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Pair-rule expression of the *Drosophila fushi tarazu* gene: a nuclear receptor response element mediates the opposing regulatory effects of *runt* and *hairy*

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SUMMARY

The segmentation genes *runt* and *hairy* are required for the proper transcriptional regulation of the pair-rule gene *fushi tarazu* during the blastoderm stage of *Drosophila* embryogenesis. The expression of different *fushi tarazu* reporter genes was examined in *runt* and *hairy* mutant embryos, as well as in *runt* over-expressing embryos in order to identify DNA elements responsible for mediating these regulatory effects. The results indicated that *runt* and *hairy* act through a common 32 base-pair element. This element, designated as fDE1, contains a binding site for a small family of orphan nuclear receptor proteins that are uniformly expressed in blastoderm embryos. The pair-rule expression of reporter gene constructs containing multimerized fDE1 elements depends on activation by *runt* and repression by *hairy*. Examination of reporter genes with mutated fDE1 elements provided further evidence that this element mediates both transcriptional activation and repression. Genetic experiments indicated that the opposing effects of *runt* and *hairy* were not due solely to cross-regulatory interactions between these two genes and that fDE1-dependent expression is regulated by factors in addition to *runt* and *hairy*.

Key words: *Drosophila*, FTZ-F1, DHR39, *fushi tarazu*, *hairy*, *runt*

INTRODUCTION

Generation of the expression patterns of the *Drosophila* pair-rule segmentation genes is one of the most remarkable displays of the on/off regulation of gene expression in developmental biology. The *fushi tarazu* (*ftz*) gene provides a well-studied, yet not understood, example of this regulation. This gene is expressed in a seven-striped, pair-rule pattern along the anterior-posterior axis during the blastoderm stage of embryogenesis (Hafen et al., 1984; Weir and Kornberg, 1985). At their initial peak levels of expression, these stripes are approximately 4 cells wide and are separated by 4-cell-wide interstripes where there is little to no expression. Studies on the *ftz cis*-regulatory region demonstrate that transcriptional regulation is central to the generation of this pattern. The initial pair-rule expression of *ftz* is mediated by the zebra element (Hiromi and Gehring, 1987). This element, which extends from –699 to +73 basepairs relative to the transcription start site, is composed of multiple smaller elements (Fig. 1). These elements confer either activation, repression or, in some cases, both activation and repression (Dearolf et al., 1989a; Topol et al., 1991). Several transcriptional regulators that interact with the zebra element have been identified. Two examples of proteins that are thought to activate *ftz* transcription are the homeodomain protein CAUDAL (CAD) and the GAGA factor (Dearolf et al., 1989b; Topol et al., 1991). An example of a repressor is TRAMTRACK (TTK, also called FTZ-F2). TTK binds to sites that are required for repressing precocious transcription during the nuclear division cycles that precede the cellular blastoderm stage (Brown et al., 1991). Two other intriguing proteins that interact with sites in the *ftz* zebra element are FTZ-F1 and DHR39 (also called FTZ-F1β), two related proteins that are orphan members of the nuclear receptor superfamily (Ueda et al., 1990; Ohno and Petkovich, 1992; Ayer et al., 1993). These two proteins bind sites that are referred to as fDE (*ftz* dual element) elements because they are associated with both activation and repression (Dearolf et al., 1989a; Topol et al., 1991). However, mutagenesis of the high-affinity FTZ-F1-binding site in the fDE1 element was reported to affect activation but not repression of *ftz* transcription (Ueda et al., 1990).

Despite these studies, no clear picture of how the zebra element generates a periodic expression pattern has emerged. This lack of understanding is particularly surprising given the regulatory candidates identified by genetic analysis of segmentation. Two strong candidates for direct regulators of *ftz* are the pair-rule genes *hairy* and *runt*. Both genes encode nuclear proteins that are likely to participate directly in transcriptional regulation. HAIRY is a member of the basic helix-loop-helix (bHLH) family of transcriptional regulators (Rushlow et al., 1989), and RUNT contains a DNA-binding and protein dimerization motif referred to as the Runt-domain (Kagoshima et al., 1993). The pair-rule stripes of *runt* and *hairy* are precisely complementary to each other and shifted in phase relative to those of *ftz*. At the mid-blastoderm stage, the *ftz* stripes overlap with the posterior half of the *runt* stripes

and the anterior half of each juxtaposed *hairy* stripe (Kania et al., 1990). As cellularization proceeds and the stripes narrow from 4 to 2 cells wide, *ftz* expression is extinguished in the posterior half (the *hairy* expressing half) of each stripe. These phasings provide an indication of *runt*'s positive role, and *hairy*'s negative role on *ftz* expression. These opposing regulatory effects are confirmed by analysis of mutants. Embryos that lack *runt* show reduced expression and premature elimination of *ftz* stripes, whereas mutations in *hairy* lead to a broadening of *ftz* stripes during these same stages (Carroll and Scott, 1986; Howard and Ingham, 1986; Ingham and Gergen, 1988).

This paper investigates the regulation of *ftz* transcription by *runt* and *hairy*. We examined the responses of a collection of *ftz/lacZ* reporter genes in *runt* and *hairy* mutant embryos as well as in embryos that overexpress *runt* in order to identify DNA elements that mediate regulation by these pair-rule genes. We found that activation by *runt* as well as repression by *hairy* are mediated through the 32-base pair fDE1 sequence element that was previously identified as the site of action of the FTZ-F1 and DHR39 orphan nuclear receptor proteins. Genetic evidence indicates that these opposing effects are independent of the cross-regulatory interactions between *runt* and *hairy*, and further demonstrates that other spatially restricted factors also contribute to the regulation of this element.

MATERIALS AND METHODS

The *hs/runt Drosophila* strain as well as the methods for embryo manipulation and in situ hybridization are described by Tsai and Gergen (1994). The standard *hs/runt* treatment included a 20-minute heat shock at 37°C, followed by a 20-30 minute recovery period prior to processing for in situ hybridization. Three different *hs/runt* lines were tested, all gave similar results. The parental strain for the establishment of the *hs/runt* line, *y* w^{67c23} was used as the wild-type control for the effects of heat-shock treatment. The segmentation mutations used were: $runt^{LB5}$, *Df (2R)* $eve^{1.27}$, $hairy^{K1}$, $hairy^{i22}$ and $ftz^{w20.}$ These mutations produce no mRNA, or produce mRNA with expression characteristics that allow unambiguous determination of embryo genotypes by in situ hybridization. The sources of the reporter gene constructs were : for *ftz/lacC*, *ftz/lacA*, *UPHZ* (Hiromi and Gehring, 1987); for *3′pstXA*, *89-2B*, *5′16* (Dearolf et al., 1989a); for *5-5my* (Ueda et al., 1990); for *3′oligoX*, *3′fDE1*, *5′fDE1*, *5′oligoX* (Topol et al. 1991).

RESULTS

Regulation of the *ftz* pair-rule pattern by *runt* and *hairy*

The *ftz* mRNA expression pattern undergoes a series of dynamic alterations during the blastoderm stage of *Drosophila* embryogenesis (Hafen et al., 1984; Weir and Kornberg, 1985).

