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The zygotic control of *Drosophila* pair-rule gene expression

II. Spatial repression by gap and pair-rule gene products

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Summary

We examined gene expression patterns in certain single and double pair-rule mutant embryos to determine which of the largely repressive pair-rule gene interactions are most likely to be direct and which interactions are probably indirect. From these studies we conclude that: (i) *hairy*$^+$ and *even-skipped* (*eve*$^+$) regulate the *fushi tarazu* (*ftz*) gene; (ii) *eve*$^+$ and *runt*$^+$ regulate the *hairy* gene; (iii) *runt*$^+$ regulates the *eve* gene; but, (iv) *runt* does not regulate the *ftz* gene pattern, and *hairy* does not regulate the *eve* gene pattern. These pair-rule interactions are not sufficient, however, to explain the periodicity of the *hairy* and *eve* patterns, so we examined specific gap gene mutant combinations to uncover their regulatory effects on these two genes. Our surprising observation is that the *hairy* and *eve* genes are expressed in embryos where the three key gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), and *knirps* (*kni*) have been removed, indicating that these gap genes are not essential to activate the pair-rule genes. In fact, we show that in the absence of either *hb*$^+$ or *kni*$^+$, or both gap genes, the *Kr*$^+$ product represses *hairy* expression. These results suggest that gap genes repress *hairy* expression in the interstripe regions, rather than activate *hairy* expression in the stripes. The molecular basis of pair-rule gene regulation by gap genes must involve some dual control mechanisms such that combinations of gap genes affect pair-rule transcription in a different manner than a single gap gene.

Key words: gap genes, pair-rule genes, blastoderm, *Drosophila* embryogenesis.

Introduction

Analyses of terminal phenotypes and of segmentation gene expression patterns in various mutant embryos has defined the overall regulatory hierarchy of the segmentation genes. Generally, each class of segmentation genes interacts to specify the finer expression pattern of the next group of genes. Thus, the maternal coordinate genes affect each other (Frohnhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988) and the gap genes (Gaul and Jäckle, 1987), which interact (Jäckle *et al.* 1986) to control pair-rule gene patterns (Carroll and Scott, 1986; Ingham *et al.* 1986; Frasch and Levine, 1987), which interact to specify the patterns of the segment polarity genes (DiNardo and O'Farrell, 1987; Martinez-Arias and White, 1988). Given the extent of these fundamental pattern-regulating gene interactions, it is a large task to determine the nature of the regulatory circuitry that operates between segmentation genes and to identify the *trans*- and *cis*-acting factors that are responsible for the pattern of gene expression.

The present approaches aimed at elucidating these factors include formal genetic analyses, studies on the effect of ectopic expression of putative regulatory proteins (Ish-Horowicz and Pinchin, 1987), analyses of *cis*-acting elements that control pair-rule gene expression (Hiromi *et al.* 1985; Hiromi and Gehring, 1987; Howard, 1988), studies on the effects of inhibiting segmentation protein synthesis (Edgar *et al.* 1986, 1989), and *in vitro* biochemical experiments (Hoey and Levine, 1988). This combination of approaches is expected to resolve the complex problem of how a crude pattern in the unfertilized egg is translated into a periodic pattern of segments.

In the accompanying paper, we showed that most zygotically required regulators of the pair-rule genes have apparently been identified and that the initial activation of the pair-rule genes does not depend upon other zygotic genes (Vavra and Carroll, accompanying paper). However, even with most of the key genes identified, it is difficult to demonstrate whether removing one gene from the system directly or indirectly perturbs the expression of others or if an observed interaction reflects positive or negative control. To address these difficulties we have analyzed pair-rule gene expression in selected single, double, and triple mutant embryos to uncover which zygotic genes are likely to act directly upon the *hairy*, *eve* and *ftz* genes. Our results, combined with other recent studies on pair-

rule gene interactions (Ingham and Gergen, 1988) and protein synthesis inhibition experiments (Edgar *et al.* 1989), support the view that certain pair-rule genes are extensively negatively regulated, i.e. specific maternal, gap and pair-rule proteins repress pair-rule genes. It appears that, at least for the *ftz* and *hairy* genes, their striped patterns are more the result of repression of gene expression in the interstripe regions than a regional activation of individual stripes.

Materials and methods

Antibodies

We have examined pair-rule gene expression in whole-mount cellular blastoderm embryos by filtered fluorescence imaging (Karr and Kornberg, 1989; Carroll *et al.* 1988) after immunoperoxidase staining with polyclonal antibodies specific for the *ftz* (Carroll and Scott, 1985), *eve* (Frasch *et al.* 1987; antibody gift of M. Frasch and M. Levine) *hairy* (Carroll *et al.* 1988), and *Krüppel* (Gaul and Jäckle, 1987; antibody gift from U. Gaul). This technique gives sharp images of protein localization and was used to double-label embryos to examine relative expression patterns or to unambiguously identify the genotype of an individual embryo derived from crosses that yield a variety of mutant progeny.

Stocks and Crosses

The null allele stocks used to generate the pair-rule double mutant embryos were: Df(1) $runt^{B57}$/FM6y^{+}Y^{mal102} (kindly provided by Peter Gergen), Df(2R) *eve* 1.27 *cn bw sp*/SM6a, and h^{7h94} e^{s}/TM3.

Runt; *eve* and *runt*; *hairy* double mutant embryos were generated by mating *runt* males to heterozygous *eve* or *hairy* virgin females. The double heterozygous F_1 females were then mated to either heterozygous *eve* or *hairy* males, yielding one-sixteenth double mutant embryos.

Stocks used in gap mutant analysis were Kr^{1} *cn bw*/*CyO* (a null allele), and the double mutant $kni^{1lD48}hb^{7m48}$ *cu sr* e^{s} *ca*/TM3 *Sb Ser*, generously provided by R. Lehmann and C. Nüsslein-Volhard. The triple gap mutant embryos were produced at a frequency of one-sixteenth from heterozygous *hb*, *kni*; *Kr* parents.

Results

Interactions between pair-rule genes

Several previous studies have analyzed the effect of individual pair-rule mutations on the expression of other pair-rule genes (Howard and Ingham, 1986; Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988). From these experiments, a general picture emerged of the pair-rule gene hierarchy that placed *hairy*, *runt* and *eve* at the top, with other pair-rule genes (e.g. *ftz*) being downstream from them. For example, *ftz* expression is altered by mutations in *h*, *runt* or *eve*, but *ftz* mutants have no impact on expression of *h*, *runt* or *eve*.

