

Kinetic analysis of segmentation gene interactions in *Drosophila* embryos

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SUMMARY

A major challenge for developmental biologists in coming years will be to place the vast number of newly identified genes into precisely ordered genetic and molecular pathways. This will require efficient methods to determine which genes interact directly and indirectly. One of the most comprehensive pathways currently under study is the genetic hierarchy that controls *Drosophila* segmentation. Yet, many of the potential interactions within this pathway remain untested or unverified. Here, we look at one of the best-characterized components of this pathway, the homeodomain-containing transcription factor Fushi tarazu (Ftz), and analyze the response kinetics of known and putative target genes. This is achieved by providing a brief pulse of Ftz expression and measuring the time required for genes to respond. The time required for Ftz to bind and regulate its own enhancer, a well-documented interaction,

is used as a standard for other direct interactions. Surprisingly, we find that both positively and negatively regulated target genes respond to Ftz with the same kinetics as autoregulation. The rate-limiting step between successive interactions (<10 minutes) is the time required for regulatory proteins to either enter or be cleared from the nucleus, indicating that protein synthesis and degradation rates are closely matched for all of the proteins studied. The matching of these two processes is likely important for the rapid and synchronous progression from one class of segmentation genes to the next. In total, 11 putative Ftz target genes are analyzed, and the data provide a substantially revised view of Ftz roles and activities within the segmentation hierarchy.

Key words: *Drosophila*, *fushi tarazu*, Segmentation, Transcription

INTRODUCTION

The involvement of transcriptional cascades in development is becoming increasingly apparent. Well-known examples include the hierarchical interactions underlying hematopoiesis and adipogenesis in vertebrates (reviewed in Shivdasani and Orkin, 1996; Fajas et al., 1998), and the ecdysone and segmentation gene pathways in *Drosophila* (reviewed in Rivera-Pomar and Jackle, 1996; Thummel, 1996). Gene expression within these cascades is predominantly controlled at the level of transcript initiation, and is based on interactions between sequence-specific transcription factors and their *cis*-acting response elements. Two types of regulatory relationships, direct and indirect, can be defined. Direct interactions occur independently of intermediary gene regulation but need not involve direct molecular contact between the regulator and its target gene promoter. Indirect interactions require the activation or repression of intermediary genes, the products of which act on the target gene in question.

The segmentation gene hierarchy of *Drosophila* is one of the best-characterized developmental cascades (reviewed in Ingham and Martinez Arias, 1992; St Johnston and Nusslein-Volhard, 1992). Its role is to pattern the anterior-posterior body axis by converting maternal information, largely in the form of transcription factor gradients, into repeating segmental units.

This requires the sequential regulation of several gene classes. First, gap genes respond to the graded distribution of maternal products, forming bands of expression that span several segment primordia. Then, gap gene proteins regulate expression of the pair-rule genes generating periodic stripes, approximately a segment in width, in different portions of alternate segmental primordia. Finally, pair-rule proteins interact in various combinatorial codes to direct the expression of segment polarity genes. Segment polarity genes are expressed in stripes in different subregions of each future segment. These stripes provide the blueprint for segmental patterning. In addition to these hierarchical interactions, there are a large number of interactions that occur within each class of segmentation genes and substantial feedback occurs between classes (e.g. Harding et al., 1986; Jackle et al., 1986; Ingham and Gergen, 1988; Hulskamp et al., 1990; Kraut and Levine, 1991). This cross-regulation significantly increases the complexity of the regulatory network.

Genetic studies have provided a great deal of information on the function of this hierarchy. In particular, genetic screens are believed to have identified most of the loci involved (Nusslein-Volhard and Wieschaus, 1980; Schupbach and Wieschaus, 1986; Perrimon et al., 1989) and subsequent epistasis studies have defined the regulatory relationships between many of these genes. Although the contribution of these studies is profound, many important issues remain unclear. For example,

in very few cases do we know whether genetic interactions represent direct or indirect regulatory interactions.

The approach most often used to distinguish between direct and indirect interactions is to identify binding sites for the putative regulator within the promoters of genetically defined target genes, and to then analyze expression of normal and mutated forms of the promoter in vivo (e.g. Jiang et al., 1991; Schier and Gehring, 1992; Schier and Gehring, 1993; Capovilla et al., 1994). Although this combination of molecular and genetic approaches has been used successfully, it is quite tedious, and problems in its execution and interpretation have been encountered. For example, the functional specificity of transcription factors in vivo cannot always be predicted by the DNA-binding properties that they exhibit in vitro. In fact, there is increasing evidence that protein-protein interactions often play a decisive role in determining binding site specificity (Chan et al., 1994; Copeland et al., 1996; Reichardt et al., 1998). Additional complications encountered in these studies include redundancy of *cis*-acting binding sites and their *trans*-acting factors and the size and complexity of most developmentally regulated promoters (e.g. Howard and Struhl, 1990; Tautz, 1992; Klingler et al., 1996; Han et al., 1998).