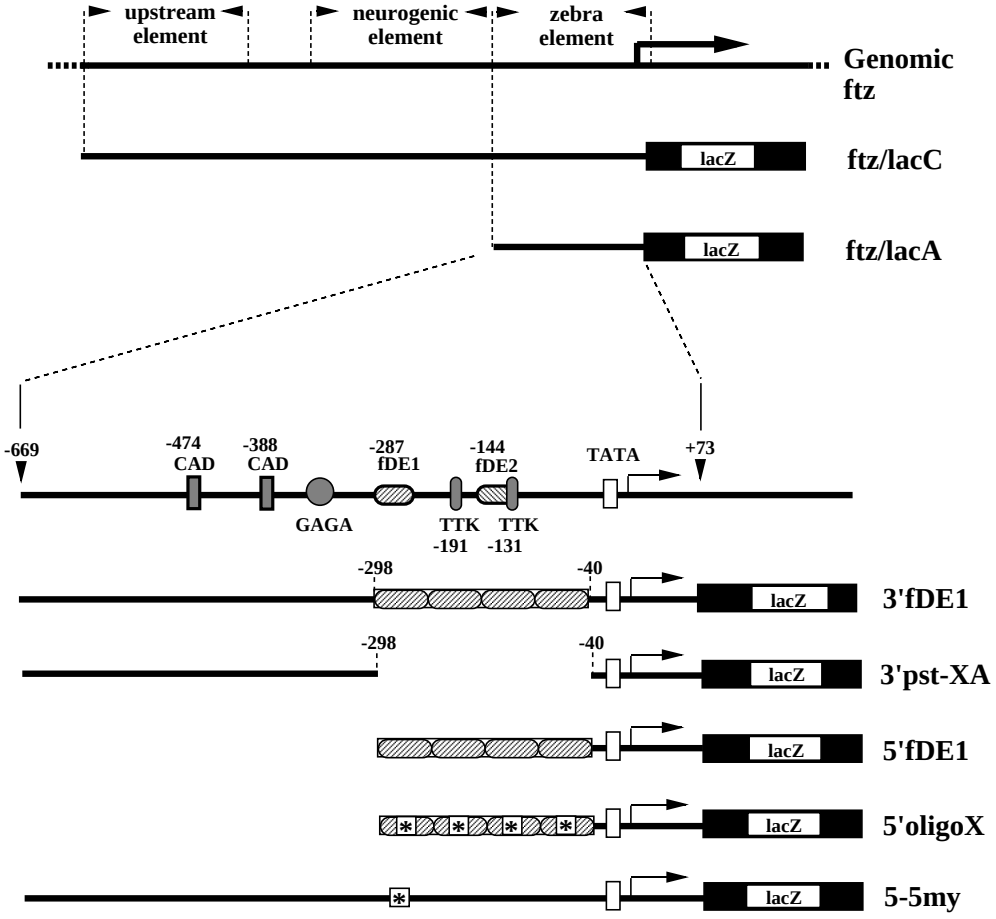

Fig. 1. The *ftz cis*-regulatory region. The *ftz* gene is represented by the solid line at the top. The arrow represents the beginning of the transcription unit. The relative positions of the three major upstream *cis*-regulatory elements are indicated. The extent of upstream DNA contained within the *ftz/lacC* and *ftz/lacA* reporters is shown below this with the *lacZ* gene indicated as a box. The bottom part of the figure shows an expanded map of the *ftz* zebra element and several other reporter gene constructs. Solid horizontal lines represent *ftz* regulatory sequences. The positions of binding sites for the CAD, GAGA and TTK factors are indicated by shaded rectangles, circle and ovals, respectively. The two fDE elements that contain binding sites for the related FTZ-F1 and DHR39 proteins are indicated by striped ovals. Stars indicate mutant fDE1 elements. The 32 base-pair sequence that comprises the fDE1 element is as follows: GCACCGTCTCA*AGGTC*GCCGAGTAGGAGAAGC. In the *5′oligoX* reporter gene, the 15 underlined nucleotides in this fDE1 sequence are replaced with: CTCAACCCTAACGGT. In the *5-5my* reporter gene, the 5 nucleotides in this fDE1 sequence that are italicized are replaced with the sequence CTGCA. All of these reporter gene constructs utilize the *ftz* TATA box region (hollow rectangle) and basal promoter region extending from −44 to +73. For details on the construction of these reporter gene constructs see Hiromi and Gehring (1987), Dearolf et al. (1989), Topol et al. (1991) and Ueda et al. (1990).

In wild-type embryos that have just completed the 13th nuclear division, two broad domains of expression emerge over the regions where the 2nd and 5th pair-rule stripes will form. As the nuclei elongate, this pattern evolves first into a four-banded and ultimately into a seven-striped pattern. The stripes are all approximately equal in intensity by the time the nuclei are fully elongated and separated by the invaginating plasma membrane (Fig. 2A). At this stage, the first six stripes are 4 cells wide and the seventh stripe is broader. As cellularization proceeds the *ftz* stripes narrow. Expression in the posterior half of each stripe is extinguished, resulting in stripes that are 2 cells wide by the completion of cellularization.

In *runt* mutants, alterations in *ftz* expression are apparent by the time the seven-striped pattern emerges. The initial difference is reduced intensity of stripe 3. The expression of this stripe as well as stripes 1 and 6 becomes greatly reduced as cellularization proceeds (Fig. 2B). These effects are more pronounced in the ventral regions of the embryo. By the completion of cellularization only stripes 4 and 7 remain. The repressive effects of *hairy* also become apparent at the mid-blastoderm stage. In *hairy* mutants, the stripes form normally, but then broaden as cellularization proceeds. These expanded stripes retract around the end of cellularization, leaving a set of weak, incomplete stripes in what would normally correspond to the *ftz* interstripe regions (Fig. 2D). These ectopic interstripes continue to be expressed during germband extension while the original *ftz* stripes narrow to a width that is only slightly broader than in wild type (not shown).

Ectopic expression experiments further establish the opposing regulatory effects of *runt* and *hairy* on *ftz*. Ish-Horowicz and Pinchin (1987) found that expression of *hairy* under the control of heat-inducible *Drosophila* hsp70 promoter completely abolished the expression of *ftz*. We generated *Drosophila* lines containing a similarly heat-inducible *hs/runt* transgene in order to further investigate *runt*'s mechanism of action (Tsai and Gergen, 1994). Transient heat-shock treatment induces uniform accumulation of Runt in all somatic cells of *hs/runt* transformants (Tsai and Gergen, 1994). This in turn leads to activation of *ftz* ; the pattern depends on the stage of the embryo and the severity of the heat shock. Short heat-shock treatment during the early blastoderm stage leads to stable, broadened stripes. More extreme treatment causes *ftz* to be expressed in a broad band that extends from 15% to 65% egg length (Fig. 2C, 0% egg length is the posterior pole). Interestingly, *hs/runt* treatment does not lead to significant *ftz* expression outside this region. This may indicate the existence of spatially restricted repressors of *ftz* that prevent activation by the uniformly expressed *hs/runt* transgene. Alternatively, this may reflect the localized expression of other factors that are necessary for the *runt*-dependent activation.