Recently, an extensive study was made of *hairy*, *runt*, *eve* and *ftz* RNA expression in certain double-mutant pair-rule combinations (Ingham and Gergen, 1988). The analysis of epistatic relationships helps to reveal which genes are likely to be directly involved in the regulation of other genes in the pathway. The observations presented here on pair-rule protein patterns overlap with the results of Ingham and Gergen (1988) on pair-rule RNA patterns. Because these interactions and the patterns of certain mutant combinations are critical to the subsequent discussion of gap gene control, we will present all of our results dealing with the regulation of *h*, *eve* and *ftz* protein expression and emphasize those details and mutant combinations that may differ from the previous observations of RNA patterns. We generally agree with the conclusions of Ingham and Gergen (1988) as to the nature of individual pair-rule regulatory interactions.

Gene expression in single pair-rule mutants

The wild-type *hairy*, *ftz* and *eve* protein patterns at the cellular blastoderm stage of embryogenesis consist of seven transverse stripes encircling the embryo and, in the case of the *hairy* gene, an additional dorsal anterior patch of expression (Fig. 1A–C). The *ftz* and *eve* stripes are in alternating domains while the *hairy* stripes are offset such that the six posterior ones transiently overlap the *ftz* stripes by about one cell and all seven *hairy* stripes overlap with each *eve* stripe (Carroll *et al.* 1988). Loss of $runt^{+}$ activity changes *h*, *eve* and *ftz* expression (Fig. 1D–F). The *hairy* pattern partly expands with the first *hairy* stripe spreading posteriorly, while the interband between stripes 3 and 4 accumulates some protein, and stripes 6 and 7 are stronger and nearly fused (Fig. 1D). Note that the *hairy* pattern is still fairly periodic, so although loss of $runt^{+}$ derepresses *hairy* expression, *runt* is only one of probably several negative regulators of *hairy*. We will symbolize these gene interactions with an arrow indicating positive regulation and a cross-hatch indicating negative control, i.e.

runt ⊣ *hairy*

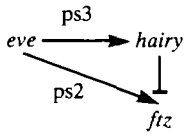
ftz expression is reduced in $runt^{-}$ embryos with the first, third, fifth, and sixth stripes narrowing or almost disappearing (Fig. 1E). The *ftz* protein pattern complements the *hairy* protein pattern and is likely to result from the initial effect of $runt^{-}$ on the *hairy* pattern and the subsequent effect of *hairy* on *ftz* (Howard and Ingham, 1986; Carroll and Scott, 1986; and see Fig. 1N below), i.e.:

runt ⊣ *hairy* ⊣ *ftz*

eve expression is only slightly affected during the cellular blastoderm stage in $runt^{-}$ embryos, the principal early defects are a reduced fifth *eve* stripe with a slight spreading of the other stripes (Fig. 1F; see also Frasch and Levine, 1987). During early gastrulation, the *eve* pattern spreads more dramatically but we are primarily concerned here with the initial periodic blastoderm pattern. Thus, *runt* appears to be a relatively late repressor of *eve* (its effects on the fifth stripe are not explained by this interaction; we do not understand the early effect of $runt^{-}$ on *eve* expression), therefore:

runt ⊣ *eve*

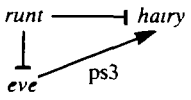
Loss of eve^+ activity affects both *hairy* and *ftz* expression. The strongest effect on *hairy* involves the second stripe which is greatly reduced in eve^- embryos (Fig. 1G) while the other stripes are generally narrower and irregularly spaced. *ftz* expression is lost in eve^- embryos from the region where the first stripe would normally form and there are shifts in the regularity of stripe width and spacing (Fig. 1H). To monitor how loss of eve^+ function changes *h* and *ftz* expression, we double-labelled eve^- embryos with *hairy* and *ftz* antibodies. There is a gap about 8 nuclei in width where little or no *hairy* or *ftz* protein accumulates, posterior to this gap the combined pattern is largely periodic (Fig. 1I). We conclude that eve^+ is required to activate *ftz* and *hairy* in parasegments (ps) 2 and 3 respectively, though this role may change after cellularization when the *ftz* (Carroll and Scott, 1986) and *hairy* patterns decay rapidly in an eve^- embryo (data not shown). Thus,



We also point out that *eve* is the earliest detectable pair-rule protein (at cycle 12) and exhibits a broad early band of expression during cycle 14 over PS1–3 (Frasch *et al.* 1987; our unpublished observations). This early band of expression may be necessary for *hairy* and *ftz* accumulation in these parasegments (see Discussion).

Gene expression in double pair-rule mutants

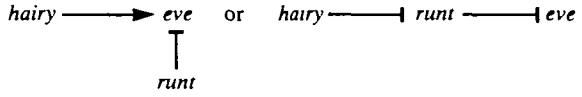
In order to better determine which of these pair-rule interactions could be direct and which are probably indirect, we examined gene expression in double pair-rule mutants. In $runt^-$; eve^- double mutant embryos, the *hairy* protein pattern exhibits elements of both single mutants, for example, loss of stripe 2 and fusion of stripes 6 and 7 (Fig. 1J). We conclude, then, that both *runt* and *eve* regulate *hairy*, thus:



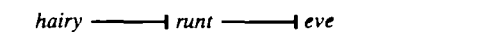
Note that since *runt* and *eve* are the only known pair-rule regulators of *hairy*, the pattern shown in Fig. 1J is very informative because it is the consequence of *hairy* regulation by genes that are above the pair-rules in the segmentation hierarchy, i.e. the gap genes (see below).

The *ftz* pattern is also strongly affected in $runt^-$; eve^- embryos; however, because of the strong influence of *hairy* on *ftz*, we cannot determine from this combination whether *runt* might regulate *ftz* more directly (Fig. 1K).

To better assess the role of $runt^+$ in *ftz* regulation and the role of $hairy^+$ in *eve* regulation, we have examined double mutants of *hairy* and *runt*. Our logic is as follows: loss of $hairy^+$ function reduces *eve* expression, mostly in the first, second, fifth and sixth stripes (Fig. 1L). This effect of *hairy* could be due to a positive requirement for *hairy* or due to a requirement for *hairy* to control *runt*, a negative regulator of *eve*, i.e.:

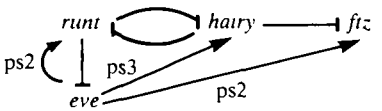


To resolve between these possibilities, we examined *eve* expression in $hairy^-$; $runt^-$ embryos (we confirmed the identity of these embryos by double labelling) and observed that *eve* expression is the same in the double mutant as in the $runt^-$ embryo (Fig. 1O), indicating that the *hairy* effect is indirect, therefore:



Ingham and Gergen (1988) have shown that *hairy* is required to repress *runt*.