These drawbacks emphasize the need for alternative methods capable of distinguishing between direct and indirect interactions in an in vivo setting. Examples of currently employed methods include in vivo cross-linking of proteins to their target sites (Gilmour and Lis, 1986; Gould et al., 1990; Walter et al., 1994), in vivo footprinting (Huibregtse and Engelke, 1989; Mirkovitch and Darnell, 1991) and, in *Drosophila*, visualization of proteins bound to polytene chromosomes (Zink and Paro, 1989; Urness and Thummel, 1990). Another approach used has been to monitor the temporal response of putative target genes following pulsed expression of the regulator. In this case, the distinction between direct and indirect targets is based on the assumption that direct targets respond immediately while indirect targets respond with a delay due to the time required for intermediary gene expression. Studies of this type have been used to identify likely targets of several pair-rule proteins (Ish-Horowicz and Pinchin, 1987; Ish-Horowicz et al., 1989; Morrissey et al., 1991; Manoukian and Krause, 1992, 1993; John et al., 1995; Saulier-Le Drean et al., 1998). However, the time required between direct gene interactions has yet to be rigorously determined.

The best-documented example of a direct regulatory interaction amongst *Drosophila* segmentation genes is the action of the pair-rule protein Fushi tarazu (Ftz) upon its own promoter. Ftz is a homeodomain-containing transcription factor required for the formation of alternate segmental regions (Wakimoto and Kaufman, 1981; Wakimoto et al., 1984) referred to as even-numbered parasegments. The function of Ftz as a direct regulator of its own promoter comes from a number of elegant studies. These include promoter deletion analyses, biochemical studies and molecular genetic approaches (Hiromi et al., 1985; Hiromi and Gehring, 1987; Ish-Horowicz et al., 1989; Pick et al., 1990; Schier and Gehring, 1992, 1993). The most convincing of these studies was the demonstration that mutations in a number of Ftz-binding sites identified in a minimal *ftz* enhancer element disrupt autoregulation (Schier and Gehring, 1992). More

importantly, it was shown that the effect of these mutations could be reversed by expressing a mutated version of Ftz that preferentially binds the mutated sites.

Molecular and genetic data indicate that the segment polarity gene *engrailed* (*en*) is also likely to be a direct target of Ftz (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Desplan et al., 1988; Han et al., 1989; Florence et al., 1997). Ftz-dependent enhancer elements have been identified in both 5'-flanking and intron sequences, and both contain Ftz-binding sites. Deletion of the Ftz-binding sites in an intron-derived reporter abolished *ftz*-dependent expression in vivo (Florence et al., 1997). Genetic data, although less conclusive, suggest that another possible target of Ftz is the segment polarity gene *wingless* (*wg*) (Ingham et al., 1988). Interestingly, the action of Ftz on this gene is negative, suggesting that Ftz might be able to function as both an activator and repressor of transcription. Other possible targets, suggested by genetic analyses, include the pair-rule genes *paired* (*prd*) (Baumgartner and Noll, 1990), *even-skipped* (*eve*) (Kellerman et al., 1990) and *runt* (*run*) (Klingler and Gergen, 1993).

Here, we use pulses of ectopic Ftz expression in vivo to test the kinetics of these and other putative target genes. The response time for *ftz* autoregulation is used as a standard. We find that the responses of putative target genes fall within two easily distinguished temporal windows, one that overlaps with the autoregulatory response and a second that occurs with a <10 minute delay. We argue that these different temporal windows reflect the responses of direct and indirect targets, respectively. Interestingly, we find that both activated and repressed target genes respond with the kinetics of direct target genes, indicating that Ftz does indeed have the ability to function as both an activator and repressor of transcription. Furthermore, we show that both processes proceed in parallel due to the matching of protein synthesis and degradation rates, which are the rate-limiting processes between successive gene interactions. Our data also provide important new insights into the complexity of Ftz functions required for the specification and differentiation of even-numbered parasegments.

MATERIALS AND METHODS

Fly stocks and mutant alleles

Two HSFtz lines, a heterozygous line (245-A) carrying the inducible transgene on the 3rd chromosome (Fitzpatrick et al., 1992) and a homozygous line (*hsf2*) carrying the transgene on the 2nd chromosome (Struhl, 1985), were used in this study. Two null alleles of *ftz*, *ftz*^{9H34} (Jurgens et al., 1984) and *ftz*^{w20} (Lewis et al., 1980a,b; Wakimoto and Kaufman, 1981), were used to examine target gene expression patterns in a *ftz* mutant background. Both caused identical changes in the expression of analyzed targets. To identify homozygous mutant embryos, mutant chromosomes were balanced over a *TM3* balancer chromosome marked with a *hunchback* β -gal reporter (Driever et al., 1989). The allele of *prd* used for mutant analysis was *prd*^{2.45.17} (Frigerio et al., 1986). *prd*⁻, HSFtz embryos were obtained as segregates from a stock of phenotype *prd*^{2.45.17}/*CyO*; *HSFtz*/*TM3*, *Sb*. To identify embryos homozygous for *prd*^{2.45.17}, a *CyO* balancer carrying the *hunchback* β -gal reporter was used (Driever et al., 1989).