Reporter genes containing *ftz cis*-regulatory elements are

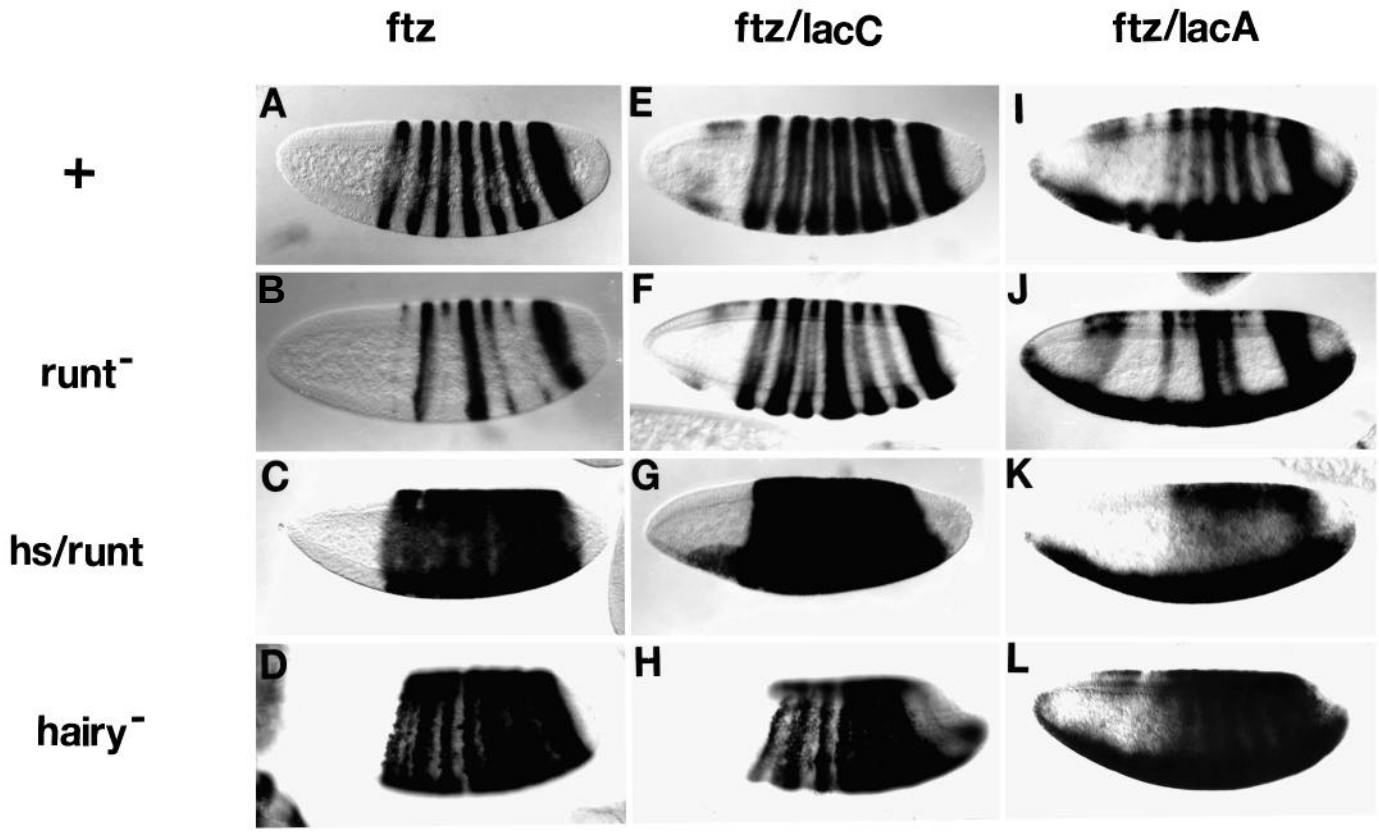

Fig. 2. Regulation of the *ftz* zebra pattern by *runt* and *hairy*. The expression of the normal *ftz* mRNA is shown in A-D. Embryos carrying the *ftz/lacC* or *ftz/lacA* transgenes are shown in E-H and I-L, respectively. (A,E,I) The wild-type patterns; (B,F,J) *runt* mutants; (C,G,K) *hs/runt* embryos; (D,H,L) *hairy* mutants. The embryos were hybridized with antisense RNA probes that detect *ftz* or *lacZ* mRNAs. *runt* and *hairy* mutant embryos were identified by in situ hybridization with antisense RNA probes. In all views, anterior is to the left and the dorsal side is on top. All the embryos shown are at the late blastoderm stage.

also regulated by *runt* and *hairy*. The *ftz/lacC* reporter gene contains all three of the major *cis*-elements that have been described for *ftz* (the upstream enhancer, the neurogenic and the zebra elements), whereas the related *ftz/lacA* gene contains only the zebra element (Hiromi et al., 1985, see Fig. 1). Both of these genes express *lacZ* mRNA in a seven-striped pattern in wild-type mid-blastoderm-stage embryos (Fig. 2E,I). Expression of both genes is reduced in *runt* mutant embryos (Fig. 2F,J). Significantly, both reporter genes are also overexpressed in *hs/runt* embryos (Fig. 2G,K) and in *hairy* mutant embryos (Fig. 2H,L). These results demonstrate that *runt* and *hairy* regulate *ftz* transcription. They further show that DNA elements that mediate these opposing regulatory effects are contained within the zebra element.

Although the overall expression of the endogenous *ftz* gene and these reporter genes are similar, there are notable differences. Some of these may be due to differences in mRNA stability. For example, the increased stability of *lacZ* mRNA relative to *ftz* mRNA probably contributes to the reduced efficiency of interstripe repression of the reporter genes (Fig. 2A,E,I). A similar explanation may account for the relative perdurance of the seven-stripe pattern of *ftz/lacC* in *runt* mutant embryos (Fig. 2F). However, differences in mRNA stability cannot explain differences in the expression of the two reporter genes. One intriguing difference is the interstripe repression of *ftz/lacC* in *hairy* mutant embryos (Fig. 2H). This is not observed with *ftz/lacA* (Fig. 2L). Thus elements that are within *ftz/lacC* but that lie outside of the zebra element contribute to interstripe repression in a manner that is independent of the function of *hairy*. A second intriguing finding is the relative weakness of stripe 1 in the *ftz/lacA* pattern (Fig. 2I). This suggests that elements required for activation of this stripe lie outside of the zebra element. Although these observations indicate that previous views of *ftz* regulation are somewhat oversimplified, they do not alter the important conclusion that the zebra element responds to the opposing regulatory effects of *runt* and *hairy*.

The *ftz* fDE1 element mediates regulation by both *runt* and *hairy*

To further investigate the regulation of the *ftz* zebra element, we examined the expression of several modified zebra element reporter gene constructs in various mutant backgrounds. The results of these experiments lead us to focus on a previously described element within the *ftz* zebra element, the fDE1 element. A clear example of the importance of this element is provided by two related constructs, *3′pstXA* and *3′fDE1*. *3′pstXA* is a derivative of *ftz/lacA* in which the proximal half of the zebra element, including the fDE1 element is removed (Dearolf et al., 1989a, see Fig. 1). In early blastoderm embryos *3′pstXA* is expressed in a broad contiguous band throughout most of the presegmental region, excluding the region where stripe 1 should form. This expression is stronger in ventral regions. During the process of cellularization two broad 'stripes' become superimposed on this pattern (Fig. 3A). Based on their positions, we interpret the anterior stripe as a fusion of stripes 4 and 5, and the posterior stripe as stripe 7. The stripe 4/5 fusion does not split to form distinct stripes and the rest of the *3′pstXA* pattern never shows evidence of pair-rule periodicity.

In contrast, the related reporter gene *3′fDE1* is expressed in a pair-rule pattern (Fig. 3B). The *3′fDE1* construct is identical to *3′pstXA* except that it also contains four copies of the 32 base-pair fDE1 element (Topol et al., 1991; see Fig. 1). The addition of these fDE1 elements appears to impose both repression and activation upon the 'basal' pattern generated by *3′pstXA*. An especially notable example of fDE1-dependent repression is the splitting of stripes 4 and 5 for *3′fDE1* (Fig. 3B) but not for *3′pstXA* (Fig. 3A). Previous studies by Topol et al. (1991) found that *3′fDE1* expression was repressed by *hairy*. These previous studies assayed accumulation of β-galactosidase protein and detected loss of interstripe repression in germband-extended embryos. We examined the response of *3′fDE1* to *hairy* using the more sensitive in situ hybridization procedure. Fusions between stripes 2 and 3, stripes 4 and 5, and stripes 6 and 7 are observed in blastoderm-stage embryos (Fig. 3E). In contrast, there is no difference in *3′pstXA* expression in wild type versus *hairy* mutants (not shown). These findings confirm and extend previous observations and lead to two important conclusions: (1) the fDE1 element is required for repression in interstripe regions, and (2) this repression depends on the function of *hairy*.