ftz expression in a $hairy^-$ embryo spreads, leaving only a few narrow strips of unlabelled cells (Fig. 1N). In the $hairy^-$; $runt^-$ double mutant embryo, the *ftz* pattern is similar to that of the $hairy^-$ embryo, indicating that the *runt* requirement shown in Fig. 1E is indirect (*via* the *runt* effect on *hairy*), therefore the whole circuit, including the observations on regulation of *runt* expression by Ingham and Gergen (1988) (who showed that *eve* is also required for early activation and late repression of *runt*), is:

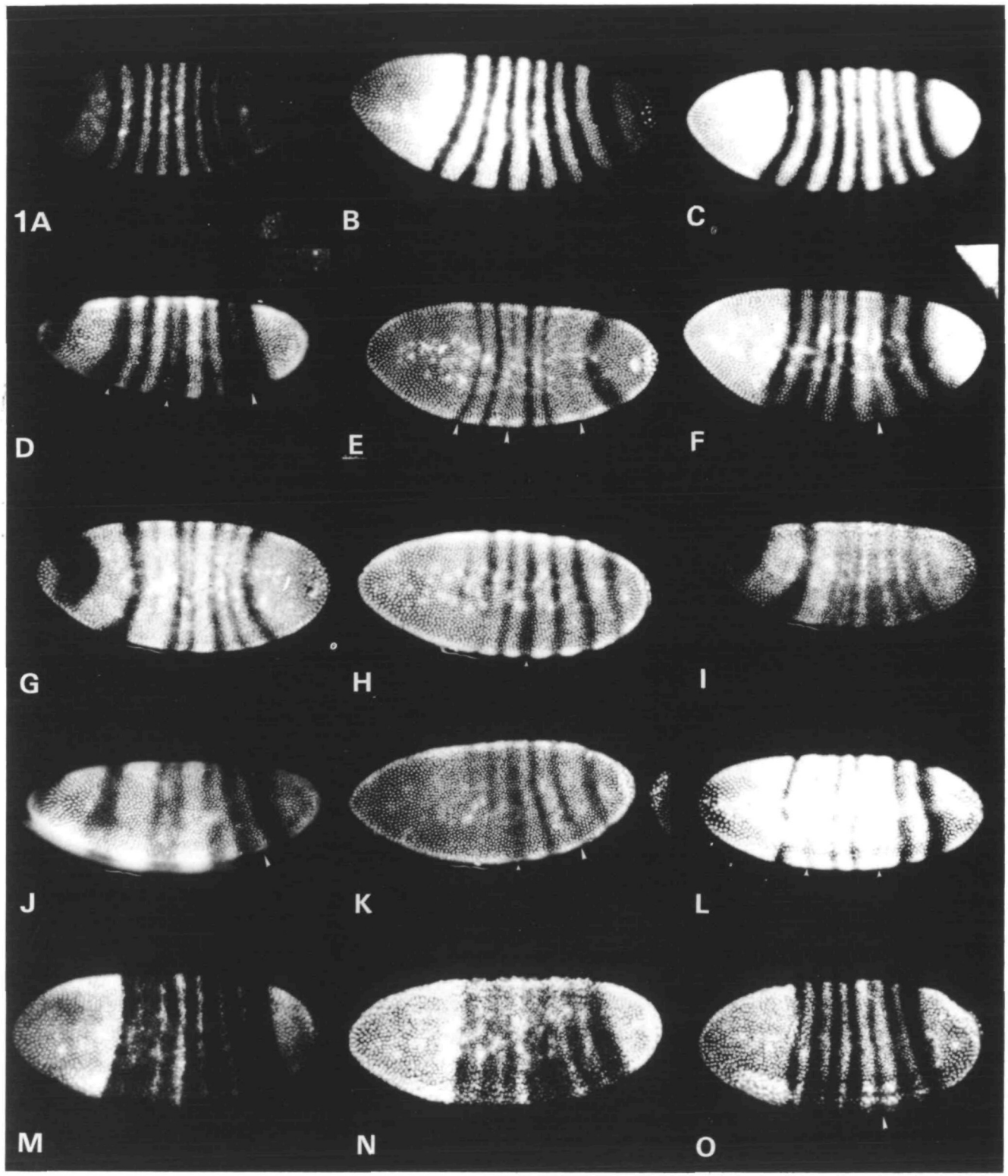


Since pair-rule genes do not feed back upon the gap genes and our aneuploid screen turned up only one new zygotic locus so far, we have no evidence of any intermediary genes that could explain our observations; thus, we suggest that the interactions diagrammed above could represent direct regulation of pair-rule gene expression at either the DNA, RNA or protein levels.

Gap gene regulation of hairy, ftz *and* eve

It is critical to note that the basic periodic patterns of *hairy* and *eve* are only moderately perturbed by pair-rule mutations (see for instance *hairy* expression in the $eve^-/runt^-$ double mutant embryo in Fig. 1J, and *eve* expression in the $runt^-/hairy^-$ embryo in Fig. 1O). This suggests that the genes above the pair-rule level, namely the zygotic gap genes, regulate their initial periodicity.

Gap genes have strong effects on the expression of *ftz* (Carroll and Scott, 1986; Ingham *et al.* 1986), *hairy* (Ingham *et al.* 1986; Carroll *et al.* 1988; Howard, 1988) and *eve* (Frasch and Levine, 1987). However, it has been difficult to distinguish which effects could be direct *versus* those that may be indirect. It has been shown that, in *Krüppel*$^-$ (*Kr*) and *knirps*$^-$ (*kni*) embryos, *ftz* expression is in a pattern that is complementary to that of *hairy* expression, while in *hunchback*$^-$ (*hb*) embryos *ftz* does not strictly follow *hairy* in the posterior of the embryo (Carroll *et al.* 1988; Ingham *et al.* 1986; Howard, 1988). From these observations, we had concluded



that *ftz* regulation is mostly independent of the three gap genes, *hb*, *Kr* and *kni*.

