Ftz modulates Runt-dependent activation and repression of segment-polarity gene transcription

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Summary

A crucial step in generating the segmented body plan in *Drosophila* is establishing stripes of expression of several key segment-polarity genes, one stripe for each parasegment, in the blastoderm stage embryo. It is well established that these patterns are generated in response to regulation by the transcription factors encoded by the pair-rule segmentation genes. However, the full set of positional cues that drive expression in either the odd- or even-numbered parasegments has not been defined for any of the segment-polarity genes. Among the complications for dissecting the pair-rule to segment-polarity transition are the regulatory interactions between the different pair-rule genes. We have used an ectopic expression system that allows for quantitative manipulation of expression levels to probe the role of the primary pair-rule transcription factor Runt in segment-polarity gene regulation. These experiments identify *sloppy paired 1* (*slp1*) as a gene that is activated and repressed by Runt in a simple combinatorial parasegment-dependent manner. The combination of Runt and Odd-paired (Opa) is both necessary and sufficient for *slp1* activation in all somatic blastoderm nuclei that do not express the Fushi tarazu (Ftz) transcription factor. By contrast, the specific combination of Runt + Ftz is sufficient for *slp1* repression in all blastoderm nuclei. We furthermore find that Ftz modulates the Runt-dependent regulation of the segment-polarity genes *wingless* (*wg*) and *engrailed* (*en*). However, in the case of *en* the combination of Runt + Ftz gives activation. The contrasting responses of different downstream targets to Runt in the presence or absence of Ftz is thus central to the combinatorial logic of the pair-rule to segment-polarity transition. The unique and simple rules for *slp1* regulation make this an attractive target for dissecting the molecular mechanisms of Runt-dependent regulation.

Key words: Runx, Hox, Zic, Segmentation, *sloppy paired 1*, *engrailed*, *wingless*, *Drosophila*

Introduction

Extensive genetic and molecular studies have elucidated the major principles of the pathway that generates the segmented body plan of the *Drosophila* embryo. Broad gradients of maternal information are decoded in three successive steps by zygotically expressed segmentation genes, each step occurring with a finer level of spatial resolution. Transcriptional regulation is central to establishing the expression patterns of different segmentation genes at each step. Indeed, it is well established that the final step of the segmentation hierarchy, the pair-rule to segment-polarity transition relies on combinatorial regulation by the pair-rule transcription factors. Furthermore, it is clear that different combinatorial rules are used to generate the odd- and even-numbered stripes of several key segment-polarity genes. However, for none of these segment-polarity genes is there a full understanding of the positional cues responsible for expression of either the odd- or even-numbered stripes. One reason for this is that the transcription factors encoded by the primary pair-rule genes *even-skipped* (*eve*), *hairy* (*h*) and *runt* (*run* – FlyBase), also have important roles in pair-rule gene regulation (Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988; Manoukian and Krause, 1992; Tsai and Gergen, 1994; Tsai and Gergen, 1995). This complication makes it difficult to identify the exact roles of these three factors in segment-polarity gene regulation and has obscured our understanding of the combinatorial rules underlying the pair-rule to segment-polarity transition.

One pivotal player in the pair-rule to segment-polarity transition is Runt, the founding member of the Runx family of transcription factors. Runx proteins function both as activators and repressors of transcription in multiple developmental pathways (Coffman, 2003; Komori, 2002; Shapiro, 1999; Speck et al., 1999; Wheeler et al., 2000). Indeed, Runt has separable roles in three developmental pathways, sex determination, segmentation and neurogenesis, within the first few hours of *Drosophila* embryogenesis (Duffy and Gergen, 1994). Ectopic expression experiments indicate a role for Runt in establishing polarity within each parasegment (Manoukian and Krause, 1993). The four-cell wide *run* stripes overlap the anterior half of each *ftz* stripe and the posterior half of each *eve* stripe. The contrasting positive and negative regulatory effects of *run* on *ftz* and *eve*, respectively, contribute to the graded activity of these two genes within each parasegment. However, Runt has additional effects on segment-polarity gene expression beyond modulating *ftz* and *eve* expression. For example, the odd-numbered *en* stripes are repressed by Runt,

even in cells that express Eve (Tracey et al., 2000). The immediate response of *en* to transient induction of a heat-inducible *hs-runt* transgene strongly suggests this repression is direct. Additional insights on Runt function have been obtained in other experiments with *hs-runt* transgenes (Li and Gergen, 1999; Pepling and Gergen, 1995; Tsai et al., 1998). However, the difficulty in reproducibly controlling the precise level and timing of expression makes this approach less than ideal for further dissecting the role of Runt and other pair-rule transcription factors in segment-polarity gene regulation.

We have recently taken advantage of an alternative strategy to investigate the segmentation gene network, and in particular the regulatory functions of Runt. This strategy uses *Drosophila* lines that maternally express the yeast transcriptional activator GAL4 to drive expression of GAL4-responsive *UAS* transgenes concomitant with the onset of zygotic transcription during the blastoderm stage of embryogenesis. The transgene construct used to express GAL4 maternally contains the *nanos* promoter and the 3′ untranslated region of an α-tubulin mRNA and is thus referred to as an *NGT* transgene (*nanos-GAL4-tubulin*). Importantly, the expression level can be quantitatively and reproducibly manipulated by using *NGT* lines that drive different levels of GAL4 expression (Tracey et al., 2000). Experiments with this system have confirmed the potent activity of Runt as a repressor of the odd-numbered *en* stripes. Indeed the lethality associated with *NGT*-driven Runt expression has provided the basis for a genetic dissection of *en* repression (Wheeler et al., 2002).

We have used this approach to systematically examine the responses of pair-rule and segment-polarity genes to different levels of Runt. After *en*, the second most sensitive segmentation gene target of Runt is the *slp1* transcription unit of the *sloppy paired* locus. We find that the combinatorial rules needed to generate two-cell wide *slp1* stripes in the posterior half of each parasegment are simpler than the rules needed to generate the single-cell wide stripes of the segment-polarity genes *en* and *wg*. Runt is required for *slp1* activation in odd-numbered parasegments. This Runt-dependent activation involves cooperation with the zinc-finger transcription factor encoded by the pair-rule gene *opa*. Indeed, the simple combination of Runt + Opa is sufficient for *slp1*activation in all somatic blastoderm cells that do not have Ftz. We furthermore find that repression of *slp1* in the anterior half of the even-numbered parasegments requires Ftz. Ftz not only blocks Runt-dependent activation in these cells, but the combined action of Runt and Ftz is sufficient for *slp1* repression in all blastoderm nuclei. Thus, Runt is switched from an activator to a repressor of *slp1* by the Ftz homeodomain transcription factor. Additional experiments indicate that Ftz also modulates the activity of Runt on the segment-polarity genes *wg* and *en*. However, in the case of *en* the combination of Runt + Ftz gives activation rather than repression. These results provide important new insights into the context-dependent activity of Runt in segmentation and also provide a valuable framework for dissecting the mechanisms of transcriptional activation and repression by Runt.

Materials and methods

Drosophila strains and transgenes

Stocks carrying the *opa[1]* and *ftz[11]* mutations were obtained from the Bloomington Stock Center. The temperature-sensitive *run[YP17]* mutation (Gergen and Wieschaus, 1986) corresponds to the *run[29]* allele listed in the Bloomington Stock Center. The *y w[67c23]* strain used to generate all transgenic lines was used as the wild-type control strain for in situ hybridization experiments.