Activation mediated through the fDE1 element also contributes to the pair-rule pattern of the *3′fDE1* reporter gene. In wild-type embryos, the expression of *3′fDE1* stripes 2, 3 and 6 is stronger than in the comparable regions of the *3′pstXA* pattern (Fig. 3A,B). Analysis of the effects of *runt* further demonstrates that the fDE1 elements are involved in activation. Elimination of *runt* has no discernible effect on the expression of *3′pstXA* (not shown). In contrast, stripes 2, 3 and 6 of *3′fDE1* are eliminated in *runt* mutant embryos (Fig. 3C). Further, *hs/runt* has no effect on *3′pstXA* expression (not shown), whereas *3′fDE1* is activated in a manner similar to *ftz/lacA* (Fig. 3D, compare with Fig. 2K). Based on these observations we conclude that (1) the fDE1 elements contribute to transcriptional activation and (2) this activation is responsive to regulation by *runt*.

The above results point to the importance of the fDE1 element, but do not eliminate the possibility that other sequences in the *ftz* zebra element are required for mediating the regulatory effects of *runt* and *hairy*. To address this, we examined the expression of a simpler reporter gene, *5′fDE1*, which contains four fDE1 elements upstream of the *ftz* basal promoter (Fig. 1). This construct is expressed at lower levels than either *ftz/lacA* or *3′fDE1*. It is initially expressed in a weak contiguous band that is detected only in ventral regions. This resolves into a pair-rule pattern that, although weak, is usually apparent by the completion of cellularization (Fig. 3G).

5′fDE1 expression is regulated by *runt* and *hairy*. There is little expression in *runt* mutant embryos (Fig. 3H). Conversely, overexpression of *runt* induces the fusion of *5′fDE1* stripes in late blastoderm-stage embryos (Fig. 3I). Finally, in *hairy* mutants, there is a fusion of stripes 2 and 3, stripes 4 and 5, and stripes 6 and 7 (Fig. 3J). As a control for the above experiments, we examined the expression of a related reporter gene, *5′oligoX* (Fig. 1). This construct is identical to *5′fDE1*, except that the fDE1 elements are mutated (Topol et al., 1991). This construct is only sporadically expressed in wild-type embryos (Fig. 3F). This expression is not detectably enhanced in *hairy* mutants or in *hs/runt* embryos. These results with *5′fDE1* and

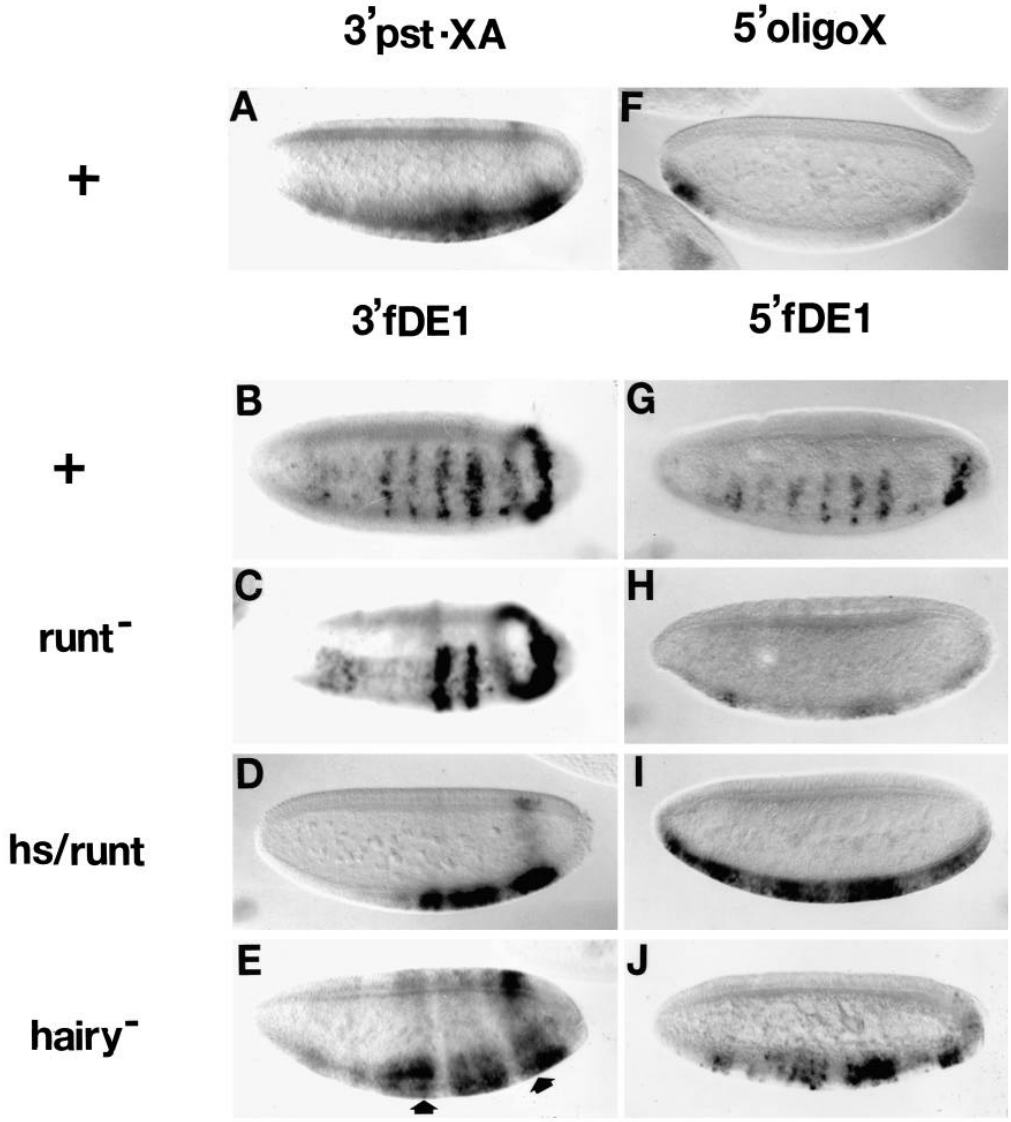

Fig. 3. The *ftz* fDE1 element mediates regulation by both *runt* and *hairy*. The top two embryos show expression of the *3′pstXA* (A) and *5′oligoX* (F) reporter genes in wild-type embryos. The other embryos on the left (B-E) carry the *3′fDE1* transgene, and the other embryos on the right (G-J) contain the *5′fDE1* transgene. (B,G) Expression of these two reporters in wild-type; (C,H) *runt* mutant embryos; (D,I) *hs/runt* embryos; (E,J) *hairy* mutant embryos. The arrows in E indicate the regions where stripes 3 and 6 should be. These are two regions where the differences between the *hairy* mutant and *runt*, *hairy* double mutant (Fig. 4B) patterns are most obvious. The embryos in B,C and G are rotated to show a ventrolateral aspect. This provides a clearer view of the expression pattern which is stronger in the ventral regions. This preferential ventral expression is observed with all of the *ftz/lacZ* reporter genes used in this study (e.g. Fig. 2I). The reason for this is not understood, but similar phenomena are also observed with other *lacZ* reporter genes generated in *rosy* P-element transformation vectors (Harding et al., 1989).

5′oligoX confirm the findings with *3′fDE1* and *3′pstXA*. They demonstrate that the fDE1 element is responsible for activation and repression, and that these responses depend on the activities of *runt* and *hairy*, respectively.

The fDE1 element responds to other regulatory cues

Several aspects of the *3′fDE1* and *5′fDE1* expression patterns suggest that the fDE1 element responds to other regulatory cues in addition to *runt* and *hairy*. Initial evidence comes from the phasing of the fDE1-dependent stripes. In late blastoderm-stage embryos, the *3′fDE1* stripes lie within the posterior half of the *runt* stripes. This is similar to the normal phasing of the intact *ftz* gene, and is consistent with the positive role that *runt* has on fDE1-dependent expression. However, this does not explain why *3′fDE1* is not expressed in the anterior halves of the *runt* stripes. Repression in these regions is not due to *hairy*, because *hairy* is not expressed in these cells (Kania et al., 1990). This suggests either that there is another repressor that is expressed in the anterior half of the *runt* stripes, or conversely that activation requires another positive factor whose activity is limited to the posterior half of the *runt* stripes.