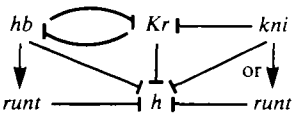
In order to better understand the role of these three gap genes in establishing the periodic patterns of *hairy* and *eve*, we have examined their expression in certain combinations of gap mutants. In Kr^{-} embryos, *eve* (Fig. 2A) and *hairy* (Fig. 2B) expand into very similar patterns with the middle of the embryo expressing two large blocks of each protein (about 10–12 nuclei in width) separated by a band of unlabelled cells (about 3–4 nuclei in width). The *ftz* pattern is complementary to the *hairy* pattern (Fig. 2C). While the expansion of the *eve* and *hairy* domains could indicate a basic negative control of these genes by *Kr*, there are many

Fig. 1. Expression of the *hairy*, *ftz*, and *eve* pair-rule genes in pair-rule mutant embryos. Cellular blastoderm-stage embryos were stained with *hairy*-, *ftz*- or *eve*-specific antibodies as described in Materials and methods. The dark areas represent regions of staining. Anterior is to the left, ventral towards the bottom of each picture, all photos are taken at a magnification of 50×. (A–C) Wild-type embryos stained with *hairy* (A), *ftz* (B), or *eve* (C) antibodies. Each pattern consists of seven fairly regularly spaced stripes encircling the embryo, *hairy* (A) is also expressed in a dorsal anterior patch. (D–F) Homozygous Df(1) $runt^{B57}$ embryos. (D) *hairy* protein expression expands to include more cells on the posterior edge of the first stripe, cells in the interband between stripes 3 and 4, and stripes 6 and 7 are nearly fused (arrows). (E) *ftz* protein expression diminishes where the *hairy* protein stripes have expanded, note stripes 1, 3, 5 and especially the sixth stripe (arrows). (F) *eve* protein expression begins to expand, except in the fifth stripe, which is strongly reduced (arrow). (G–I) Homozygous Df(2R) $eve^{1.27}$ embryos. (G) *hairy* protein expression is strongly reduced in the region of stripe 2 (bracket), stripes 3 and 4 are poorly resolved and all stripes are narrowed and spaced unevenly. (H) *ftz* protein expression is reduced in the region of stripe 1 and ventrally in stripe 2 (bracket), stripe 4 is wider than normal, all stripes are spaced unevenly. (I) Double staining with *hairy* and *ftz* shows a large 8-nucleus-wide gap where neither product accumulates (bracket). (J,K) Homozygous $runt^-$; eve^- embryos. (J) *hairy* protein expression exhibits elements of both the $runt^-$ and eve^- patterns shown in (D) and (G) (bracket and arrow). (K) The *ftz* protein pattern also exhibits elements of both the $runt^-$ (E) and eve^- (H) patterns (bracket and arrows). (L,M) Homozygous $hairy^{7H94}$ embryos. (L) *eve* protein expression is reduced in the second and fifth stripes (arrows), while (M) *ftz* protein expression expands to include most cells of the normal $hairy^+$ domain. (N,O) Homozygous $hairy^-$; $runt^-$ embryos. (N) The *ftz* protein pattern resembles that of the $hairy^-$ single mutant (M) and not the $runt^-$ pattern (E). (O) The *eve* protein pattern resembles the $runt^-$ single mutant pattern (F) and not the $hairy^-$ pattern (L), the arrow points to the reduced fifth stripe.

alternative explanations to consider that are best explored after examining mutant combinations.

In hb^-, kni^- embryos, *hairy* is not expressed over most of the anterior segment primordia (about 40–65 % egg length, Fig. 2E) but has spread out on the posterior part of the kni^+ domain (about 20–35 % egg length). We infer from this pattern that kni^+ is required to keep *hairy* off (either directly or indirectly *via* the *runt* gene) in this posterior region of the embryo and that some gene(s) still keeps *hairy* off in the unstained middle third of the embryo. The best candidate for this latter activity is Kr^+ which, while normally expressed in PS 4–7, spreads anteriorly in hb^- embryos and posteriorly in kni^- embryos, approximately that region where *hairy* is not expressed in a hb^-, kni^- embryo (Jäckle *et al.* 1986; Box C diagrammed in Fig. 3). To determine whether Kr^+ is responsible for keeping *hairy* off over the middle third of the hb^-, kni^- embryo, we constructed triple mutants that were hb^-, kni^-; Kr^-. In these embryos, *hairy* is expressed across most of the posterior two-thirds of the embryo; that is, the additional removal of Kr^+ has derepressed *hairy* (compare Fig. 2H with Fig. 2E; Box C in Fig. 3). Therefore, Kr^+, in the absence of the hb^+ and kni^+ gene products, appears to negatively regulate *hairy*.

This repressive function of Kr^+ is also apparent in single mutant embryos that are either hb^- or kni^- (summarized in Fig. 3). In hb^- embryos, Kr^+ expression expands anteriorly (Jäckle *et al.* 1986; Gaul and Jäckle, 1987), while *hairy* expression shifts anterior to and is lost within the new anterior *Kr* domain (Box A, Fig. 3; Carroll *et al.* 1988). In kni^- embryos, Kr^+ expression expands posteriorly (Jäckle *et al.* 1986; Gaul and Jäckle, 1987), while *h* expression spreads out posterior to, and is lost within, the new posterior *Kr* domain (Box B, Fig. 3; Carroll *et al.* 1988). These observations, and the effect of removing of Kr^+ in a hb^-, kni^- genetic background, suggest that *Kr* can behave as a repressor of *h* expression and may explain the loss of *hairy* expression in certain regions of hb^- or kni^- embryos. However, since *hairy* is expressed outside of the *Kr* domain in both of these gap mutants, the ectopic *hairy* expression could be due to either a direct negative requirement for hb^+ and kni^+ or an indirect effect of the gap genes on *hairy* mediated through the *runt* gene. The spatial restriction of *hairy* in the $runt^-/eve^-$ embryos suggests that there are other negative regulatory functions for gap genes but we cannot identify them from these experiments. Other gap genes such as *giant* (Carroll and Scott, 1986; Petschek *et al.* 1987; Frasch and Levine, 1987) and the terminal gene *tailless* (Mahoney and Lengyel, 1987; Frasch and Levine, 1987) are also required to establish the pair-rule patterns. We do not have enough information at this stage to decipher the regulatory functions of these two genes, or of the new gene we have uncovered on chromosome arm 2L (Carroll and Vavra, 1989), in establishing pair-rule gene patterns. In summary, the three gap gene interactions with *hairy* and each other (Jäckle *et al.* 1986) are:



The *eve* pattern in hb^-, kni^- embryos differs considerably from the *hairy* pattern in that it extends from parasegments 1–13 (about 15–70 % egg length, Fig. 2D) and is at much lower than wild-type levels. And, in contrast to *hairy*, the additional removal of Kr^+ has no discernible effect on *eve* expression in a hb^-, kni^- background (compare Fig. 2G with Fig. 2D). hb^+ and kni^+ appear to be required for spatial repression of *eve* into stripes, while Kr^+ appears to have little influence on *eve* under these circumstances. Also, it appears that hb^+ and kni^+ may be necessary for the proper level of *eve* expression since *eve* protein does not accumulate to normal levels in hb^-, kni^- or the triple mutant embryos. We cannot tell from these experiments whether the control of *eve* by hb^+ and kni^+ involves repression or activation, or through combinatorial interactions, both mechanisms (see Discussion).