The GAL4-drivers *P{GAL4-nos.NGT}11* (*NGT11*), *P{GAL4-nos.NGT}40* (*NGT40*) and *P{GAL4-nos.NGT}A* (*NGTA*) have been described previously (Tracey et al., 2000; Wheeler et al., 2002). Homozygous *NGT40* females produce approximately twice the levels of maternal GAL4 activity as females homozygous for either *NGT11* or *NGTA*. Females homozygous for both *NGT40* and *NGTA* produce ~1.5 times more activity than homozygous *NGT40* females, whereas females heterozygous for both *NGT40* and *NGTA* produce ~0.75× the activity of homozygous *NGT40* females.

The *P{UAS-runt.T}14* (*UAS-runt[14]*), *P{UAS-runt.T}232* (*UAS-runt[232]*) and *P{UAS-runt.T}15* (*UAS-runt[15]*) transgenes have been described previously (Li and Gergen, 1999; Tracey et al., 2000). The third chromsome-linked *P{UAS-runt.T}13* (*UAS-runt[13]*) transgene is comparable in activity with *UAS-runt[232]*. The *P{UAS-opa.VZ}36* (*UAS-opa[36]*) transgene insertion was created by standard germ line transformation using the p:Δ2-3 helper plasmid. This transposon construct was generated by first digesting pNB40:opa[C] (Benedyk et al., 1994) with *Bst*EII and *Bgl*II to remove vector sequences containing the SP6 promoter and 5′ untranslated leader of the *Xenopus* β-globin gene as well as 177 nucleotides of the *opa* 5′ untranslated leader. The digested plasmid was treated with Klenow polymerase and re-circularized with DNA ligase. A 2.8 kb *Eco*RI fragment from this modified *opa* construct was then excised and re-cloned into pUAS-T (Brand and Perrimon, 1993). The second chromosome-linked *P{UAS-opa.VZ}14* (*UAS-opa[14]*) insertion, as well as the third chromosome linked *P{UAS-opa.VZ}10* (*UAS-opa[10]*) and *P{UAS-opa.VZ}12* (*UAS-opa[12]*) insertions, were obtained by mobilization of *UAS-opa[36]* (Robertson et al., 1988). Based on the lethality associated with different levels of *NGT*-driven expression we estimate that *UAS-opa[12]*, *UAS-opa[14]* and *UAS-opa[10]* are expressed at 2.5-, 3- and 4-fold higher levels, respectively, than *UAS-opa[36]*. The *UAS-ftz[261]* line was provided by U. Lohr and L. Pick.

Embryo manipulation and in situ hybridization

Embryos were collected as described (Tsai and Gergen, 1994). For experiments with temperature-sensitive mutations, embryos were collected for 1 hour at 25°C, grown for 4.5 hours at the permissive temperature of 18°C, and then shifted to the non-permissive temperature of 30°C for 20 minutes immediately prior to fixation for in situ hybridization.

In situ hybridization with digoxigenin-labeled antisense RNA probes was carried out as described (Klingler and Gergen, 1993) with the following modifications: embryos were digested with Proteinase K (30 μg/ml in PBT=PBS + 0.1% Tween-20) for 3 minutes followed by inactivation with glycine (2 mg/ml) in PBT. To further reduce non-specific binding, embryos were also pre-treated in a 10% (v/v) solution of heat inactivated goat serum for 1 hour prior to incubation with the anti-digoxigenin antibody.

The protocol for double-label in situ hybridization using biotin- and digoxigenin-labeled RNA probes was adapted from that described by O'Neill and Bier (O'Neill and Bier, 1994), with the following modifications: embryos were digested with Proteinase K (50 μg/ml in PBT) for 2.5 minutes; hybridization was carried out at 65°C overnight; post-hybridization washes were carried out in 1% goat serum, 0.3% deoxycholate, 0.3% triton-X in PBS in place of BSA/PBT; and the immunohistochemical detection reaction of HRP-conjugated antibodies with peroxidase and diaminobenzidine was stopped by washing the embryos four times in HRP buffer (50 mM citric acid, 50 mM ammonium acetate, pH 5).

The plasmid templates used to generate digoxigenin-labeled riboprobes for *odd-skipped* (*odd*), *paired* (*prd*) and *slp1* are described in Wheeler et al. (Wheeler et al., 2002). The templates for *en* and *ftz*