The *3′fDE1* pattern in embryos that lack both *runt* and *hairy* provides further evidence that the fDE1 element responds to other factors. These double mutant embryos exhibit a better pattern than is observed in either single mutant. In *runt* single mutants, the only significant expression is in stripes 4, 5 and 7 (Fig. 3C). Removal of *hairy* now leads to expression in stripes 2, 3 and 6 (Fig. 4B). Thus, there must be factors in addition to *runt* that activate *3′fDE1* expression in these stripes. From the viewpoint of repression, the fusion between stripes 2 and 3, stripes 4 and 5 and stripes 6 and 7 is less severe and the intensity of stripes 3 and 6 is lower in the double mutant embryos (Fig. 4B) than in *hairy* single mutant embryos (Fig. 3E), indicating there are factors in addition to *hairy* that repress *3′fDE1* expression.

The novel patterns observed in these double mutant embryos further show that the cross-regulatory interactions between *runt* and *hairy* cannot fully account for the regulatory effects of these genes on *ftz* expression. If the only role of *runt* was to prevent repression by *hairy*, then in embryos that lack *hairy* the presence or absence of *runt* would have no effect. Similarly, if the only role of *hairy* was to block activation by *runt*, then the patterns in *runt* single mutants and *runt*, *hairy*

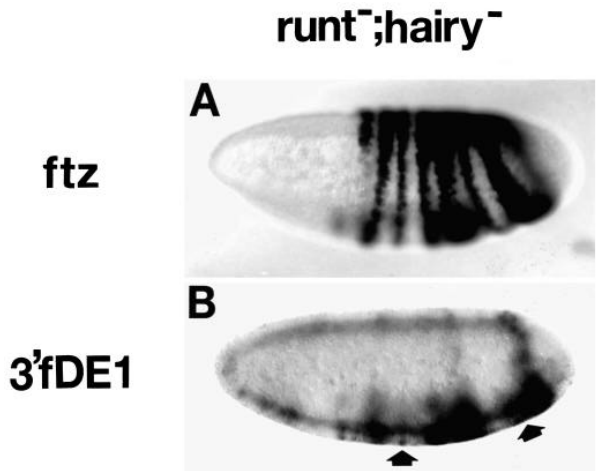

Fig. 4. The fDE1 element receives other periodic cues. (A,B) The expression of *ftz* and *3′fDE1* in embryos that are mutant for both *runt* and *hairy*. The *ftz* pattern is to be compared with that observed in embryos mutant only for *runt* (Fig. 2B) or *hairy* (Fig. 2D). The *3′fDE1* pattern is also restored compared to that in each single mutant embryo. Expression in the regions corresponding to stripes 2, 3 and 6 is increased over the level observed in *runt* mutant embryos (see Fig. 3C). The fusion between stripes 2 and 3, stripes 4 and 5, and stripes 6 and 7 is less significant than in *hairy* mutants (Fig. 3E). The arrows in B indicate the regions where stripes 3 and 6 should be. These are two regions where the differences between the *runt, hairy* double mutant and *hairy* single mutant (Fig. 3E) patterns are most obvious.

mutants would be the same. The patterns in double mutant embryos are distinct from either of the single mutants. Thus *runt* and *hairy* have independent regulatory inputs into *ftz* (and by extension, the fDE1 element).

The stage-specific effects of *hs/runt* provide a final line of evidence that fDE1-dependent expression is regulated by other factors. In *hs/runt* embryos that have undergone gastrulation the *ftz* stripes become broader (Fig. 5G). Similar results are obtained with the *ftz/lacC*, *ftz/lacA*, *3′fDE1*, and *5′fDE1* reporter genes (Fig. 5H-K). Most obvious is the induction of *5′fDE1* which is usually expressed at only very low levels during these stages and predominantly in mesoderm. The control reporter genes *3′pstXA* and *5′oligoX* are not affected (not shown), thus indicating the importance of the fDE1 element in mediating the response to *hs/runt*. Stripe fusion is not observed during these stages, even with extreme heat-shock treatments, indicating a restriction in the response to *hs/runt* that is a function of position along the anterior-posterior axis. This restriction depends on *hairy* as *5′fDE1* is somewhat de-repressed in *hairy* mutants (Fig. 5F). Further, overexpression of *runt* in *hairy* mutant embryos leads to an almost solid band of expression in the extending germ band (Fig. 5L). However, this *hairy*-dependent restriction of *5′fDE1* expression is probably not direct as *hairy* expression fades dramatically during gastrulation and is undetectable during these stages (Carroll et al., 1988). Thus there would appear to be another factor whose activity is regulated by *hairy* that influences fDE1-dependent transcription during this later stage of embryogenesis.

The FTZ-F1-binding site is important for activation and repression

The fDE1 element contains a binding site for two related orphan nuclear receptor proteins that are expressed during embryogenesis, FTZ-F1 and DHR39 (Ueda et al., 1990; Ohno et al., 1994). The in vivo function of this site was previously investigated by examining the expression of a *ftz/lacA* derivative in which this binding site is mutated (Fig. 1). This mutant reporter gene, *5-5my* had reduced expression of stripes 1, 2, 3 and 6, suggesting that FTZ-F1 worked primarily as an activator (Ueda et al., 1990). These previous experiments did not find evidence of a repressive role for the fDE1 element; these conclusions were also based on the patterns of β-galactosidase protein accumulation in germ-band extended embryos. Qualitatively different results are obtained when the *5-5my* pattern is examined using in situ hybridization. Although expression in stripes 1, 2, 3 and 6 is reduced, the more obvious effect is loss of repression in interstripe regions. In blastoderm-stage embryos there is significant expression between the stripes and the stripe margins are not well defined (Fig. 6A, compare with Fig. 2I). The lack of interstripe repression is even more obvious in germband-extended embryos (Fig. 6B, compare with Fig. 5C). These results indicate that the FTZ-F1-binding site in the fDE1 element mediates both positive and negative regulation.

DISCUSSION

Regulation of *ftz* by pair-rule genes

Expression of the *ftz* seven-stripe pattern is regulated by the pair-rule genes *runt* and *hairy*. These two genes are expressed in complementary patterns in the blastoderm embryo and have reciprocal effects on *ftz*. The cross-regulatory interactions between *runt* and *hairy* (Ingham and Gergen, 1988; Hooper et al., 1989) raise the possibility that the effects of one of these two genes may be indirect and due to this cross-regulation. For example, the loss of *ftz* interstripe repression observed in *hairy* mutant embryos could in principle result from the de-repression of *runt* expression that occurs in these same embryos (Klingler and Gergen, 1993). Although it is likely that this indirect effect contributes, *hairy* must have regulatory effects on *ftz* that are independent of its effects on *runt*. First, as shown above, the *ftz* (and *3′fDE1*) patterns in *hairy, runt* double mutant embryos are de-repressed compared to the patterns in embryos mutant only for *runt*. Second, ectopic expression of *hairy* leads to the elimination of *ftz* expression (Ish-Horowicz and Pinchin, 1987), but has little effect on *runt* expression (Tsai and Gergen, unpublished). Similar arguments eliminate the possibility that the positive effect of *runt* is due solely to repression of *hairy*. The expression of *hairy* is altered in blastoderm-stage *hs/runt* embryos (Manoukian and Krause, 1993; Tsai and Gergen, 1994). However, these effects are not uniform and expression of some *hairy* stripes is actually increased. Further evidence comes from the reduced expression of *ftz* (and *3′fDE1*) in *hairy, runt* double mutant embryos relative to embryos mutant only for *hairy*. Thus *runt* has activating effects on *ftz* that are independent of its regulatory effects on *hairy*.