mRNA and protein localization

Except for *ftz*, probes for in situ hybridization were prepared by PCR in two steps. The first step was to amplify a cDNA portion of the target gene from a plasmid template. The product of this reaction was then

used as template, along with the 3' primer and nucleotides to generate antisense probes. Nucleotides used in the latter reaction contained digoxigenin-labeled *dUTP* (Boehringer Mannheim). The following plasmids were used to prepare probes: *pSKrun* (provided by P. Gergen), *pJGH4* containing *hairy* cDNA (provided by D. Ish-Horowicz), *pKSII+eve121* and *prdORF2* (provided by C. Desplan), *odd 7.4 II-F* (Coulter et al., 1990), *pBSslp1* (provided by W. Ghering), *pwg-c14* (provided by N. Baker), *pBSen* and *pNB40opa* (provided by S. DiNardo), and *pBSgsb* (provided by S. Cote). *T7* and *T3* promoter primers (Promega Corporation) were used to prepare probes for *runt*, *en*, *eve*, *odd*, *slp* and *wg*. Other probes were prepared with the following primers: 5'-*GCTCGCCACTCCAATTGG-3'* and 5'-*GCAGCTGCTGCTGCTTCCGG-3'* for *hairy*, 5'-*GCCTCAGTAAGCCATGTCGC-3'* and 5'-*GGGCGGTGACATCCAGAGCC-3'* for *paired*, 5'-*GATGAACGCCCTTCATTGAGC-3'* and 5'-*TTGACCAGCTTGACTTGGC-3'* for *opa*. To prepare the *ftz* probe, the *pGEMF4* plasmid, made by subcloning a *Sall-AvaII* fragment of the *ftz* transcription unit into the vector *pGEM-1* (Promega Corporation), was first digested with *FspI*. The digested DNA was then used as a template, together with a *T7* promoter primer (Promega Corporation), to obtain *DIG*-labeled antisense cDNA by PCR.

In situ hybridization to whole-mount embryos using digoxigenin-labeled DNA probes was performed as previously described (Manoukian and Krause, 1992). Immunolocalization of proteins, and double-labeling of protein and mRNA were also performed as previously described (Manoukian and Krause, 1992). Double-labeling using anti- β -galactosidase antibodies was used to detect embryos heterozygous and wild-type for *ftz* and *prd*. Anti- β -gal antibodies were obtained from Promega Corporation and Prd antibody was provided by C. Desplan.

Determining target gene response kinetics

HSFtz embryos were collected on apple juice/agar plates for 40 minutes and aged for 2 hours and 40 minutes at 25°C. Embryos were then heat-treated for 8 minutes by submersion in water preheated to 36.5°C. Following heat-treatment, embryos were split into 8 batches and allowed to recover at 25°C. Embryos were dechorionated and fixed at 5 minute intervals thereafter, starting with a 5 minute recovery and ending with a 40 minute recovery. Once fixed, embryos were stored in MeOH at -20°C. This procedure was repeated a number of times, and the embryos representing each 5 minute recovery time pooled. Once sufficient numbers of embryos were collected, embryos representing each 5 minute interval were once again divided into aliquots for hybridization with different probes. For *ftz*, *en*, *wg* and *prd*, the experiment was repeated and the values obtained from each experiment were averaged.

Following hybridization and mounting, expression patterns were observed by microscopy and the number of altered patterns scored. For ectopically activated targets, responses were first scored as positive when ectopic expression was about 25-50% of endogenous levels. For repressed targets, unaffected stripes served as internal controls. Hence, early responses could be scored with more certainty. Stripes with about a 20% drop in relative expression levels were easily detected.

To help with counting, grids were drawn on each slide. Approximately 200-300 embryos were mounted per slide and two slides were prepared for each probe/recovery time. Percentages of embryos showing altered expression patterns were determined and plotted in relation to their time of recovery. Curves calculated in this manner overlap closely with curves generated in previous studies, where target gene responses were quantitated by enzyme reactions and colorimetry (Manoukian and Krause, 1992).

Kinetic curves were obtained for transcripts encoded by the *ftz*, *en*, *wg* and *prd* genes, and for proteins encoded by the *prd* and *en* genes. All other target gene responses were simply observed at 20 and 40 minutes post-heat shock. For heat-sensitive loci such as *run*, *eve*, *gsb* and *wg*, experiments were repeated using the heat-inducible Ftz line

hsf2 (Struhl, 1985). Responses were analyzed with 2, 4, 6 and 8 minute heat shocks. In general, all Ftz-specific responses could be reproduced with only a 2-4 minute heat shock. Non-specific responses due to heat treatment were not observed with these durations of heat shock. Specific versus non-specific effects were determined by analyzing the response of all loci in heat-shocked and non-heat-shocked Oregon R controls, treated in parallel.

RESULTS

Experimental approach

To monitor transcriptional targets of Ftz, expression patterns of putative target genes were monitored by in situ hybridization in heat-inducible Ftz (HSFtz) transgenic embryos. HSFtz embryos carry a *ftz* transgene in which the *ftz* cDNA is transcribed under control of the *hsp70* heat-inducible promoter (Struhl, 1985). When heat-pulsed, these embryos express *ftz* throughout the embryo. Two HSFtz lines (Struhl, 1985; Fitzpatrick et al., 1992), carrying different constructs inserted at different genomic locations (see Materials and methods), were used in this study. The majority of experiments were performed using a line carrying a heterozygous copy of the transgene on the third chromosome (Fitzpatrick et al., 1992). Heat pulses used for this line were for 8 minutes at 36.5°C. These conditions are optimal for the regulation of *ftz* target genes and induction of an "anti-*ftz*" cuticular phenotype, as previously described (Struhl, 1985; Ish-Horowicz et al., 1989). Under these conditions, the levels of ectopic *ftz* expressed from the transgene are lower than endogenous levels of expression (Struhl, 1985; Fitzpatrick et al., 1992).