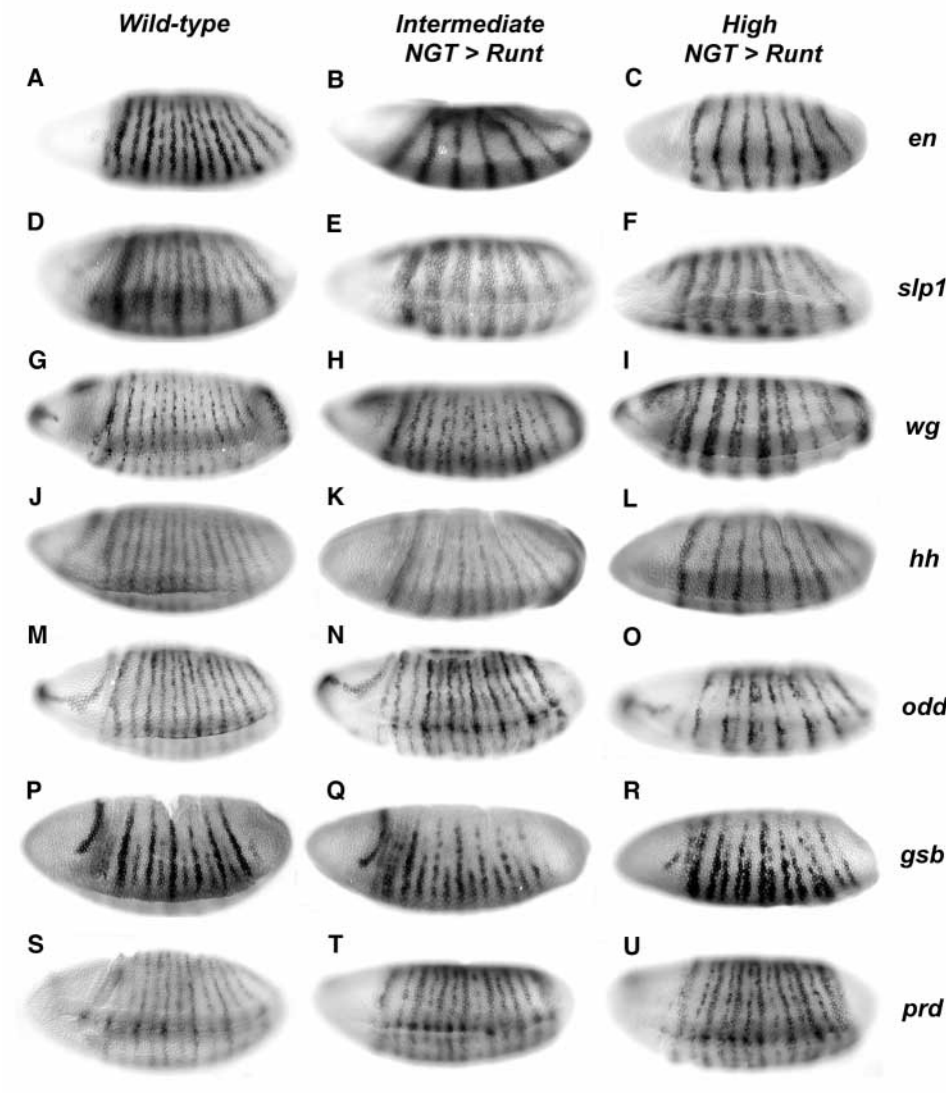

Fig. 1. *slp1* is a sensitive target of Runt. In situ hybridization reveals the segmentally repeated expression patterns of different segmentation genes in gastrula stage embryos. Embryos in this and other figures are shown anterior towards the left, dorsal side upwards. Each row shows expression of a different segmentation gene as labeled on the right. The wild-type expression patterns are shown in the left column of embryos. Embryos in the middle and right column of embryos have intermediate and high levels of *NGT*-driven ectopic Runt expression, respectively. Intermediate and high levels of ectopic Runt were obtained by mating females homozygous for *NGT11* and *NGT40*, respectively to homozygous *UAS-runt[232]* males. These are the same combinations used to demonstrate that high, but not intermediate, levels of Runt alter expression of *eve* and *ftz* (Tracey et al., 2000). Not shown in this panel are the responses of the pair-rule genes *hairy* and *odd-paired* (*opa*). *NGT*-driven Runt expression leads to stripe-specific repression of *hairy* similar to that obtained in *hs-runt* embryos, but only at high levels of expression. The *opa* pattern is unique amongst the pair-rule genes and is expressed in a broad band spanning the pre-segmental region of the embryo, rather than in a series of stripes. This pattern is not altered by ectopic Runt.

are described elsewhere (Tracey et al., 2000; Tsai and Gergen, 1994). The biotinylated *ftz* riboprobe was synthesized with Biotin-21-UTP (Clontech) in place of digoxigenin-conjugated UTP. The *gooseberry* (*gsb*) probe was synthesized with T7 RNA polymerase using *Sal*I-linearized BsH9c2 (Baumgartner et al., 1987). The *hedgehog* (*hh*) probe was synthesized with T3 RNA polymerase using *Nde*I-linearized pB:hh[4/1/8.3] (J. Mohler, personal communication). The *wg* riboprobe was synthesized with T3 RNA polymerase using an *EcoRI*-linearized p*wg*-12 template. This pBluescribe plasmid contains a 1.3 kb *Hin*dIII + *Eco*RI genomic fragment that encodes much of the 4th and 5th exons (N. Baker, personal communication).

Results

slp1 is a sensitive target of Runt

The role of Runt as a primary pair-rule gene complicates interpreting the alterations in segment-polarity gene expression that are observed in *run* mutants. Recent experiments utilizing a GAL4-based *NGT*-expression system to manipulate expression in the blastoderm embryo demonstrated that low levels of Runt repress *en* in odd-numbered parasegments without altering expression of the pair-rule genes *eve* and *ftz* (Tracey et al., 2000). This observation suggested that this approach might provide a useful tool for defining the role of Runt in regulating other segment-polarity genes. We therefore undertook a systematic survey of the response of other segmentation genes to increasing levels of *NGT*-driven Runt expression. These experiments revealed significant differences in sensitivity as well as interesting differences in the nature of the response of different genes to ectopic Runt. As found previously, the odd-numbered *en* stripes are repressed at both intermediate and high levels of ectopic runt (Fig. 1A-C). After *en*, the second most sensitive target is *slp1*. This gene shows a partially penetrant and subtle defect in the spacing of the segmentally repeated stripes in embryos with low levels of *NGT*-driven Runt (data not shown). A more pronounced alteration is obtained in embryos with intermediate levels of Runt. In these embryos the *slp1* pattern is converted from a segment-polarity-like, 14-stripe pattern (Fig. 1D) to a pair-rule-like, seven-stripe pattern (Fig. 1E). At this level, expression of other segmentation genes is normal although there are subtle changes in the spacing of the *wg* stripes (Fig. 1G,H) and a partial loss of the odd-numbered *hh* stripes (Fig. 1J,K). All three of these genes show clearer alterations at higher levels of *NGT*-driven Runt (Fig. 1F,I,L), with *wg* responding in a

manner similar to *slp1* and *hh* responding in a manner similar to *en*. High Runt levels also produce spacing defects in the expression of *odd* and *gsb*, as well as a more subtle effect on *prd* (Fig. 1O,R,U). Several of the changes observed at high levels of ectopic Runt are likely to be indirect and due to alterations in the expression of *eve*, *ftz* and *hairy* (Tracey et al., 2000; Tsai and Gergen, 1994; Tsai and Gergen, 1995). The response of *slp1* to ectopic Runt is notable both because of its sensitivity and apparent simplicity, thus suggesting that Runt plays a pivotal role in regulating *slp1* transcription.

Parasegment-specific activation and repression of *slp1* transcription by Runt

slp1 is expressed in a repeating two-cell wide stripe pattern in late blastoderm stage embryos (Fig. 2A). The phasing of this expression relative to other key pair-rule genes is shown in Fig. 2B. Parasegmental units are defined by expression of the homeodomain proteins Eve and Ftz. The initial pair-rule expression of Eve and Ftz is in complementary, four cell wide stripes. These stripes narrow during the process of cellularization as expression is lost in the more posterior cells in each parasegment. The *slp1* stripes arise in the two most posterior cells in each parasegment, the cells that are first to lose Eve and Ftz expression. Interestingly, the borders of the *slp1* stripes within each parasegment align with the borders of *run* and *hairy* expression. These two pair-rule genes are expressed in complementary patterns that are shifted by two cells relative to the *eve* and *ftz* patterns (Ingham and Gergen, 1988; Kania et al., 1990; Kosman et al., 1998). In odd parasegments, *slp1* is activated in cells that express Runt, whereas in even parasegments *slp1* is repressed in the Runt-expressing cells. As will be shown below, *slp1* activation in odd-numbered parasegments requires the specific combination of Runt and Opa, whereas repression in even-numbered parasegments involves the specific combination of Runt + Ftz. These two regulatory interactions are schematically depicted in Fig. 2B to provide a framework for interpreting the alterations produced by the various genetic manipulations described below.

The expression of *slp1* in embryos deficient for Runt consists of six irregularly spaced stripes of different widths and intensities (data not shown). These several changes reflect the altered expression of other pair-rule genes in these embryos. In order to more specifically define the role of Runt in *slp1* regulation we took advantage of the temperature-sensitive *run[YP17]* mutation. Embryos hemizygous for this mutation were allowed to develop at the permissive temperature through the early blastoderm stage when the seven-stripe patterns of the pair-rule genes are established. These embryos were then shifted to the non-permissive temperature for 20 minutes and then fixed for in situ hybridization. This transient elimination of *run* leads to loss of *slp1* expression in odd-numbered parasegments (Fig. 2C). There is also expanded *slp1* expression in even-numbered parasegments, with the exception that expression is lost in the ventral portion of parasegment 4 (Fig. 2C). The contrasting loss of activation and partial loss of repression produced by this transient reduction of *run* is schematically depicted in Fig. 2D. These results demonstrate an acute temporal requirement for Runt in *slp1* regulation and strongly suggest that Runt normally functions as both an activator and repressor of *slp1* transcription.