All the pair-rule genes examined to date are regulated by *runt* and *hairy* (Carroll and Scott, 1986; Frasch and Levine, 1987; Baumgartner and Noll, 1991). Of these, two have been shown to regulate *ftz* and thus could be responsible for mediating the effects of *runt* and *hairy*. First, *eve* can act as a

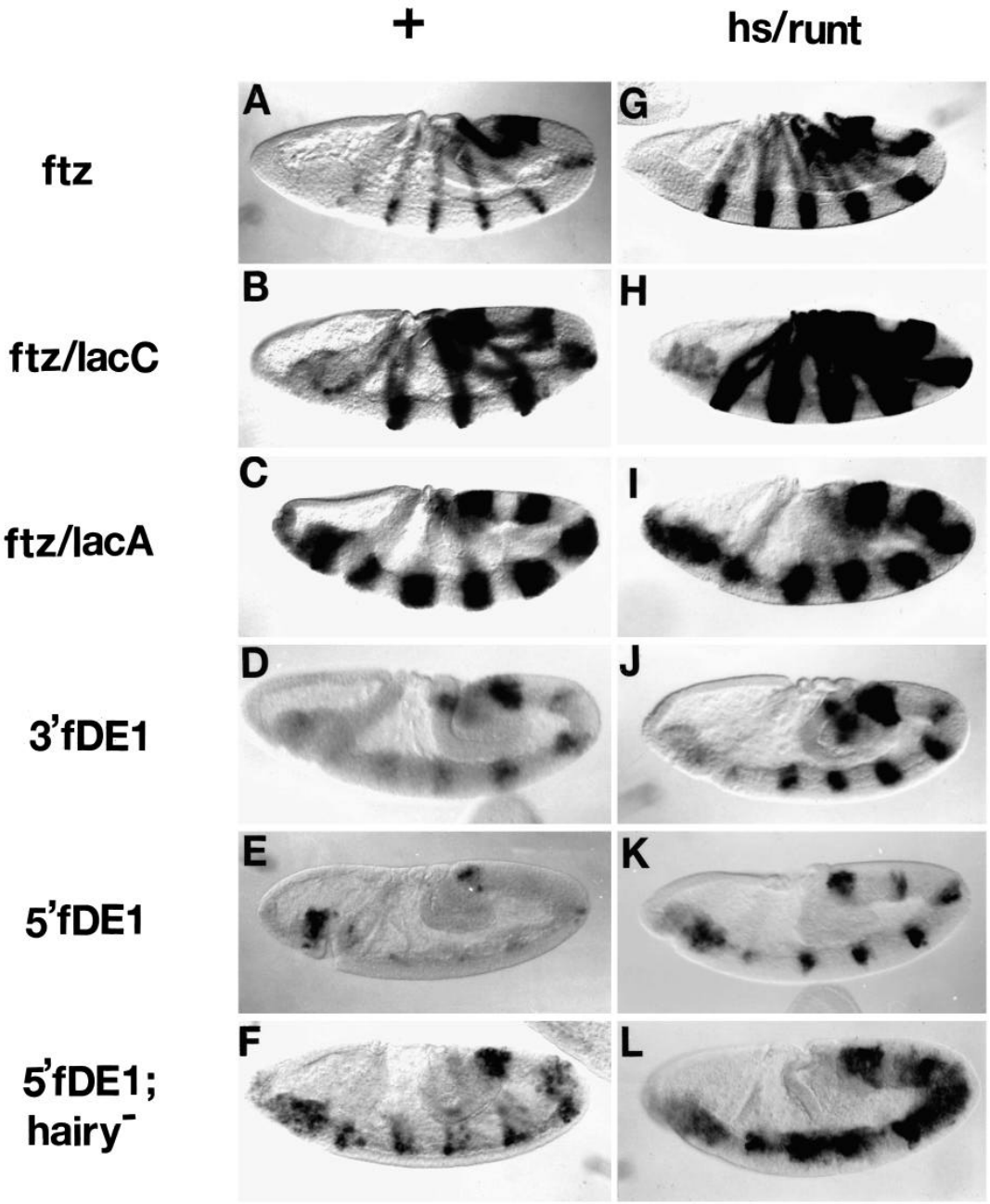

Fig. 5. Stage-specific differences of fDE1-dependent regulation. The embryos on the left are all wild-type, except for F, which is mutant for *hairy*. The embryos on the right all carry the *hs/f runt* transgene, and the embryo in L is, in addition, mutant for *hairy*. The expression patterns shown, from top to bottom, are those of *ftz* (A,G), *ftz/lacC* (B,H), *ftz/lacA* (C,I), *3′fDE1* (D,J), and *5′fDE1* (E,F,K and L), respectively.

repressor of *ftz* (Manoukian and Krause, 1992). Ectopic *runt* expression leads to a reduction in *eve* expression, but similar to the effects on *hairy*, this effect is markedly stripe specific (Manoukian and Krause, 1993; Tsai and Gergen, 1994). Further, the changes in the *ftz* pattern in *eve* mutant embryos are relatively subtle (Carroll and Scott, 1986). Thus it seems unlikely that altered *eve* expression plays a central role in mediating the regulation of *ftz* by either *runt* or *hairy*.

The other pair-rule regulator of *ftz* is *ftz* itself (Hiromi and Gehring, 1987; Schier and Gehring, 1992). Autoregulation is responsible for maintaining *ftz* expression within the 2-cell-wide stripes that remain in late blastoderm-stage embryos. These cells express *runt*, but not *hairy*. Two observations indicate that *runt*-dependent activation does not depend on this autoregulatory pathway. First, *runt*-dependent activation is mediated by the zebra element, whereas *ftz*-dependent autoregulation is mediated through the upstream enhancer (Hiromi and Gehring, 1987; Schier and Gehring, 1992). Secondly, we examined the effects of ectopic *runt* expression in embryos homozygous for the ftz^{W20} mutation. The ftz^{W20} transcript can be distinguished from the wild-type transcript based on its aberrant subcellular localization (Ingham and Gergen, 1988). In homozygous ftz^{W20} embryos, the expression of this mutant transcript is still positively regulated by *hs/runt* treatment (C. Tsai, unpublished). Therefore, activation by *runt* does not require *ftz* activity. In summary, these genetic experiments indicate that *runt* and *hairy* have regulatory inputs into *ftz* transcription that are independent of known regulatory interactions between pair-rule genes.

A puzzle with respect to *runt*'s role as an activator is the preferential loss of certain *ftz* stripes in *runt* mutant embryos. At first glance this does not correlate with the relatively

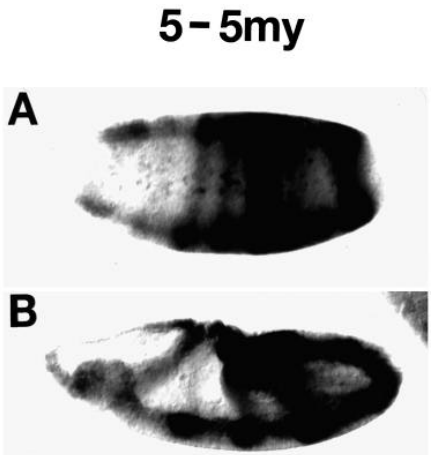

Fig. 6. The FTZ-F1 site is important for both activation and repression. (A,B) The expression patterns of the *5-5my* reporter gene at the end of blastoderm stage and at the mid-germband extension stage, respectively.

uniform activation that is observed in *hs/runt* embryos. Comparison of the *5′fDE1* and *3′fDE1* patterns provides insight towards a solution to this paradox. Expression of both reporter genes is activated by *hs/runt* and reduced in *runt* mutants. Importantly, *3′fDE1* retains stripes 4, 5 and 7 in *runt* mutants. Thus the distal portion of the zebra element in *3′fDE1* contains elements that can activate expression of these stripes in a *runt*-independent manner. Excellent candidates for this are the CAD-response elements located in the distal portion of the zebra element that are required for activation in posterior regions of the embryo (Dearolf et al., 1989b). Our interpretation is that *runt* acts relatively uniformly, and that other factors like CAD make this activation redundant in certain regions.