The second line, *hsf2* (Struhl, 1985), was used when analyzing the expression of heat-sensitive loci. This line expresses the same levels of protein as the first with half the duration of heat shock (4 minutes at 36.5°C). Non-specific effects due to heat treatment were revealed by analyzing gene expression patterns in Oregon R embryos heat shocked in parallel to HSFtz embryos. No changes in target gene expression were observed in Oregon R embryos following a 4 minute heat pulse.

Kinetics of endogenous *ftz* induction

Compelling molecular and genetic evidence has been previously presented demonstrating that Ftz directly binds and regulates expression of its own promoter (Hiromi et al., 1985; Hiromi and Gehring, 1987; Schier and Gehring, 1992). Hence, the temporal response of this interaction was measured and used as a basis of comparison for other possible direct interactions. Previous studies have shown that endogenous *ftz* stripes respond to ectopic Ftz expression by limited expansion: each stripe expands anteriorly by about one cell (Fig. 1B) (Ish-Horowicz et al., 1989). To determine the kinetics of this response, HSFtz embryos were heat treated for 8 minutes and aliquots were fixed at 5 minute intervals (see materials and methods). Enough embryos (approximately 50,000 in total) were collected so that each aliquot of staged and fixed embryos could be partitioned for staining with as many as ten different probes. In this way, possible variations in staining due to differing heat shock or fixation conditions were avoided.

A minimum of 500 embryos per 5 minute interval were examined by in situ hybridization for changes in *ftz* expression. The fraction of embryos exhibiting broadened Ftz stripes was

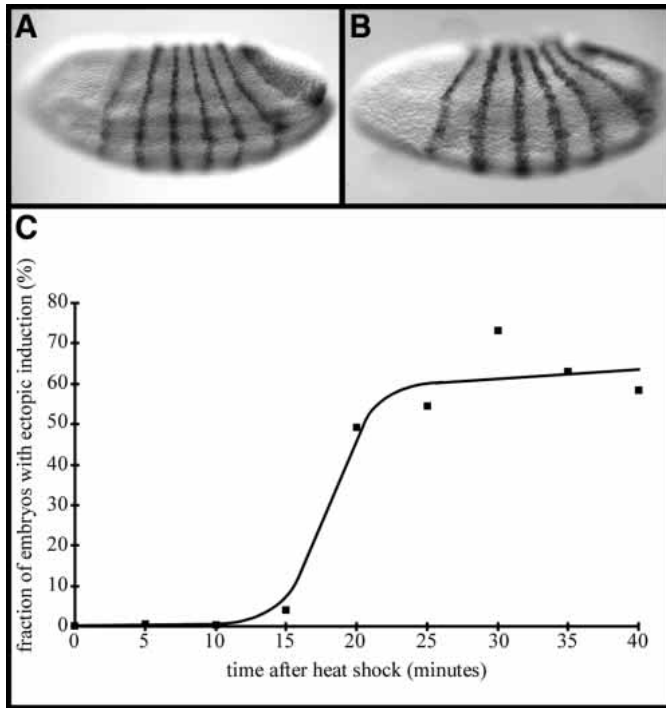


Fig. 1. Kinetics of endogenous *ftz* activation. Endogenous *ftz* mRNA in wild-type (A) and HSFtz (B) stage 7 embryos. The embryo in B was fixed 20 minutes after an 8 minute heat shock. Stripes of endogenous *ftz* expression are normally 1-2 cells wide at this stage (except stripe 7), but respond to a pulse of ubiquitous Ftz expression by expanding anteriorly an additional 1-2 cells per stripe. The kinetics of this broadening response is shown in (C). The curve shows the percentage of embryos exhibiting broadened stripes at various time points after the end of heat shock. The values shown represent the average of two separate experiments. Standard deviations for these values are between 0 and 2%.

determined. Embryos were scored as positive when the regions of broadening could be visually determined with certainty (see Materials and methods). The results of this analysis are presented in the form of a curve (Fig. 1C), which plots the percentage of embryos exhibiting expanded *ftz* stripes as a function of the time in minutes following the heat pulse. This curve represents the average of four experiments performed with two separate collection series. Embryos for each experiment were counted twice (eight counts in total) with essentially identical numbers tallied for each duplicate count.

The resultant curve shows that autoregulation, resulting in *ftz* stripe widening, occurs between 15 and 20 minutes post-heat shock. After this time, a plateau is reached with about 65% of embryos having responded. Since the HSFtz line used in this experiment is heterozygous, a maximum of 75% of the embryos examined would be expected to exhibit this response. Failure to reach this theoretical maximum at most time points is probably due to the presence of embryos that were either inaccurately staged, unfertilized or inefficiently heat shocked. One exception was the value determined for the 30 minute time point. This was a highly reproducible 72%, after which the number of embryos with widened stripes appeared to steadily decrease. While this suggests the possibility of complex

influences on the ability of Ftz to autoregulate in these cells, we are reluctant to make conclusions based on a single time point.