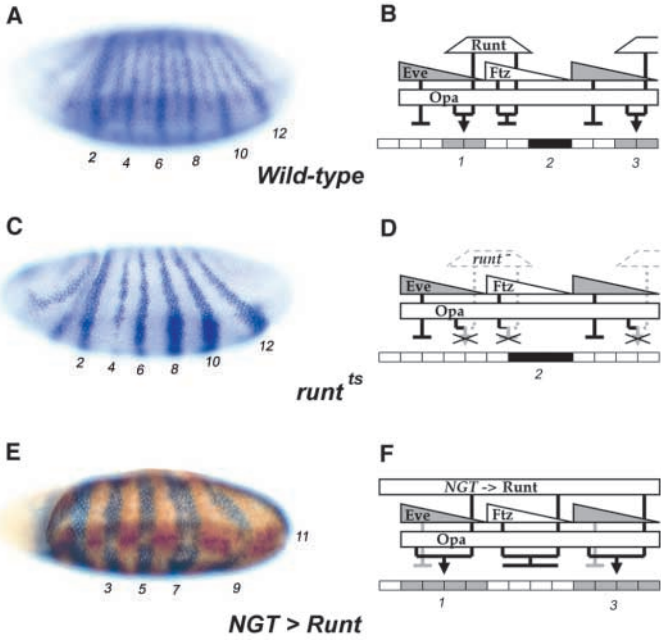

Fig. 2. Parasegment-specific activation and repression of *slp1* by Runt. (A) Wild-type *slp1* expression in a gastrula stage embryo as visualized by in situ hybridization. (B) The phasing of *slp1* expression relative to the expression of different pair-rule transcription factors. A strip of cells along the anteroposterior axis is depicted across the bottom with *slp1*-expressing cells indicated by shading. The higher expression level of the even-numbered stripes is indicated by darker shading. The four-cell wide Runt stripes are depicted above this strip as a trapezoid, reflecting the higher expression levels in the center of the stripes. By contrast, Eve and Ftz stripes are depicted as triangles with peak expression in the most anterior cells, whereas the uniform expression of Opa is depicted as a broad rectangle that spans the presegmental region. The regulatory circuitry responsible for generating the *slp1* pattern is also depicted. Activation by Runt + Opa is indicated with an arrowhead, whereas repression by either Eve, or the combination of Runt + Ftz, is indicated with a horizontal bar. (C) Transient elimination of *runt* activity in an embryo hemizygous for the temperature-sensitive *runt[YP17]* mutation leads to loss of odd-numbered *slp1* stripes and expansion of some of the even-numbered stripes. These changes are interpreted to be due to loss of Runt-dependent activation and repression as indicated in D. (E) Double in situ hybridization showing the complementary expression of *slp1* (blue) and *ftz* (brown) mRNAs in embryos with a high-level of *NGT*-driven Runt. This embryo was obtained by crossing homozygous *NGT40* females with homozygous *UAS-runt[232]* males. As indicated in F, *slp1* expression in these embryos fills the presumptive odd-numbered parasegments and is repressed throughout the even-numbered parasegments.

As described above, the 14-striped *slp1* pattern is converted into a seven-stripe pattern in the presence of intermediate as well as high levels of *NGT*-driven Runt (Fig. 1E,F). These seven stripes are broader than the two-cell wide stripes that normally comprise the posterior half of each parasegment. We used double in situ hybridization to investigate the relationship between these broadened *slp1* stripes and the expression of *ftz*, which identifies cells in even-numbered parasegments. The function of Runt as an activator of *ftz* is revealed by broadened four-cell wide stripes in gastrula stage embryos that have high

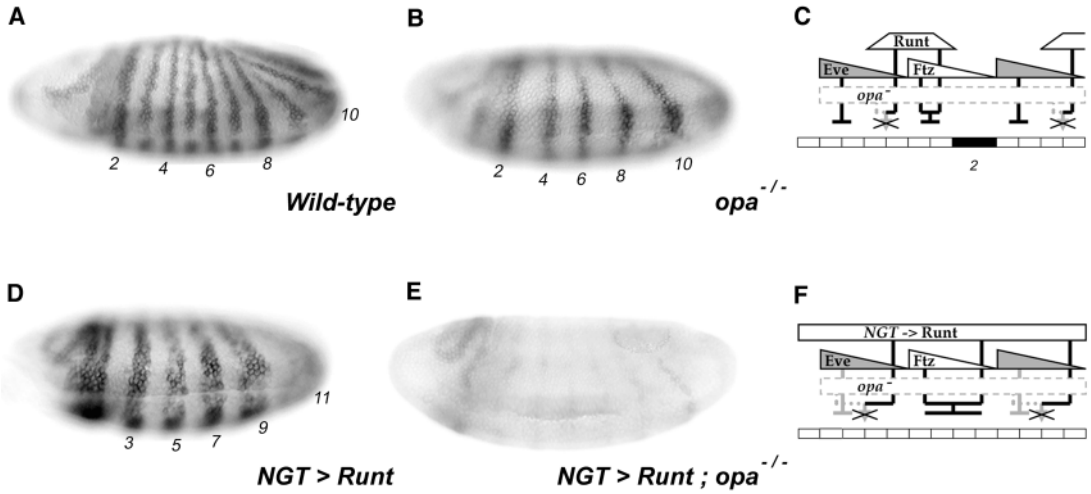

Fig. 3. Opa is required for Runt-dependent activation. Expression of *slp1* mRNA in embryos that are wild-type for *runt* (A,B), or that have high levels of *NGT*-driven Runt (D,E). Ectopic Runt in these embryos was obtained by crossing females heterozygous for both *NGT40* and *NGTA* to homozygous *UAS-Runt[232]* males. The embryos in A and D are wild type for *opa*, whereas the embryos in B and E are homozygous for the *opa[1]* mutation. (C) Schematic interpretation of the response of *slp1* to the loss of Opa in an embryo with normal Runt expression. (F) Schematic interpretation of the effects of loss of Opa in an embryo with uniform Runt expression.

levels of *NGT*-driven Runt (Fig. 2E). In wild-type embryos the *ftz* mRNA pattern is resolved to 2 cell-wide stripes by this stage. The broadened stripes of *slp1* and *ftz* in these embryos are complementary to each other (Fig. 2E). Thus, uniform expression of Runt in the blastoderm embryo leads to activation of *slp1* in all cells within odd parasegments while conversely leading to repression in all cells within even parasegments (Fig. 2F). The changes produced at these high levels of Runt may in part be indirect. Indeed, as will be shown below, the broadening of *ftz* contributes to the repression of *slp1* in even-numbered parasegments. Nevertheless, this result provides compelling evidence that Runt has a dual, parasegment-specific role in *slp1* regulation.