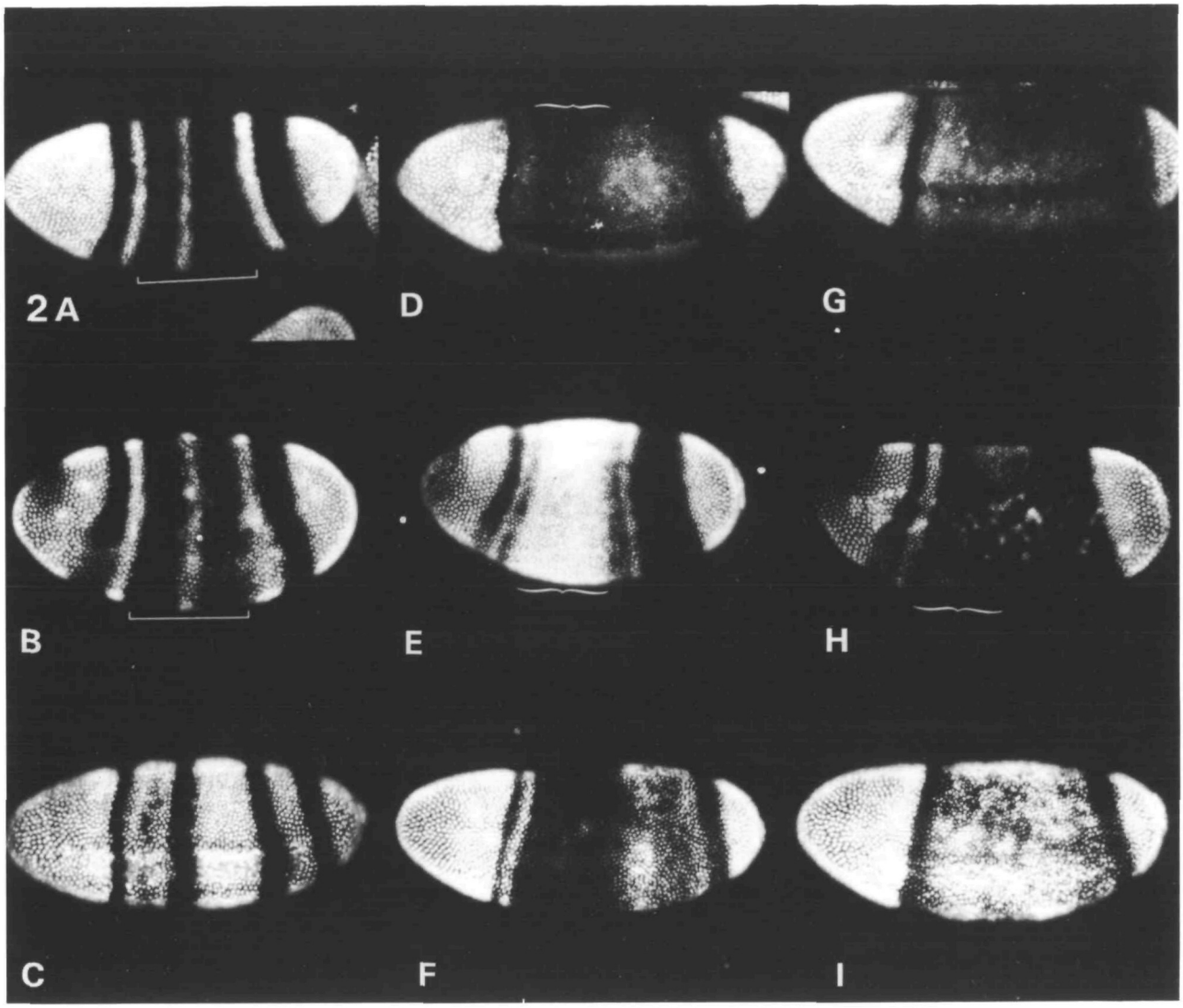


Fig. 2. Expression of the *eve*, *hairy*, and *ftz* pair-rule genes in embryos lacking one or more gap gene functions. Embryos that were mutant for the *Kr* (A–C); *hb*, *kni* (D–F); or *hb*, *kni*, and K_r (G–I) gap genes were stained with the *eve* (A,D,G), *hairy* (B,E,H), *ftz* (C,F,I) and *Kr* (D,G) antibodies. (A–C) Homozygous Kr^1 embryos. (A) *eve* protein expression expands into two large blocks in the central region of the embryo (large bracket). (B) *hairy* protein expression also expands into two large blocks in the central region of the embryo (large bracket), the level of protein accumulation within cells in those blocks is still lower than that found in cells of the anterior and posterior stripe. (C) *ftz* protein accumulation is largely complementary to the pattern of *hairy* expression in (B) above. (D–F) Homozygous hb^{7M48}, kni^{1ID48} embryos. (D) *eve* protein expression expands across most of the normally striped region of the embryo but is at a lower than normal level except at the edges of the pattern. This embryo was also labelled with *Kr* antibody (bracket) to distingish it from the embryo in (G). (E) *hairy* protein expression is lost from the middle of the embryo (bracket) but expands in the posterior striped region. (F) The *ftz* protein pattern is largely complementary to the *hairy* pattern in (E). (G–I) Homozygous Kr^1; hb^{7M48}, kni^{1ID48} embryos. (G) *eve* protein expression is very similar to that found in (D), this embryo did not label with *Kr* antibody and was therefore confirmed to be Kr^-. (H) *hairy* protein expression expands to include most of the normally striped region, the large area that was unlabelled in (E) is now expressing *hairy* (bracket). The expression is a bit weaker dorsally in the center of the broad domain. (I) *ftz* protein expression remains high at the edges of the normally striped domain but is weak and mottled between the terminal stripes.

Surprisingly, all three pair-rule genes are expressed in the triple gap mutant embryos despite the elimination of these key gap gene functions. (The *ftz* patterns in both the hb^-, kni^- embryos and the Kr^-; hb^-, kni^- embryos are complementary to the *hairy* patterns.) From this observation, we conclude that the Kr^+, hb^+, and kni^+ activities are not absolutely required to activate pair-rule gene expression. Rather, Kr^+ (and perhaps hb^+ and kni^+) appears to repress *hairy* expression, while hb^+ and kni^+ could be involved in both the spatial repression and the activation of *eve*.