The role of the fDE1 element in generating periodic patterns

It is remarkable that a 32 base-pair *cis*-regulatory element, the fDE1 element is capable of integrating sufficient spatial and temporal regulatory information to generate a periodic pattern of gene expression in blastoderm-stage *Drosophila* embryos. It was previously noted that deletion of this element leads to de-repression in *ftz* interstripe regions while also reducing the overall expression levels (Dearolf et al., 1989a; Topol et al., 1991); hence the designation as the fDE element (*ftz* Dual Element). Here we demonstrated that *runt* and *hairy* have central roles in establishing this fDE1-dependent, periodic expression pattern, and that the fDE1 element responds to other spatial regulatory cues. The reporter genes used for these experiments contain the *ftz* basal promoter, starting 40 base pairs upstream of the transcription start site and including 73 base pairs of 5′ untranslated leader sequences (Dearolf et al., 1989a). Neither *runt* or *hairy* affected expression of the *3′pstXA* and *5′oligoX* constructs, both of which contain the *ftz* basal promoter region and lack the fDE1 element. This is strong evidence that the fDE1 element is required, but does not exclude the possibility that the observed regulatory effects also involve sequences in the *ftz* basal promoter region.

The fDE1 element contains a binding site for the orphan nuclear receptor proteins FTZ-F1 and DHR39 (Ueda et al., 1990; Ohno et al., 1994). The expression of the *5-5my* reporter gene, in which this binding site is mutated, provides further evidence for the dual nature of the fDE1 element (Ueda et al., 1990). We found a reduction in the expression level of the *runt*-dependent stripes similar to that observed by Ueda et al. (1990) suggesting that activation by *runt* is mediated by FTZ-F1 and/or DHR39. However, in contrast to the previous findings, we also observed loss of interstripe repression. We presume that the difference between our results and the previous findings are due to the methods used to monitor gene expression. Although *5-5my* expression is altered relative to *ftz/lacA*, aspects of periodic activation and repression are retained. There is a second, lower affinity FTZ-F1-binding site that is proximal to the site in the fDE1 element (see Fig. 1; Ueda et al., 1990). This site falls within a region that contains the fDE2 element, an element that also confers both activation and repression (Dearolf et al., 1989a; Topol et al., 1991). It seems likely that the fDE2 element is responsible for the periodicity that is retained in the *5-5my* expression pattern. This redundancy presumably serves to fine tune the expression of *ftz*, ultimately leading to the formation of the sharp on-off expression patterns that are central to the process of segmentation. There are three putative fDE elements in the *ftz* upstream enhancer (Han et al., 1993; Schier and Gehring, 1992). These elements may well account for some of the differences between the *ftz/lacC* and *ftz/lacA* patterns. Based on the observations above, we might expect that expression of a reporter gene that contains only the upstream enhancer would be affected by *hs/runt* treatment. However, the expression of the *UPHZ* reporter gene (Hiromi and Gehring, 1987) is not significantly altered in *hs/runt* embryos (not shown). This difference may be explained by the stage specificity of the response to *hs/runt*. Expression of *UPHZ* is usually not detectable until the onset of gastrulation. This is a stage when the response of the *5′fDE1* reporter to *hs/runt* is already restricted.

Interaction of runt, hairy, and FTZ-F1 with the fDE1 element

The RUNT and HAIRY proteins have motifs indicative of sequence-specific DNA binding (Rushlow et al., 1989; Kagoshima et al., 1993). Thus a simple model would be that these two proteins directly interact with the fDE elements. RUNT contains the Runt-domain, a DNA-binding motif also found in the mammalian PEBP2αA and AML1 proteins. The mammalian Runt-domain proteins bind to the consensus sequence ACCPuCA (Satake et al., 1992; Melnikova et al., 1993). This same sequence is recognized by the *Drosophila* RUNT protein, albeit with lower affinity (Kagoshima et al., 1993; M. Pepling and JPG, unpublished). There are no good matches to this sequence in the fDE1 element. We have also not found any evidence that RUNT will bind to the fDE1 element in electrophoretic mobility shift assays experiments (C. Tsai, unpublished). HAIRY is a member of a subfamily of bHLH proteins that contain a proline in their basic region and that will bind to a canonical E-box (CAnnTG) as well as to the divergent sequences CACGAG and CACAAG (Tietze et al., 1992). The fDE1 element does not contain matches to these sequences. Further, experiments with in vitro translated protein fail to reveal any interaction between HAIRY and the fDE1 element (C. Tsai, unpublished). The DNA-binding motifs in RUNT and HAIRY are involved in mediating interactions with other proteins (Kagoshima et al., 1993; Murre et al., 1989).

Association with other proteins that were not included in our assays could facilitate direct interactions between either (both) of these proteins and the fDE element in vivo.

Two nuclear factors that are likely to be important for the regulatory phenomena that we described are FTZ-F1 and DHR39. The point mutation in the *5-5my* reporter gene was designed to specifically eliminate interaction between the FTZ-F1 protein and the fDE1 element (Ueda et al., 1990). This mutation affects both activation and repression. This strongly suggests that the opposing effects of *runt* and *hairy* are mediated by FTZ-F1 and/or DHR39. The mRNAs for FTZ-F1 and DHR39 are uniformly expressed during the blastoderm stage (Ohno and Petkovich, 1992; C. Tsai and P. Gergen, unpublished observations). How do interactions between these uniformly expressed factors and the fDE elements generate a periodic expression pattern in response to the regulatory cues provided by *runt* and *hairy*? In this regard, it is interesting to note a reported difference in the properties of FTZ-F1 and DHR39. In transient transfection experiments, FTZ-F1 behaves as an activator whereas DHR39 acts as a repressor and can interfere with FTZ-F1-dependent activation (Ayer et al., 1993; Ohno et al., 1994). This suggests a model whereby *runt* and *hairy* could regulate the fDE elements by differentially influencing the relative activities of FTZ-F1 and DHR39. There is no evidence that *runt* or *hairy* regulate the expression of FTZ-F1 or DHR39. Thus these putative interactions may best be accounted for by protein-protein interactions.

Although RUNT and HAIRY are unrelated types of transcriptional regulators, they share a feature that could provide a molecular link for their opposing regulatory properties. The C terminus of the HAIRY protein ends with the tetrapeptide sequence WRPW. This motif is required for *hairy* function (Wainwright and Ish-Horowicz, 1992) and mediates interaction with an unrelated protein, GROUCHO (Paroush et al., 1994). The C terminus of the RUNT protein ends with a related sequence WRPY (Kania et al., 1990) that is conserved in other *Drosophila* species (M. Pepling, unpublished), as well as in the mammalian Runt-domain proteins (Ogawa et al., 1993; Bae et al., 1993). This conservation strongly suggests that RUNT's WRPY motif is functionally important; perhaps involved in interacting with GROUCHO. Competitive interactions for common factors provide an attractive mechanism to account for the opposing regulatory effects of RUNT and HAIRY. Further studies on the molecular mechanism of fDE1-dependent regulation should provide insight on the intertwined activities of these two transcriptional regulators and their interactions with the FTZ-F1 family of nuclear receptors.

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REFERENCES

Ayer, S., Walker, N., Mosammaparast, M., Nelson, J.P., Shilo, B.-z. and Benyajati, C. (1993). Activation and repression of Drosophila alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element. *Nucleic Acids Res.* **21**, 1619-1627.