Runt and Opa cooperate to activate *slp1* transcription

Based on the above results we examined the role of all of the other pair-rule genes in *slp1* regulation. A somewhat surprising result from these experiments is that *slp1* expression in odd-numbered parasegments is lost in *opa* mutant embryos (Fig. 3B). The importance of Opa is surprising as expression of other segment-polarity genes is reduced, but not eliminated in *opa* mutants (Benedyk et al., 1994; Cimbora and Sakonju, 1995). Moreover, Opa is expressed at uniform levels throughout the pre-segmental region of the embryo, and thus does not provide positional information that defines the placement of *slp1* stripes relative to other pair-rule transcription factors. As shown above, the odd-numbered *slp1* stripes require Runt, and are interpreted to expand in response to ectopic Runt. We tested the requirement for Opa in this Runt-dependent activation by examining *slp1* expression in embryos that have high levels of *NGT*-driven Runt and that are also mutant for *opa*. Expression of *slp1* within the pre-segmental region is lost in these embryos (Fig. 3E). This result corroborates our interpretation that the expanded *slp1* stripes produced by *NGT*-driven Runt correspond to the odd-numbered stripes and further confirms the importance of Opa for Runt-dependent activation.

A useful feature of the GAL4 expression system is that expression levels can be varied by changing the strengths of either the GAL4 driver, or the responding UAS transgene (e.g. Li and Gergen, 1999). We took advantage of this feature to further investigate the relative roles of Runt and Opa in *slp1* regulation by generating a co-expression matrix with a panel of different *UAS-runt* and *UAS-opa* lines. Increasing the level of Opa in embryos with the same low level of *NGT*-driven Runt (Fig. 4A-C) alters *slp1* in a manner similar to that obtained by increasing Runt alone (compare Fig. 4C with Fig. 1F). Thus, Opa potentiates Runt-dependent regulation in a concentration-dependent manner. Concentration-dependent effects of Opa are also observed at both intermediate (Fig. 4D-F) and high (Fig. 4G-I) levels of *NGT*-driven Runt. In order to interpret these changes, it is useful to first consider the relatively simple, yet striking response of *slp1* to high levels of both Runt and Opa (Fig. 4I). In these embryos, *slp1* is expressed throughout the anterior head region and is nearly uniformly repressed throughout the pre-segmental region of the embryo. The anterior activation is particularly informative as none of the other pair-rule or segment-polarity gene shows this response to Runt and Opa (see below). Thus, anterior activation of *slp1* by Runt and Opa occurs in the absence of regulatory inputs from other segmentation genes. It is notable that anterior activation can be triggered either by increasing the level of Runt in embryos with constant intermediate levels of Opa (Fig. 4B,E,H), or by increasing the levels of Opa in embryos with constant intermediate levels of Runt (Fig. 4D-F). The observation that Runt and Opa are both obligatory for anterior activation, coupled with this mutual dose-dependent cooperation strongly suggests that these two factors function together in a concentration-dependent complex to activate *slp1*transcription.

The other notable response to high levels of Runt and Opa is the nearly complete repression of *slp1* throughout the presegmental region of the embryo (Fig. 4I). As described above, *slp1* and *ftz* are expressed in complementary patterns in

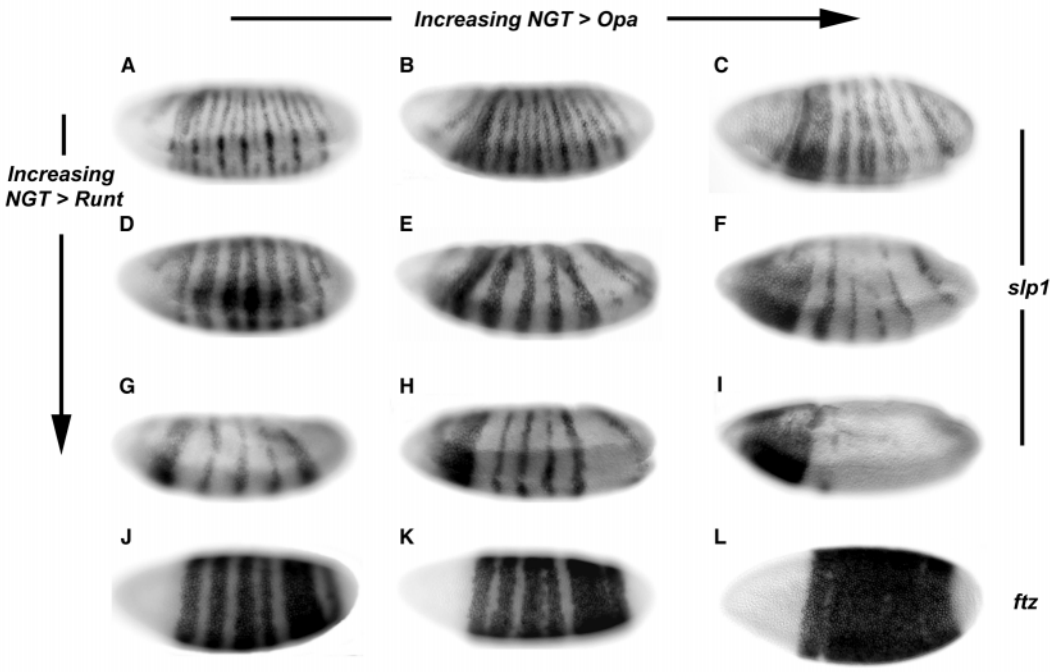

Fig. 4. Runt and Opa cooperate to activate *slp1*transcription. In situ hybridization showing the expression of *slp1* (A-I) and *ftz* (J-L) mRNAs in embryos with varying levels of *NGT*-driven Runt and Opa. In all cases, ectopic expression was obtained in crosses using females homozygous for both *NGT40* and *NGTA*. Variations in expression were obtained using different *UAS-runt* and *UAS-opa* lines. Embryos in the first row carry *UAS-runt[14]* in combination with (A) *UAS-opa[36]*, (B) *UAS-opa[12]* and (C) *UAS-opa[10]*, which are ordered in increasing strength from left to right. Embryos in the second row (D-F) carry *UAS-runt[232]* in combination with the same three *UAS-opa* transgenes. Embryos in the third and fourth rows (G-L) carry *UAS-runt[15]*, also in combination with the same three *UAS-opa* transgenes. The different levels of ectopic Runt expression are organized in increasing strength from top to bottom, *UAS-runt[232]* and *UAS-runt[15]*, giving approximately two- and fivefold increases, respectively, over the level obtained with *UAS-runt[14]* (Li and Gergen, 1999).

embryos with high uniform levels of Runt (Fig. 2E). Examination of the response of *ftz* to the co-expression of Runt and Opa indicates a perfect correlation between the elimination of *slp1* (Fig. 4G-I) and the expansion of *ftz* (Fig. 4J-L). These observations indicate that Opa potentiates the ability of Runt to activate *ftz*. Moreover, these results strongly suggest that Ftz plays a key role in *slp1* repression.

Ftz and Runt cooperate to repress *slp1*

The four-cell wide *ftz* stripes identify the even-numbered parasegments of a mid-blastoderm stage embryo. Expression of *slp1* arises in the two posterior-most cells of each of these parasegments, i.e. the cells that lose expression as the *ftz* stripes narrow during the process of cellularization. In *ftz* mutant embryos, *slp1* expression is de-repressed to produce six-cell wide stripes (Fig. 5A). Double in situ hybridization experiments with *en* and *slp1* (data not shown) indicate that this pattern is the result of de-repression in the anterior half of the even-numbered parasegments (Fig. 5B). As described above, *slp1* and *ftz* are expressed in complementary patterns in embryos that have high levels of *NGT*-driven Runt (Fig. 2E). These complementary patterns are due to repression by Ftz as *slp1* is expressed throughout the presegmental region of a *ftz* mutant embryo that has high levels of Runt (Fig. 5C). This pattern conforms precisely to the expectation for activation by the combined action of Runt and Opa (Fig. 5D). Based on these results, as well as on the close correspondence of the responses of *slp1* and *ftz* to varying levels of Runt and Opa, we conclude that Ftz prevents the activation of *slp1* by Runt and Opa.