Temporal aspects of the regulation of stripe formation

Even if one assumes that the regulatory circuit diagrams

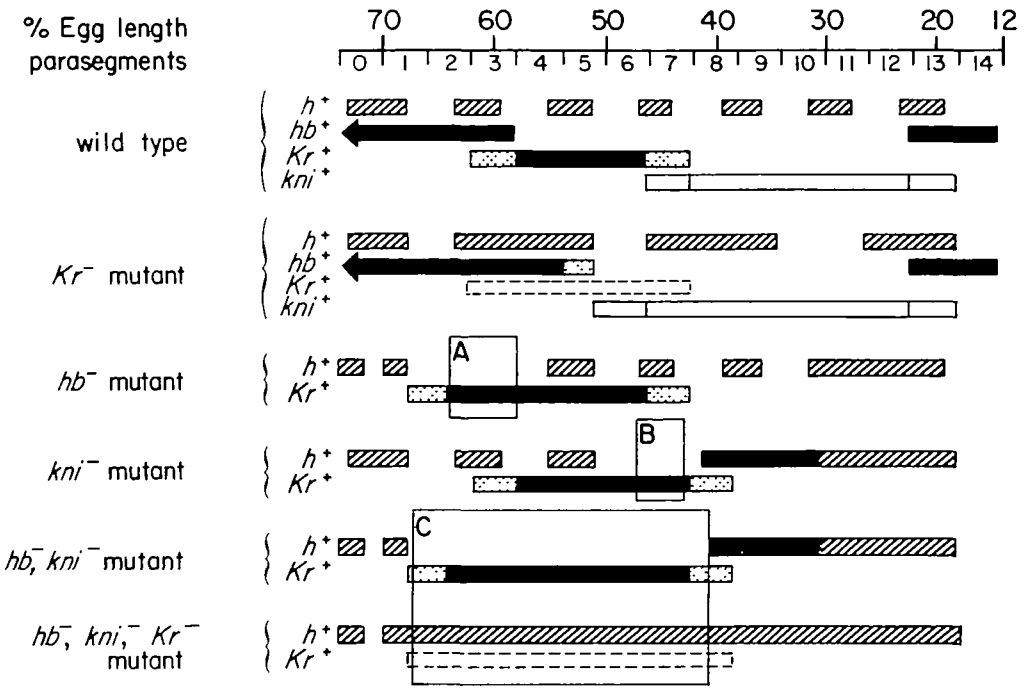


Fig. 3. The relationship between *Kr* and *hairy* gene expression in wild-type and gap mutant embryos. The positions of *hairy* stripes (striped shading) relative to gap gene domains of expression (solid shading) in various genotypes (left) are shown. There is some uncertainty as to the precise borders of gap gene expression, this is indicated by stippling. The *knirps* pattern of expression has not been reported, its presumed domain is indicated by an open box. The key relationships between *hairy* and Kr^+ expression are shown in the labelled boxes: box A, note that the expansion of Kr^+ in the hb^- embryo is accompanied by the loss of a *hairy* stripe within the new Kr^+ domain; box B, the expansion of Kr^+ in the kni^- embryo is accompanied by the loss of a *hairy* stripe in the new Kr^+ domain; and box C, the large gap in *hairy* expression in the hb^-, kni^- embryo correlates with an expanded Kr^+ domain and when Kr^+ is removed, *hairy* expression fills the formerly inactive region. Data measurements are from this work (variation ±1–2%) and from Carroll *et al.* (1988), Jäckle *et al.* (1986), Gaul and Jäckle (1987), and Akam (1987).

presented in the previous sections are correct, given the known spatial relationships between all of the segmentation genes discussed, it is still not possible to accurately predict the pattern of a particular pair-rule gene in a particular mutant embryo. Why, for instance, if $runt^+$ is a negative regulator of *hairy* doesn't *hairy* expression expand throughout a $runt^-$ embryo? Why is *eve* expression fairly normal initially in a $runt^-$ embryo? The clues to these questions lie in the asynchronous kinetics of stripe formation and in how different regulators may be present in different amounts at different stages during cycle 14.

At any given moment in the pair-rule stripe formation sequence, the degree of gap, pair-rule and autoregulatory input may vary. The best evidence for this variation is twofold. First, stripes do not form uniformly. As shown for the *ftz* (Karr and Kornberg, 1989) and *eve* proteins (Frasch *et al.* 1987) and *hairy* mRNA (Howard, 1988), the intensity and width of the pair-rule stripes changes dramatically during cycle 14. Second, the range of novel stripe phenotypes induced by injection of cycloheximide becomes more restricted as the embryo nears the cellular blastoderm stage (Edgar *et al.* 1986; 1989).

The asynchronous resolution of pair-rule stripes explains why the removal of an individual regulatory protein may not result in a predictable symmetrical change in the target gene pattern along the entire embryo. We can illustrate this point for the $runt^+$ – and eve^+–*hairy* interactions by comparing the early stages of *hairy* protein accumulation in a wild-type embryo with the eventual *hairy* protein pattern in $runt^-$ and eve^- embryos. The accumulation of *hairy* protein is not uniform across the cycle 14 embryo. The earliest stripe to appear is a broad first stripe (Fig. 4A), followed by a very broad stripe in the position of what will be the third and fourth stripes (Fig. 4B). The seventh, second, fifth and sixth stripes follow shortly afterward. The narrowing of the first stripe and the separation of the third and fourth stripes, which occurs shortly before the entire seven stripe pattern is completed (Fig. 4C) depends upon $runt^+$ since, as described earlier, the *hairy* pattern is not resolved in these regions in a $runt^-$ embryo (Fig. 4D). Similarly, the delayed appearance of the second *hairy* stripe may reflect the temporal requirement for the eve^+ product to act upon *hairy* (Fig. 1G). Taken together, the kinetics of wild-type stripe formation and the effects of the $runt^-$ and eve^- mutations indicate that there is a temporal sequence to stripe resolution and that $runt^+$ and eve^+ functions are downstream from the gap genes that initially refine the *hairy* pattern. These results explain the frequent lack of correspondence between the place where a regulatory gene is expressed and the effect of its removal on a