***engrailed (en)* activation follows the same kinetics as *ftz* activation**

The second best-characterized target of Ftz, and hence the next target that we examined, is the segment polarity gene *engrailed (en)*. Stripes of *en* initiate at the anterior edge of each parasegment (Fig. 2A). Every second stripe overlaps with the anterior portion of each *ftz* stripe and is lost in *ftz* mutant embryos (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Lawrence et al., 1987). As previously shown, ubiquitous expression of Ftz causes a broadening of expression of these *ftz*-dependent *en* stripes (Ish-Horowicz et al., 1989; Fig. 2B), making them 2-3 cells wide instead of 1-2 cells wide. This expansion occurs in the same cells as endogenous *ftz* stripe expansion. The kinetics of this response was determined in the same way as described above for endogenous *ftz*, using embryos from the same set of collections. As shown in Fig. 2C, the curve generated for *en* overlaps very closely that of the endogenous *ftz* response, suggesting that both genes are direct targets of Ftz. If *en* were regulated indirectly, a delay would be expected reflecting the time required for intermediary gene products to be expressed, to accumulate and to elicit a response.

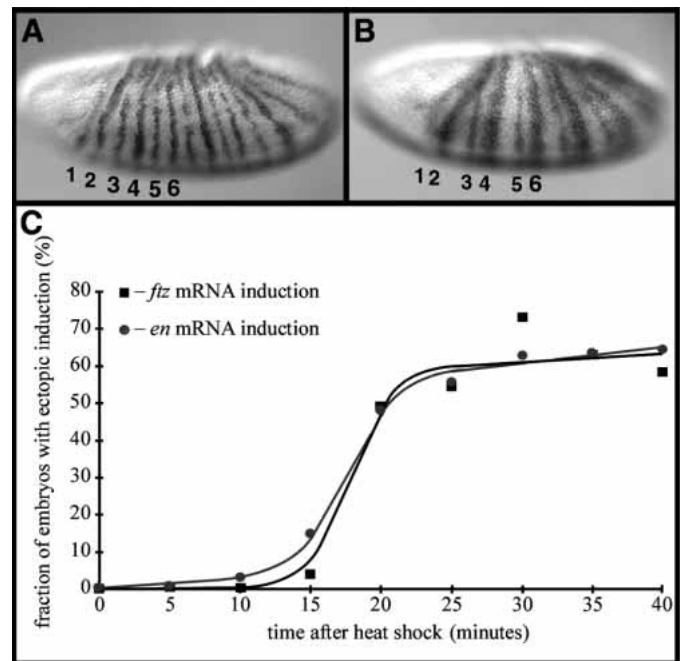


Fig. 2. The kinetics of *en* and *ftz* activation are similar. (A) Expression of *en* in a stage 7 embryo. Fourteen evenly spaced stripes are present and are each 1-2 cells wide. The first six stripes are numbered. (B) In HSFtz embryos, fixed 20 minutes after an 8 minute heat shock, even-numbered *en* stripes have expanded anteriorly by 1-2 cells. The kinetics of this response (C) overlaps that of endogenous *ftz* activation. Values for the *ftz* curve are marked as squares, and those for the *en* curve as circles. As for *ftz*, the values calculated for *en* represent the average of two separate experiments. Standard deviations for these values range between 0 and 2%.