We used ectopic expression to further investigate the role of Ftz in *slp1* regulation. *NGT*-driven Ftz represses *slp1*, but importantly only in odd-numbered parasegments (Fig. 5E). Runt expression is unaltered in these embryos (data not shown). Thus, Ftz-dependent repression of *slp1* occurs only in cells that express Runt (Fig. 5F). The observation that *slp1* expression in even-numbered parasegments is resistant to repression by Ftz indicates that some other factor is required for Ftz-dependent repression. Ftz normally represses *slp1* in Runt-expressing cells in even-numbered parasegments and ectopic Ftz leads to repression in the Runt-expressing cells in odd parasegments. This correlation strongly suggests that *slp1* is repressed by the specific combination of Runt + Ftz. We tested this hypothesis using the *NGT* system to express both Runt and Ftz throughout the embryo. Consistent with our hypothesis, co-expression of Runt and Ftz represses *slp1* throughout the embryo (Fig. 5G). This result provides a clear indication of the ability of Runt to repress *slp1*. Moreover, the reciprocal effects produced in the absence (Fig. 5C) versus presence of Ftz (Fig. 5G) provide compelling evidence that Ftz converts Runt from an activator to a repressor of *slp1* transcription.

Ftz regulates the activity of Runt on the segment-polarity genes *wg* and *en*

The above experiments were initiated due to the sensitivity and simplicity of the *slp1* response to *NGT*-driven Runt. Although *wg* is less sensitive than *slp1*, the parallel responses of these two genes (Fig. 1F,I) suggest that Ftz and Runt interact in a similar manner to regulate *wg*. The one cell-wide *wg* stripes identify the posterior-most cells within each parasegment and correspond to a subset of the *slp1*-expressing cells (Fig. 6). As observed for *slp1*, transient elimination of *run* leads to loss of *wg* expression in odd-parasegments and expanded expression in a subset of even-numbered parasegments (Fig. 7A). The

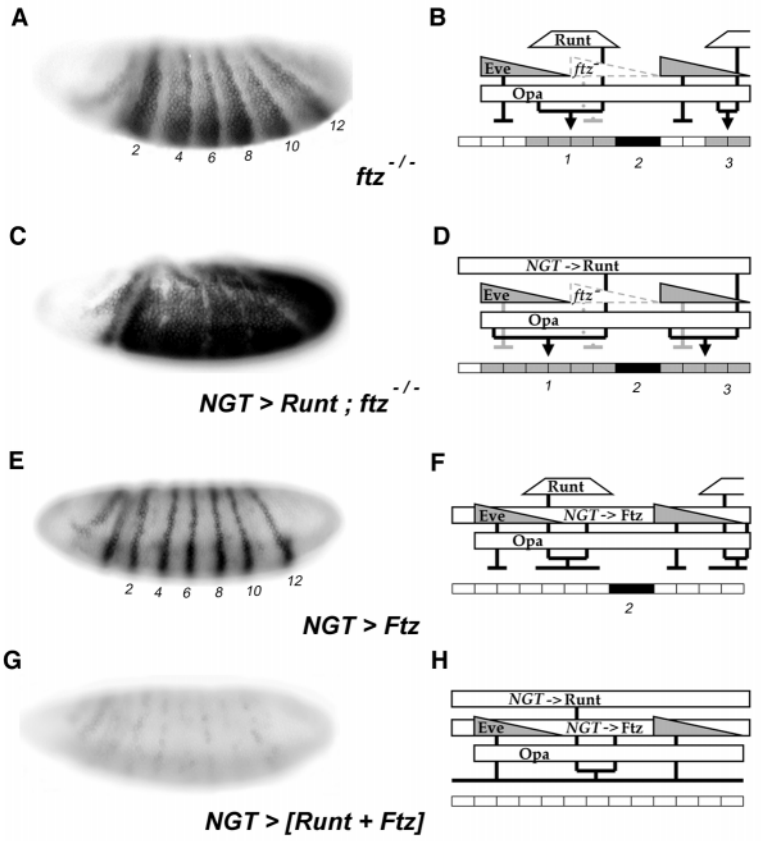

Fig. 5. Ftz converts Runt from an activator to a repressor of *slp1*. (A) Embryos mutant for *ftz* express *slp1* in the anterior half of the even-numbered parasegments, resulting in six-cell wide stripes. (B) The *ftz* mutant phenotype and the way in which this expanded expression is accounted for by Runt+Opa dependent activation. Note that *run* expression is not significantly altered in *ftz* mutant embryos at this stage (Klingler and Gergen, 1993). (C) Ectopic Runt expression in *ftz* mutant embryos activates *slp1* throughout the pre-segmental region. This embryo was obtained from a cross between a female heterozygous for *NGT40*, *NGTA* and the *ftz[11]* mutation with a male homozygous for *UAS-runt[232]* and heterozygous for *ftz[11]*. The level of *NGT*-driven Runt obtained with this combination does not fully overcome Eve-dependent repression, resulting in a few thin stripes of cells with reduced *slp1* expression. Runt+Opa-dependent activation (D) results in the *slp1* pattern shown in C. The effects of *NGT*-driven Ftz on *slp1* is shown in E and interpreted in F. The embryo in E is from a mating between homozygous *NGT40* females and homozygous *UAS-ftz[261]* males. The effects of co-expressing Runt and Ftz are shown in G and interpreted in H. The embryo in G is from a mating between homozygous *NGT40* females and males homozygous for both *UAS-runt[232]* and *UAS-ftz[261]*.

odd-numbered *wg* stripes are specifically repressed by *NGT*-driven Ftz expression (Fig. 7B), whereas co-expression of Runt and Ftz represses *wg* in both odd- and even-numbered parasegments (Fig. 7C). Thus, as found for *slp1*, Runt and Ftz specifically cooperate to repress *wg*. However, the rules for Runt-dependent activation of *wg* are more complex than for *slp1*, as *NGT*-driven co-expression of Runt and Opa is not sufficient for *wg* activation in the anterior unsegmented region of the embryo (Fig. 7D). Although the full set of rules for *wg* regulation thus remains elusive, these results demonstrate a pivotal role for Ftz in modulating the regulatory effects of Runt on *wg* expression.

Similar experiments indicate that specific interactions between Ftz and Runt are important for regulation of the segment-polarity gene *en*. However, the striking result in this case is that Runt and Ftz cooperate to activate, rather than repress transcription. *en* is normally expressed in the anterior most row of cells in each parasegment (Fig. 6). The even-numbered stripes arise in Ftz-expressing cells and require Ftz for expression (DiNardo and O'Farrell, 1987; Florence et al., 1997). Runt also plays an acute role in activation of the even-numbered *en* stripes as they are lost in response to a transient reduction in *run* (Fig. 7E). Ectopic expression experiments provide further evidence that the specific combination of Runt + Ftz gives activation of *en*. *NGT*-driven Ftz leads to expansion of the even-numbered *en* stripes (Fig.