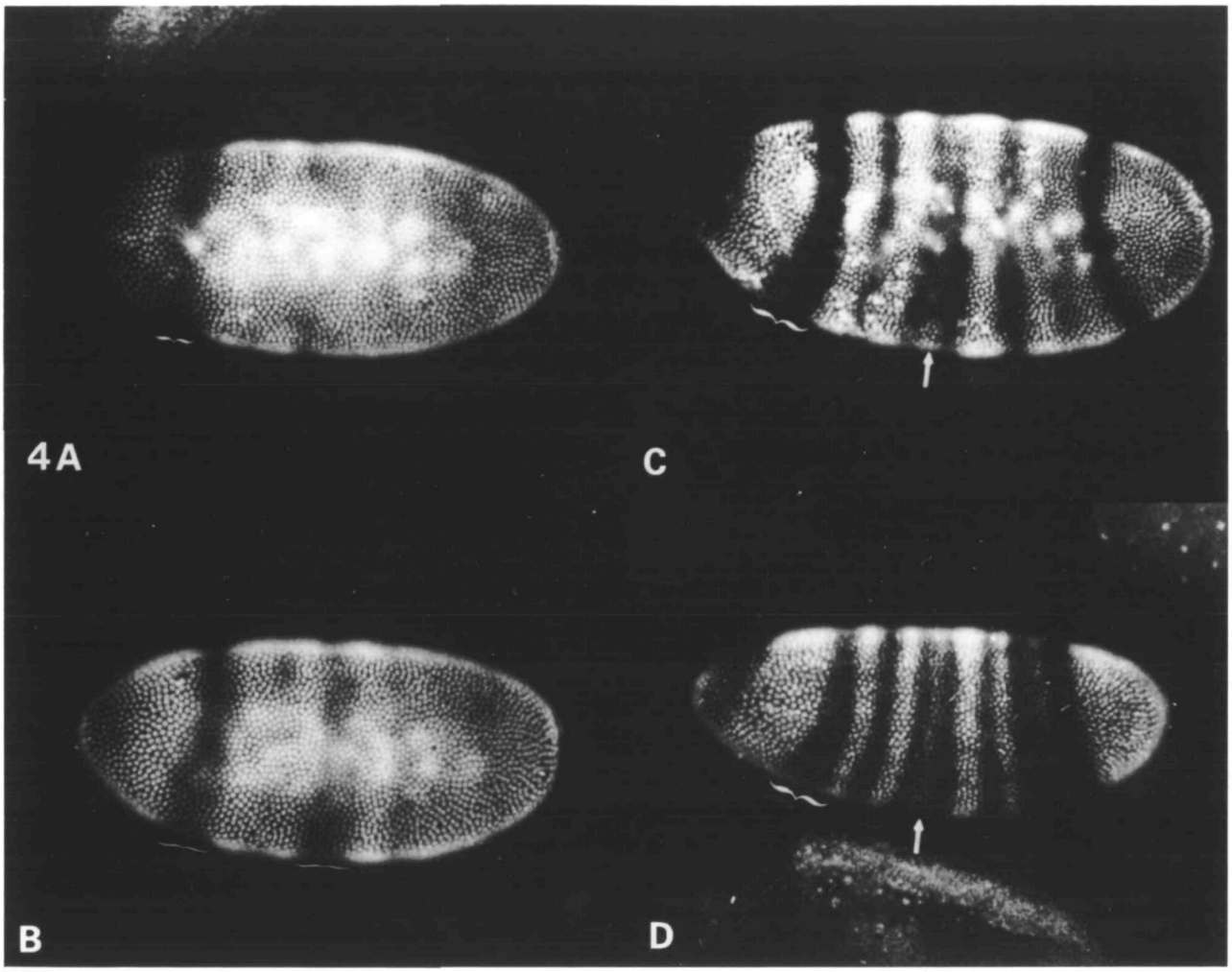


Fig. 4. The temporal sequence of *hairy* protein accumulation in normal and *runt*$^-$ embryos. (A–C) *hairy* protein expression during cycle 14. The initial visible stripe is a broad first stripe (A, bracket), followed by a broad third/fourth stripe (B, bracket). This last broad stripe begins to split before the second and sixth stripes are at full levels (C, arrow) and the first stripe narrows (C, bracket). (D) In a *runt*$^-$ embryo, the first stripe is abnormally broad (bracket) and the third and fourth stripes do not separate completely (arrow), as if the pattern arrests in the stage shown in (C).

target gene pattern. Even though each *hairy*$^+$ stripe is eventually overlapped by an *eve*$^+$ stripe and each interstripe contains a *runt*$^+$ stripe, the effects of these latter two genes are restricted to certain regions of the embryo by the preceding set of gap regulatory activities.

Discussion

From this analysis of pair-rule gene expression in known segmentation mutant embryos, we have drawn three main conclusions about the genes that establish the periodic patterns of pair-rule gene expression. First, several previously described pair-rule interactions are probably indirect, indicating that there are fewer *trans*-acting regulators of individual pair-rule genes than studies of single mutants may have suggested. Second, those pair-rule gene interactions that may be direct often involve repression of other pair-rule genes. Finally, the three key gap genes *hb*, *Kr* and *kni* are not essential to activate genes such as *hairy*; on the contrary, when expressed alone in a cell, *Kr*$^+$ appears to repress *hairy* expression. These observations have several implications for understanding how pair-rule genes are regulated by *trans*-acting factors.

Regulation of pair-rule genes by known zygotic segmentation genes: Pair-rule gene interactions

We have described the logic behind the epistasis tests of pair-rule regulatory interactions. Our interpretations support the conclusions of Ingham and Gergen (1988) and extend those of several previous studies (Howard and Ingham, 1986; Carroll and Scott, 1986; Ingham *et al.* 1986; Frasch and Levine, 1987) that have dissected the pair-rule regulatory circuit. All evidence suggests that most interactions involve negative regulation with *hairy*$^+$ acting as a negative regulator of *ftz* (Howard and Ingham, 1986; Carroll and Scott, 1986; Ish-Horowicz

and Pinchin, 1987) and *runt* (Ingham and Gergen, 1988), *runt*$^+$ as a negative regulator of *eve* (Frasch and Levine, 1987) and *hairy* (Ingham and Gergen, 1988), and *eve*$^+$ as both an early activator of *hairy*, *ftz* and *runt*, and a late repressor of *ftz* and *runt* (Ingham and Gergen, 1988). The net regulatory effects of each gene are not equivalent. Based upon the severity of the gene expression pattern perturbations in each mutant, *hairy*$^+$ appears to be a stronger or earlier-acting repressor of *runt* and *ftz* than *runt*$^+$ is of *hairy* and *eve*.

Based upon double mutant patterns, we also conclude that the requirements for *hairy* in *eve* regulation (Frasch and Levine, 1987) and *runt* in *ftz* regulation (Carroll and Scott, 1986; Frasch and Levine, 1987) are probably indirect and are mediated *via runt* and *hairy* in these two cases, respectively.