Bae, S.C., Yamaguchi-Iwai, Y., Ogawa, E., Maruyama, M., Inuzuka, M., Kagoshima, H., Shigesada, K., Satake, M. and Ito, Y. (1993). Isolation of PEBP2αB cDNA representing the mouse homolog of human acute myeloid leukemia gene, *AML1*. *Oncogene* **8**, 809-814.

Baumgartner, S. and Noll, M. (1991). Network of interactions among pair-rule genes regulating *paired* expression during primordial segmentation of Drosophila. *Mech. Dev.* **33**, 1-18.

Brown, J. L., Sonoda, S., Ueda, H., Scott, M. P. and Wu, C. (1991). Repression of the Drosophila *fushi tarazu* (*ftz*) segmentation gene. *EMBO J.* **10**, 665-674.

Carroll, S. B., Laughon, A. and Thalley, B. S. (1988). Expression, function, and regulation of the *hairy* segmentation protein in the Drosophila embryo. *Genes Dev.* **2**, 883-890.

Carroll, S. B. and Scott, M. P. (1986). Zygotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early Drosophila embryogenesis. *Cell* **45**, 113-126.

Dearolf, C. R., Topol, J. and Parker, C. S. (1989a). Transcriptional control of Drosophila *fushi tarazu* zebra stripe expression. *Genes Dev.* **3**, 384-398.

Dearolf, C. R., Topol, J. and Parker, C. S. (1989b). The *caudal* gene product is a direct activator of *fushi tarazu* transcription during Drosophila embryogenesis. *Nature* **341**, 340-343.

Frasch, M. and Levine, M. (1987). Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in Drosophila. *Genes Dev.* **1**, 981-995.

Hafen, E., Kuroiwa, A. and Gehring, W. J. (1984). Spatial distribution of transcripts from the segmentation gene *fushi-tarazu* during Drosophila embryonic development. *Cell* **37**, 833-841.

Han, W., Yu, Y., Altan, N. and Pick, L. (1993). Multiple proteins interact with the *fushi tarazu* proximal enhancer. *Mol. Cell. Biol.* **13**, 5549-5559.

Harding, K., Hoey, T., Warrior, R. and Levine, M. (1989). Autoregulatory and gap-gene response elements of the *even-skipped* promoter of Drosophila. *EMBO J.* **8**, 1205-1212.

Hiromi, Y. and Gehring, W. J. (1987). Regulation and function of the Drosophila segmentation gene *fushi tarazu. Cell* **50**, 963-974.

Hiromi, Y., Kuroiwa, A. and Gehring, W.J. (1985). Control elements of the Drosophila segmentation gene *fushi tarazu. Cell* **43**, 603-613.

Hooper, K. L., Parkhurst, S. M. and Ish-Horowicz, D. (1989). Spatial control of *hairy* protein expression during embryogenesis. *Development* **102**, 489-504.

Howard, K. and Ingham, P. W. (1986). Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy* and *engrailed* in the Drosophila blastoderm. *Cell* **44**, 949-957.

Ingham, P. W. and Gergen, P. (1988). Interactions between the pair-rule genes *runt*, *hairy*, *even-skipped* and *fushi tarazu* and the establishment of periodic pattern in the Drosophila embryo. *Development* **104 Supplement**, 51-60.

Ish-Horowicz, D., and Pinchin, S. M. (1987). Pattern abnormalities induced by ectopic expression of the Drosophila gene *hairy* are associated with repression of *ftz* transcription. *Cell* **51**, 405-415.

Kagoshima, H., Shigesada, K., Satake, M., Ito, Y., Miyoshi, H., Ohki, M., Pepling, M. and Gergen, J.P. (1993). The Runt-domain identifies a new family of heteromeric transcriptional regulators. *Trends in Genetics* **106**, 338-341.

Kania, M. A., Bonner, A. S., Duffy, J. B. and Gergen, J. P. (1990). The Drosophila segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. *Genes Dev.* **4**, 1701-1713.

Klingler, M. and Gergen, J.P. (1993). Regulation of *runt* transcription by Drosophila segmentation genes. *Mech. Dev.* **43**, 3-19.

Manoukian, A.S. and Krause, H.M. (1993). Control of segmental asymmetry in Drosophila embryos. *Development* **118**, 785-796.

Manoukian, A.S. and Krause, H.M. (1992). Concentration-dependent activities of the *even-skipped* protein in Drosophila embryos. *Genes Dev.* **6**, 1740-1751.

Melnikova, I.N., Crute, B.E., Wang, S. and Speck, N.A. (1993). Sequence specificity of the Core-binding factor. *J. Virology* **67**, 2408-2411.

Murre, C., McCaw, P.S. and Baltimore, D. (1989). A new DNA-binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* **56**, 777-783.

Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake,

M., Shigesada, K. and Ito, Y. (1993). PEBP2/PEA2 represents a new family of transcription factor homologous to the products of the Drosophila *runt* and the human *AML1*. *Proc. Nat. Acad. Sci., USA.* **90**, 6859-6863.

Ohno, C.K. and Petkovich, M. (1992). FTZ-F1β, a novel member of the Drosophila nuclear receptor family. *Mech. Dev.* **40**, 13-24.

Ohno, C.K., Ueda, H. and Petkovich, M. (1994). The Drosophila nuclear receptors FTZ-F1α and FTZ-F1β compete as monomers for binding to a site in the *fushi tarazu* gene. *Mol. Cell. Biol.* **14**, 3166-3175.

Paroush, Z., Finley, R.L. Jr., Kidd, T., Wainwright, S.M., Ingham, P.W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation and sex-determination, and interacts directly with Hairy-related bHLH proteins. *Cell*, in press.

Rushlow, C.A., Hogan, A., Pinchin, S.M., Howe, K., Lardelli, M. and Ish-Horowicz, D. (1989). The Drosophila *hairy* protein acts both in segmentation and bristle patterning and shows homology to N-myc. *EMBO J.* **8**, 3095-3103.

Satake, M., Inuzuka, M., Shigesada, K. Oikawa, T. and Ito, Y. (1992). Differential expression of subspecies of polyomavirus and murine leukemia virus enhancer core binding protein, PEBP2, in various hematopoietic cells. *Jpn. J. Cancer Res.* **83**, 714-722.

Schier, A. F. and Gehring, W. J. (1992). Direct homeodomain-DNA interaction in the autoregulation of the *fushi tarazu* gene. *Nature* **356**, 804-807.

Tietze, K., Oellers, N. and Knust, E. (1992). Enhancer of split^{D}, a dominant mutation of Drosophila, and its use in the study of functional domains of a helix-loop-helix protein. *Proc. Nat. Acad. Sci., USA* **89**, 6152-6156.

Topol, J., Dearolf, C. R., Prakash, K., Parker, C. S. (1991). Synthetic oligonucleotides recreate Drosophila *fushi tarazu* zebra-stripe expression. *Genes Dev.* **5**, 855-867.

Tsai, C. and Gergen, J.P. (1994). Gap gene properties of the pair-rule gene *runt* during Drosophila segmentation. *Development* **120**, 1671-1683.

Ueda, H., Sonoda, S., Brown, J,.L., Scott, M.P. and Wu, C. (1990). A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. *Genes Dev.* **4**, 624-635.

Wainwright, S.M. and Ish-Horowicz, D. (1992). Point mutations in the Drosophila *hairy* gene demonstrate *in vivo* requirements for basic, helix-loop-helix, and WRPW domains. *Mol. Cell. Biol.* **12**, 2475-2483.

Weir, M. P. and Kornberg, T. (1985). Patterns of *engrailed* and *fushi tarazu* transcripts reveal novel intermediate stages in Drosophila segmentation. *Nature* **318**, 433-439.

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