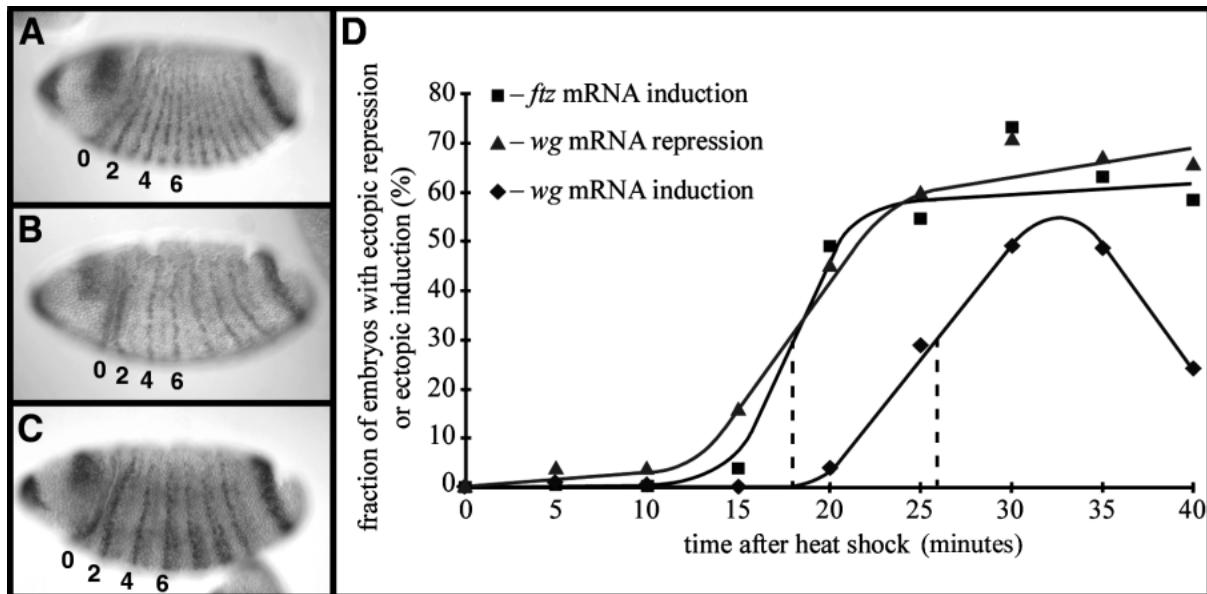


Fig. 3. Direct and indirect responses of *wg*. Expression of *wg* is shown in wild-type (A) and HSFtz (B,C) stage 6 embryos. Two different changes in expression were observed in response to ectopic Ftz. The earliest response is characterized by repression of odd-numbered *wg* stripes (B). Even-numbered stripes (designated with numbers) are also weakly repressed. In embryos fixed later (35 minutes post-heat shock) (C), odd-numbered stripes appear to expand anteriorly into odd-numbered parasegments. The kinetics of *wg* repression (triangles) and *wg* activation (diamonds) are shown in (D) and compared with the response of endogenous *ftz* (squares). The mid-points of response curves are indicated by vertical dashed lines. The values indicated represent the average of two separate experiments. Standard deviations for these values (not shown) range between 0 and 2%.

Two responses of *wingless* (*wg*) in HSFtz embryos

In contrast to *en*, the segment polarity gene *wingless* (*wg*) has been identified genetically as a negative target of Ftz (Ingham et al., 1988). This negative interaction has also been demonstrated in HSFtz embryos (Ish-Horowicz et al., 1989). Although all *wg* stripes are repressed in these embryos, the predominant effect is on odd-numbered stripes which, as shown in Fig. 3B, are completely repressed following Ftz induction. Repression of even-numbered stripes is much less efficient.

To assess whether this repression is direct, we determined the kinetics of repression as measured above for *ftz* and *en* activation. The differential repression of odd- versus even-numbered stripes of *wg* was a helpful tool and internal control for recognizing affected embryos. The kinetic curve showing the percentage of embryos affected following heat shock is presented in Fig. 3D. Again, this curve follows very closely that of *ftz* autoregulation, with the midpoint of both curves occurring at 18 minutes post-heat shock. This indicates that repression of *wg* by Ftz is also likely to be direct, and that Ftz can act as both an activator and repressor of transcription.

Repression was not the only response exhibited by *wg* in HSFtz embryos. Weak activation within most of each odd-numbered parasegment was also detected (see Fig. 3C). Fig. 3D shows that the kinetic curve of this activation response is considerably delayed relative to the kinetics of the other three responses measured thus far. This suggests that *wg* activation results from an indirect genetic interaction. A likely intermediary gene in this response is the *paired* gene (*prd*). *prd* is genetically required for the proper initiation of all 14 *wg* stripes (Ingham et al., 1988; Copeland et al., 1996) and all 14 *wg* stripes expand rapidly in HSPrd embryos (Copeland et al., 1996).

Prd functions as an intermediate between Ftz and *wg*

To test whether the *prd* gene acts as an intermediate in the positive response of *wg* to ectopic Ftz, we determined the spatial and temporal responses of *prd* in HSFtz embryos. If *prd* does function as an intermediary gene, its expression should be induced in odd-numbered parasegments where *wg* activation is later observed. Moreover, the induction of *prd* transcripts should occur with the same rapid kinetics as the *ftz*, *en* and early *wg* responses. Shown in Fig. 4B is the *prd* expression pattern detected 20 minutes after ectopic expression of Ftz. Stripes are significantly wider than those in similarly staged wild-type embryos (Fig. 4A). Using the most posterior stripe of *prd* as a landmark, it can be seen that each of the expanded stripes has broadened at its anterior edge. These regions of expansion comprise most of each odd-numbered parasegment, which is exactly where ectopic expression of *wg* occurs (Fig. 3C).

The time course of *prd* mRNA induction was assessed as described for endogenous *ftz*, *en* and *wg*. Although the slopes of the *prd* and *ftz* activation curves differ (Fig. 4E), the initial responses occur at about the same time, suggesting that the interaction between Ftz and *prd* is also direct. The differences in the slopes of the two curves are likely due to the autoregulatory nature of the *ftz* response: for a short time, Ftz is expressed from both heat shock and endogenous promoters, and then maximal expression is sustained via autoregulation at the endogenous locus. In contrast, *prd* activation takes place in regions of the embryo where neither *ftz* (Ish-Horowicz et al., 1989) nor *prd* (Morrissey et al., 1991) autoregulates. Hence, *prd* transcripts do not accumulate as quickly as those of *ftz* and soon disappear due to degradation of the ectopically expressed Ftz activator.