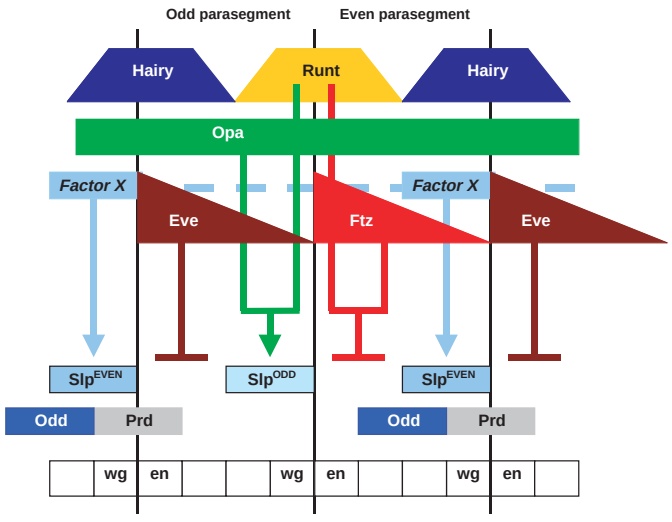

Fig. 6. The pair-rule to segment-polarity transition. The contrasting Runt-dependent activation and repression of *slp1* in different cells within the pre-segmental region of the blastoderm embryo is fully explained by the overlapping expression of Ftz, as indicated in this diagram. Also depicted are the expression domains of the pair-rule transcription factors Odd and Prd, which overlap the *slp1*stripes (Morrissey et al., 1991; Mullen and DiNardo, 1995). Combinatorial regulation by Eve, Runt, Opa and Ftz accounts for all aspects of *slp1* regulation, except for activation in even-numbered parasegments. The minimal spatial domain of activity of a Factor X that is proposed to be responsible for this aspect of *slp1* expression is depicted in pale blue. Factor X-dependent activation may also contribute to the expanded *slp1* expression obtained by transient elimination of *eve* or *run*. The possibility that Factor X is active in other cells within the pre-segmental region is indicated by the broken blue line. The strip of cells along the anteroposterior axis drawn at the bottom of this diagram shows the relationship of *wg* and *en* expression in each parasegment to that of *slp1* and the pair-rule transcription factors.

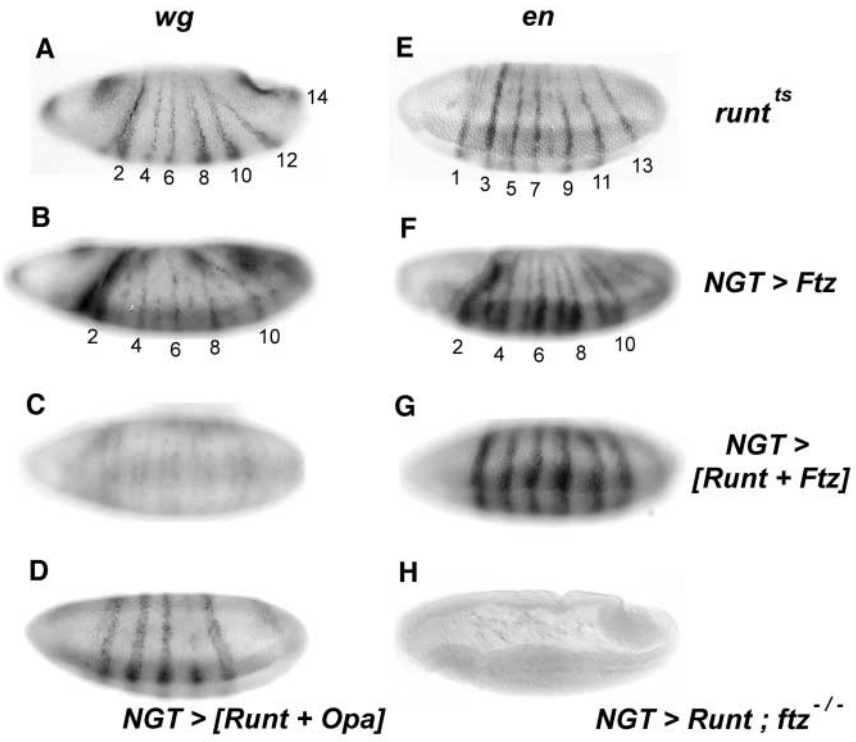

Fig. 7. Ftz modulates Runt-dependent activation and repression of *wg* and *en*. Expression of *wg* (A-D) and *en* (E-H) in response to different perturbations in Runt and Ftz activity. (A,E) Response of these two genes to transient elimination of *run* in a temperature-shifted *run[YP17]* embryo. (B,F) Response to *NGT*-driven Ftz. These embryos are from a mating of homozygous *NGT40* females to homozygous *UAS-ftz[261]* males. (C,G) *NGT*-driven co-expression of Runt and Ftz blocks *wg* expression, while producing a pair-rule pattern of *en* expression. (D) Response of *wg* to *NGT*-driven co-expression of Runt and Opa. This embryo is from a mating of females homozygous for both *NGT40* and *NGTA* to males homozygous for the *UAS-runt[13]* and *UAS-opa[14]* transgenes. This combination results in a *slp1* pattern similar to the embryos in Fig. 4F,H. (H) Response of *en* to *NGT*-driven Runt in embryos that are also homozygous for the *ftz[11]* mutation. This embryo is from the same cross used to generate the embryo in Fig. 5C.

7F), presumably into cells that normally express Runt but not Ftz. More provocative is the pair-rule like expression of *en* in response to *NGT*-driven co-expression of both Runt and Ftz (Fig. 7G). The *en* activation in these embryos is Ftz dependent as all expression is lost in *ftz* mutant embryos that have the same levels of *NGT*-driven Runt (Fig. 7H). The observation that co-expression of Runt and Ftz does not produce activation in the head suggests that another spatially restricted factor also participates in *en* activation. The non-uniform expression of *en* in the pre-segmental region of these embryos could be due to non-uniform expression of this additional activator and/or to regulatory inputs from a repressor such as *odd* (Mullen and DiNardo, 1995). Thus, as found for *wg*, additional regulatory inputs are used to refine the response of *en* to Runt and Ftz. These complications aside, the contrasting response of *en* to *NGT*-driven Runt in the presence (Fig. 7G) versus absence (Fig. 7H) of Ftz provides compelling evidence that Ftz plays a pivotal role in modulating the Runt-dependent regulation of *en*. Taken together with the results on *slp1* and *wg*, these experiments indicate that the combination of Runt + Ftz is interpreted in a gene-specific manner to give either activation or repression of segment-polarity gene transcription.