Gap gene regulation of pair-rule gene expression: the evidence for spatial repression

The most important conclusion drawn from our analysis of *hairy* gene expression in different gap mutant combinations is that some gap genes, particularly the *Kr*$^+$ product, repress *hairy* expression. Three pieces of evidence support this claim. First, *hairy* expression in a *runt*$^-$; *eve*$^-$ embryo shows several gaps in the pattern, and since *runt* and *eve* are the only known pair-rule regulators of *hairy*, the gap genes must be responsible for the remaining spatial restriction of the *hairy* pattern (Fig. 1J). Second, in the absence of *hb*$^+$ and/or *kni*$^+$, loss of *hairy* expression occurs in the region where the *Kr*$^+$ domain expands (Fig. 2E and Fig. 3). Finally, removing *Kr*$^+$ along with *hb*$^+$ and *kni*$^+$ derepresses *hairy* expression over the posterior two-thirds of the embryo (Fig. 2H); that is, in the absence of these three gap genes, the *hairy* pattern is nearly uniform and the gene is strongly active. We do not believe that these interactions are indirect (for example, mediated *via* the *runt* gene) because of the close correspondence of ectopic *Kr*$^+$ expression with *hairy* repression and because the *hairy* pattern is strongly modulated even in the absence of *runt*$^+$ and *eve*$^+$ (Fig. 1J). These results are significant because they challenge some current notions about pair-rule gene regulation that emphasize transcriptional activation by gap genes.

Do gap genes activate pair-rule stripes?

There are two different sets of experiments that support a gap-gene-driven region-specific activation mechanism for pair-rule genes. The first involves the characterization of a series of alleles of the *hairy* gene that express only a subset of the normal seven *hairy* blastoderm stripes (Howard *et al.* 1988). Four alleles were described that consisted of progressively larger deletions of the 5′ DNA flanking the *hairy* promoter. As the amount of 5′ flanking sequence was reduced, certain *hairy* stripes disappeared from the spatial mRNA pattern. Apparently, the lost *cis*-acting elements are required for the expression of various stripes in specific regions of the embryo and respond to regionalized cues in the blastoderm nuclei. The simplest explanation would be that gap proteins acted upon these elements to activate the different *hairy* stripes. The second set of experiments involve demonstrations that certain elements of the *eve* stripe pattern can be generated by placing 5′ flanking DNA of the *eve* gene upstream of the B-galactosidase reporter gene, and that different deletions within this DNA result in deletion of different *eve*-Bgal stripes (Harding *et al.* 1989; Gato *et al.* 1989). These studies suggest that different 5′ elements respond to regional cues that activate *eve* transcription.

Or do gap genes repress pair-rule interstripes?

It is also possible that gap genes could modulate pair-rule gene expression by transcriptional repression. In this model formulated by B. Edgar and G. Odell (personal communication), there are two possible modes for repression to operate. In *combinatorial* repression, two different gap gene products work together to repress pair-rule transcription, while in *competitive* repression individual gap gene products act as repressors but their repressive effects are offset by competition from those gap genes that overlap them. Each gap gene domain then has a peak of repression that is narrower than the whole domain.

One set of experiments that led to this model involved cycloheximide injection into blastoderm embryos, which demonstrated that the polar and periodic repression of *ftz* (Edgar *et al.* 1986), *hairy*, *eve* and *runt* (Edgar *et al.* 1989) mRNA expression could be blocked by inhibiting protein synthesis, as was the turnover of their normally very short-lived mRNAs. This indicated that the short-lived regulators of pair-rule genes might be repressors and not necessarily activators of transcription. The combination of spatial repression and rapid RNA degradation is still a relatively simple explanation for the seven-stripes pattern, but in the case of the *hairy*-regulated *ftz* gene, this appears to be the central mode of its spatial regulation.

Our new data support a gap gene repression mechanism for *hairy* stripes over a regional activation mechanism because of three key observations. First, because *hairy* expression expands at high levels across a *hb*$^-$, *kni*$^-$; *Kr*$^-$ embryo, the idea that these genes are essential activators of *hairy* expression must be incorrect. Second, *Kr*$^+$ expression in the absence of *hb*$^+$ and/or *kni*$^+$ is always associated with repression of *hairy* expression. And third, the restriction of the *hairy* pattern in an embryo lacking the specific *hairy* regulatory genes *eve*$^+$ and *runt*$^+$ reflects spatial repression in several regions of the embryo that must be due to gap genes. We interpret the pattern (Fig. 1J) of *hairy* expression in the middle of a *runt*$^-$/*eve*$^-$ embryo as suggesting competitive repression between *hb*$^+$ and *Kr*$^+$, and *kni*$^+$ and *Kr*$^+$. This is because the stripes that do form (a weak third and fourth) appear to be positioned near the edges of the *Kr* domain, overlapping with the *hb*$^+$ domain on the anterior edge and the presumed *kni*$^+$ domain on the posterior edge and separated by an interstripe towards the center of the *Kr* domain. Not all individual region-specific regulators necessarily act negatively upon *hairy*, the function of *eve*$^+$ may involve activation of the second *hairy* stripe.

If spatial repression by gap genes is occurring, how does one explain the patterns of the 5′ *cis*-acting mutants of *hairy* (Howard *et al.* 1988) or of the *eve-Bgal* gene fusions (Gato *et al.* 1989; Harding *et al.* 1989)? In the case of the *hairy cis*-acting deletion mutants, we can offer an alterative explanation for the loss of *hairy* stripes besides the lack of positive regulation. In h^{m3}/h^{m3} embryos, for example, the third and fourth *hairy* stripes, which form over the normal *Kr* domain, are missing. While this might be due to the deletion of a Kr^{+}-driven enhancer element, the lack of expression could also be due to the loss of gap regulatory elements that would modulate *Kr* repression. That is, perhaps the h^{m3}/h^{m3} embryos lack stripes three and four because of *Kr* repression, not because of a failure to activate these stripes.

We can find evidence for similar dual gap gene control of *eve* stripes/interstripes in the experiments described by Gato *et al.* (1989) (see Fig. 5 therein) in that when these authors examined the pattern of the relevant *eve-Bgal* stripes in hb^{-}, Kr^{-}, or even gt^{-} mutants, no stripe disappeared, the patterns merely shifted. If a given stripe required activation by a single gap gene, then removing that gene should delete the stripe. This was not observed. At present, we cannot tell which genes repress or activate *eve* expression. The spreading of eve^{+} in hb^{-}, kni^{-} embryos suggests some level of spatial repression but the accompanying lower level of expression may suggest a requirement for hb^{+} and kni^{+} in *eve* activation. We believe that the very different effects of the hb^{-}, kni^{-} and triple gap mutant backgrounds on *eve* and *hairy*, and the demonstration that *eve* is auto-regulated (Frasch *et al.* 1988; Harding *et al.* 1989; Gato *et al.* 1989), provide evidence that the regulatory wiring of *hairy* and *eve* may not be quite so similar as their spatial overlap may have suggested.

In order to determine how *hairy* and *eve* are regulated, it will be crucial to determine whether gap proteins interact with sequences upstream of the pair-rule genes and if there are any functional associations between the gap proteins or their target sites. To understand the entire program of pair-rule gene regulation, the regulatory effect of each *trans*-acting protein will need to be determined in the context of the other regulatory proteins that may function competitively or combinatorially on the different pair-rule genes.

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