigma], and 5 ml of heptane, following standard protocols. Sin-
ingle- and double-antibody labeling experiments were carried out in Eppendorf tubes, as described in Skeath and Carroll (1992) except as noted below.

For staining embryos using peroxidase, Vector's ABC elite kit was used and the following changes were made to the peroxidase staining protocol (Skeath and Carroll 1992): After 30 min of washes in 100 mM Tris-HCl (pH 6.8) and before the development of the peroxidase reaction (see Skeath and Carroll 1992), embryos were preincubated for 2 min either in a solution containing 100 mM Tris-HCl (pH 6.8) and 0.5 mg/ml of diaminobenzidine (DAB, for a brown reaction product) or in a solution containing 100 mM Tris-HCl (pH 6.8), 0.5 mg/ml DAB, and 0.03% [wt/vol] Co$^{2+}$ and Ni$^{2+}$ ions (for a black reaction product). After 2 min of preincubation, the solution was removed and the stain was developed by the addition of 100 mM Tris-HCl (pH 6.8), 0.5 mg/ml of DAB, and 0.002% H$_2$O$_2$. The reaction was monitored visually by placing ~20 μl of embryos on a microscope slide and observing the reaction through a dissecting scope. When the stain had developed to the desired intensity, the reaction was stopped by the addition of 5 μl of 20% sodium azide. All other steps were performed as described previously (Skeath and Carroll 1992).

For detection of ac protein, monoclonal antibody (mAb) 990E5F1 was used at a 1:3 dilution; for detection of lsc protein, a rat anti-lsc antibody (generously provided by Fernando Jimenez, Centro de Biologia Molecular, Madrid, Spain) was used at a 1:500 dilution, for detection of en protein, mAb NiV409 (kindly provided by Nipam Patel, Carnegie Institute of Embryology, Baltimore, MD), which recognizes both en and invected, was used at a 1:1 dilution, and for detection of gsb-distal protein, rat mAb16F12 and mAb10E10 (generously provided by Anne Ungar and Bob Holmgren, Northwestern University, Evanston, IL) were used together each at a 1:5 dilution. For sequentially double-labeling embryos for ac protein and either lsc, en, or gsb protein, embryos were first stained with the ac antibody and then stained with one of the other three antibodies. Double-labeling embryos for ac and lsc were done by use of two sequential peroxidase staining reactions, whereas double-labeling embryos for ac and either en or gsb was done by use of a peroxidase staining reaction for ac followed by an alkaline-phosphatase staining reaction for en or gsb (see Skeath and Carroll 1992). To identify unambiguously early gsb and ndk embryos, we balanced each stock over balanced chromosomes that express lacZ under the control of the ftz stripe element. Embryos were then doubly or triply labeled for ac and lacZ or for ac, lacZ, and en. Mutant embryos were identified as those that did not express lacZ. Df(1)scsl2 or embryos were identified unambiguously by double-labeling embryos for ac and lsc. Mutant embryos were identified as those that expressed no lsc protein.

In situ hybridization was carried out as described in Tautz and Pfeifle (1989). For detection of sc RNA expression, embryos were hybridized in a total volume of 100 μl of hybridization solution containing 17 ng of a digoxigenin-labeled DNA probe from the 1.5-kb EcoRI fragment of a sc cDNA (kindly provided by the late Carlos Cabrera). For the simultaneous detection of both sc and wg RNA expression, embryos were hybridized [100 μl total volume] with the sc probe [17 ng] and with 17 ng of a digoxigenin-labeled DNA probe from the 1-kb EcoRV–HindIII fragment of a wg cDNA (Rijsewijk et al. 1987). Detection of lacZ RNA was carried out as described in Tautz and Pfeifle (1989), as modified by Jiang et al. (1991).

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