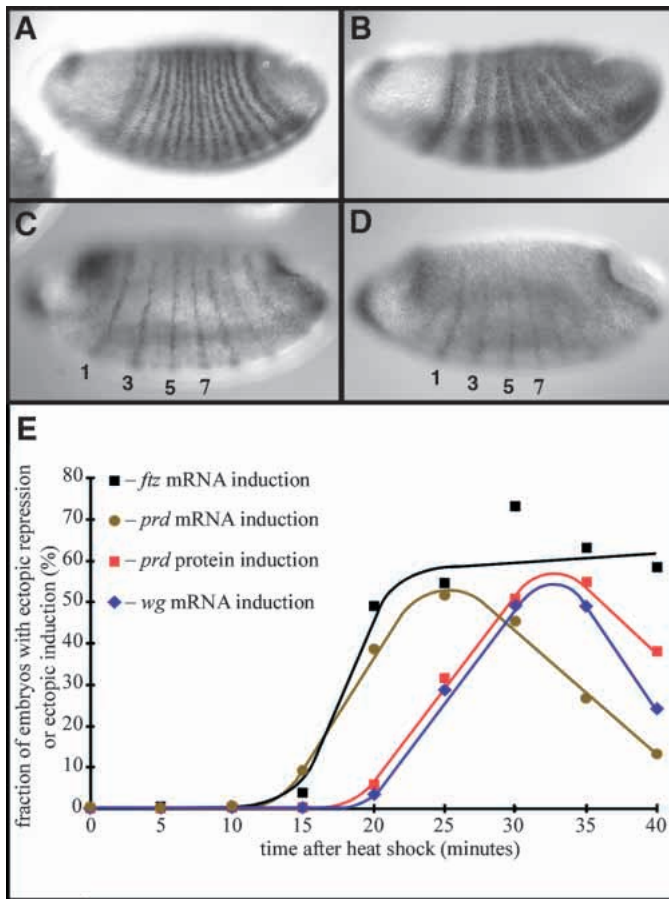


Fig. 4. *prd* is required for activation of *wg*. Expression patterns of *prd* mRNA are shown in wild-type (A) and HSFtz (B) stage 7 embryos. Secondary stripes of *prd* expand anteriorly into odd-numbered parasegments, 20 minutes after an 8 minute heat shock. These *prd*-expressing cells are the same cells that express ectopic *wg* 35 minutes after heat shock, consistent with *prd* acting as an intermediary activator. In *prd*⁻ embryos (C), even-numbered *wg* stripes are missing and odd-numbered stripes, the first four of which are numbered, are weak. In HSFtz;*prd*⁻ embryos (D), ectopic expression of *wg* fails to occur. These results are consistent with Prd acting as a requisite activator of *wg*. (E) Comparison of the induction curves of *prd* (brown circles) and *wg* (purple diamonds) transcripts relative to the curve for *ftz* autoregulation (black squares). Also shown is the time required for accumulation of Prd protein within the nucleus (red squares). It can be seen from the curves that *prd* responds with the kinetics of a direct target, and that the time course of nuclear Prd accumulation precedes that of *wg* transcript accumulation by 1-2 minutes. The values represent an average of 2 experiments. Standard deviations of these values (not shown) range between 0 and 4%.

The role of *prd* as an intermediary factor in *wg* activation was tested further by examining *wg* expression in a HSFtz;*prd*⁻ background. In the absence of *prd*, *wg* stripes should no longer expand. As shown in Fig. 4D, ectopic Ftz does indeed fail to activate ectopic *wg* in the absence of *prd*. The expression pattern in HSFtz;*prd*⁻ embryos is essentially identical to the pattern of *wg* expressed in *prd*⁻ embryos (Fig. 4C): odd-numbered stripes are weak and even-numbered stripes are essentially absent. This result is consistent with the proposed

role of *prd* as a direct activator of *wg* and as a genetic intermediate between Ftz and *wg* during ectopic stripe broadening.

Direct regulation of *wg* by Prd

Previous studies (Copeland et al., 1996), and the results presented above, support the idea that the *prd* protein (Prd) is a direct activator of *wg*. To verify this, we analyzed the nature of the temporal delay between *prd* and *wg* activation. Specifically, we examined the temporal accumulation of Prd protein with respect to *prd* and *wg* transcripts. If the interaction between *prd* and *wg* is direct, then we would expect that much of the interval between accumulation of the two transcripts would be occupied by synthesis and nuclear transport of the *prd* protein.

Nuclear expression of Prd was monitored immunohistochemically at various intervals following ectopic Ftz induction and quantitated as described above (see also Materials and methods). The kinetic curve for ectopic Prd induction is shown in Fig. 4E. This curve closely resembles that of *wg* mRNA activation except that it is shifted by 1-2 minutes to the left (earlier). This indicates that most of the delay observed between the accumulation of *prd* and *wg* transcripts (about 8 minutes) is consumed by the synthesis and localization of *prd* protein. The time required (~6-7 minutes) may be fairly typical of other segmentation proteins expressed at this stage. Indeed, the delay between detection of *en* transcript and protein responses was also 6-7 minutes, with curves that were virtually identical to those of *prd* transcripts and protein (data not shown).