Discussion

slp1 regulation and the pair-rule to segment-polarity transition

The differential combinatorial effects of Runt and Ftz on segment-polarity gene regulation described above emerged as a result of analyzing the sensitive and relatively simple response of *slp1* to ectopic Runt. The *slp1* transcription unit is one of two redundant genes that comprise the *slp* locus (Grossniklaus et al., 1992). This locus was initially characterized as having a pair-rule function in the segmentation gene hierarchy based on a weak pair-rule phenotype associated with loss of *slp1* function (Grossniklaus et al., 1992; Nusslein-Volhard et al., 1984). The *slp1* and *slp2* genes are expressed in similar patterns during early embryogenesis. Embryos deficient for both *slp1* and *slp2* have an unsegmented lawn cuticle phenotype similar to that produced by *wg* mutations (Grossniklaus et al., 1992). This raises the question of whether it is most appropriate to consider *slp* as a pair-rule or segment-polarity locus. In the most straightforward interpretation of the segmentation hierarchy, the role of the pair-rule genes is to establish the initial metameric expression patterns of the segment-polarity genes. The initial expression of the key segment polarity genes *en* and *wg* is normal in gastrula stage embryos that are deleted for both *slp1* and *slp2* (data not shown). The expression of *wg* begins to become abnormal and is lost during early germband extension. These observations are consistent with the proposal of Grossniklaus and colleagues that *slp* expression identifies cells that are competent to maintain *wg* expression subsequent to the blastoderm stage (Cadigan et al., 1994). Based on these observations, we conclude that *slp1* and *slp2* are most appropriately classified as segment polarity genes, not pair-rule genes.

The expression of *slp1* (and *slp2*) differs from several other segment-polarity genes in that the metameric pattern is comprised of two-cell wide, rather than single-cell wide stripes. These two cell-wide stripes comprise the posterior half of each parasegment (Fig. 6). As shown above, *slp1* activation in odd-numbered parasegments requires the cooperative action of Runt and Opa, whereas in even-numbered parasegments Runt works together with Ftz to repress *slp1* expression. The simple rules involving these three factors fully account for *slp1* regulation in all of the Runt-expressing cells in the blastoderm embryo (Fig. 6) but also raise a question regarding the positional cues that regulate *slp1* expression in cells that do not express Runt.

There are four other pair-rule transcription factors that could be involved in *slp1* regulation: Eve, Hairy, Odd and Prd. The phasing of the pair-rule expression domains of these factors are shown in Fig. 6. Expression of both Odd and Prd overlaps the *slp1* stripes in a manner that suggests that neither of these factors provides positional information crucial for *slp1* regulation. Consistent with this, there are no substantial changes in the early 14-striped *slp1* pattern in embryos mutant for either *odd* or *prd* (data not shown). By contrast, elimination of either Eve or Hairy leads to changes in both the number and spacing of the *slp1* stripes. However, as these are both primary pair-rule genes some of these changes are certainly indirect and due to alterations in Runt and Ftz expression (Carroll and Scott, 1986; Ingham and Gergen, 1988; Klingler and Gergen, 1993).

Several lines of evidence indicate that Eve has a direct role in *slp1* repression. Experiments with the temperature-sensitive *eve[ID19]* mutation indicate that transient elimination of Eve at the cellular blastoderm stage leads to expanded six cell-wide *slp1* stripes because of de-repression in the anterior two cells of each odd-numbered parasegment (data not shown). These two are the cells with the highest level of Eve, indicating that the primary role of Eve at this stage is to repress *slp1* expression (Fig. 6). Complementary experiments with an inducible *hs-Eve* transgene reveal that ectopic Eve blocks *slp1* activation in both odd- and even-numbered parasegments (data not shown). This result not only confirms Eve's role as a repressor, but also reveals a crucial difference between Eve- and Ftz-dependent repression. As shown above, Ftz-dependent repression is restricted to odd-numbered parasegments unless Runt is also ectopically expressed (Fig. 5). This same restriction is observed in experiments with *hs-Ftz* transgenes (data not shown), indicating that the difference between Eve and Ftz is not due to the mode of ectopic expression. Taken altogether these results indicate that Eve and Ftz normally have comparable roles in repressing *slp1* transcription in the anterior half of the odd- and even-numbered parasegments, respectively, in late blastoderm stage embryos (Fig. 6). The key distinction in the regulation of *slp1* by these two homeodomain transcription factors is the critical role that Runt plays in Ftz-dependent repression.

One aspect of *slp1* expression not accounted for by the above rules is the factor (or combination of factors), referred to here as factor X, that is responsible for *slp1* activation in the posterior half of the even-numbered parasegments (Fig. 6). Activation in these cells is blocked either by the combination of Runt+Ftz (Fig. 5G) or by ectopic Eve (data not shown). Runt and Ftz are co-expressed anterior to these even-numbered stripes and presumably play a role in defining the anterior margin of these stripes. Conversely, Eve is expressed posterior to these cells and probably has a role in defining the posterior margins of these stripes. The sole pair-rule transcription factor that remains as a candidate for Factor X is Hairy, which is expressed in the posterior half of even-numbered parasegments (Fig. 6). However, we do not think that factor X is Hairy for several reasons. All of the evidence to date indicates that Hairy functions as a repressor (Barolo and Levine, 1997; Ish-Horowicz and Pinchin, 1987; Jimenez et al., 1997; Poortinga et al., 1998; van Doren et al., 1994). Furthermore, *NGT*-driven expression of Hairy does not lead to *slp1* activation in anterior blastoderm cells similar to that produced by the co-expression of Runt and Opa (data not shown). Identification of factor X is clearly important for a complete understanding of *slp1* regulation.

Context-dependent activities of Runt

Previous studies indicated that Runt has roles in both activating and repressing transcription of different target genes in the *Drosophila* embryo (Kramer et al., 1999; Tracey et al., 2000; Tsai and Gergen, 1994; Tsai and Gergen, 1995). The results presented above provide additional compelling evidence for this dual activity and also provide insight on factors that contribute to this context-dependent regulation. The dramatic effects of Ftz on Runt-dependent *slp1* regulation clearly demonstrate that one important component of context is the specific combination of other transcription factors that are present in a cell. Indeed, the unique and relatively simple rules for *slp1* regulation make this an especially attractive target for dissecting the molecular mechanisms whereby Ftz converts Runt from an activator to a repressor of transcription. It seems likely that the rules governing the Runt-dependent regulation of *slp1* will provide a foundation for understanding the regulation of *wg* and *gsb*, two segment-polarity genes that are expressed in a subset of *slp*-expressing cells and that respond to Runt in a manner similar, but not identical to *slp1*.

Our results also point to a second important component of context-dependent regulation by Runt. The specific combination of Runt + Ftz, which represses *slp1*, does not always give repression as these same two factors work together to activate *en* in some of these same cells at the same stage of development. Thus, cellular context alone cannot fully account for the regulatory differences and there must be a target-gene specific component of context-dependent regulation. A similar gene-specific example of context-dependent regulation has recently been described for the Runx protein Lozenge (Canon and Banerjee, 2003). In this case, the presence of binding sites for the Cut homeodomain protein helps to stabilize a complex that leads to repression of *deadpan* transcription in the same cells in which Lozenge is responsible for activation of *Drosophila Pax2*. In a strict parallel of this model, we would speculate that the *slp1* regulatory region contains binding sites for some factor that helps to stabilize a repressor complex that includes the Runt and Ftz proteins. In a reciprocal, and not mutually exclusive model, perhaps there are binding sites for a factor in the *en* regulatory region that helps to stabilize a Runt- and Ftz-dependent transcriptional activation complex. Further studies on the *en* and *slp1* cis-regulatory regions are needed in order to address these questions at the molecular level. This future work is crucial for understanding the context-dependent activity of Runt and thus the molecular logic of the control system that underlies the pair-rule to segment-polarity transition in *Drosophila* segmentation.

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