These data do not exclude the possibility that there are genes in addition to *prd* that are required for ectopic activation of *wg*. However, if such gene products are required, their rates of synthesis or removal do not appear to supercede the temporal limitations imposed by the synthesis of Prd.

Primary pair-rule genes do not respond directly to ectopic Ftz

The pair-rule genes *hairy* (*h*), *runt* (*run*) and *even-skipped* (*eve*) were previously designated as primary pair-rule genes, as they were believed to regulate, and not to be regulated, by the other pair-rule loci (Carroll and Scott, 1986; Howard and Ingham, 1986; Ingham and Gergen, 1988; Pankratz et al., 1990). However, the results of more recent genetic analyses suggest that *eve* and *run* may be targets of Ftz (Kellerman et al., 1990; Klingler and Gergen, 1993). Given the conflicting nature of these results, we analyzed expression patterns of the primary pair-rule genes in HSFtz embryos to clarify their regulatory relationships. Since we have consistently observed primary and secondary responses that peak 20 and 35 minutes post-heat shock (this study and Manoukian and Krause, 1992; Manoukian and Krause, 1993; Saulier-Le Drean et al., 1998), we examined the primary pair-rule gene expression patterns at these time points. The more efficient Ftz-expressing line, *hsf2* (Struhl, 1985), was used due to the sensitivity of *eve* and *run* to longer (>4 minutes) heat shocks.

Fig. 5B shows a typical example of *eve* expression 20 minutes after induction of ectopic Ftz. This pattern shows no obvious differences from the wild-type pattern shown in Fig. 5A. Likewise, expression of *h* and *run* were also unaffected 20 minutes after induction of Ftz (not shown). This was true for

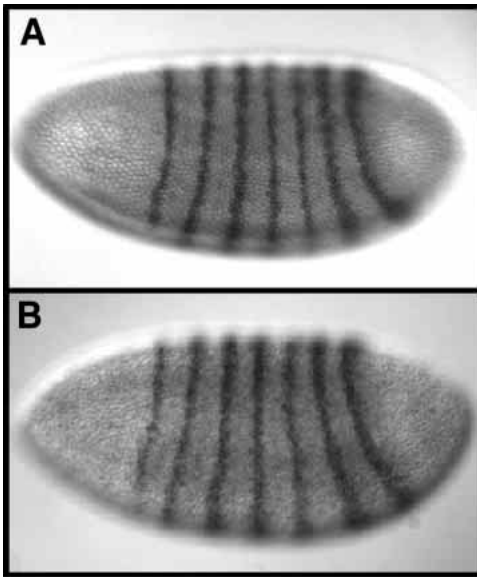


Fig. 5. Expression of *eve* is unaffected by ectopic Ftz. Expression of *eve* is shown in wild-type (A) and *hsf2* (B) stage 5 (late) embryos. The embryo in B was fixed 20 minutes after a 4 minute heat shock. No significant differences in the two expression patterns were observed.

all stages (5-7) examined. Thus, no evidence was found to support the possibility that these genes are direct targets of Ftz.

Somewhat surprisingly, no differences in primary pair-rule gene expression patterns were apparent at the later recovery time either (data not shown), indicating a lack of both direct and indirect responses. Similarly, no primary or secondary responses were observed for another pair-rule gene *odd-skipped* (data not shown). Thus, only a subset of genes investigated in this study respond to ectopic Ftz, further demonstrating the specificity of this assay and Ftz function.

***prd*, *odd-skipped* (*odd*) and *sloppy-paired* (*slp*) are direct targets of Ftz**

To determine the regulatory relationships between Ftz and the other non-primary pair-rule genes, we analyzed the expression of *odd-skipped* (*odd*) and *sloppy-paired* (*slp*) in HSFtz embryos fixed 20 and 35 minutes post Ftz induction. Expression patterns of these genes were also examined in *ftz* mutant embryos to obtain genetic confirmation of the interactions observed.

Like *ftz*, *en* and *prd*, *odd* appears to be directly activated by Ftz. In stage 5 embryos, ectopic expression of Ftz causes rapid expansion of *odd*, from its initiating pattern of six stripes (Fig. 6A), to near homogeneous expression across the germband (Fig. 6B). In stage 6 embryos, *odd* is normally expressed in 14 evenly expressed stripes (see Fig. 6D). Ectopic Ftz causes an intensification of the primary *odd* stripes at this stage (Fig. 6E). These stripes are derived from the original 7 stripes that overlap *ftz* stripes. In stage 7 embryos, these primary stripes are not only intensified, but expand anteriorly as well (from about 1 cell wide to 2 cells wide; data not shown). The percentage of embryos responding to ectopic Ftz, at all stages tested, was about the same as the percentage of embryos that show *ftz* autoregulation, *en* and *prd* activation and *wg* repression. Thus,

Ftz appears to be an activator of *odd* at all stages tested. This positive relationship between Ftz and *odd* is consistent with the differences in *odd* expression observed in *ftz* mutant embryos. Stripes of *odd* appear to be diminished in intensity in stage 5 embryos (Fig. 6C), and primary stripes are weak or missing in stage 6 (Fig. 6F) and 7 (not shown) embryos.

Unlike *prd* and *odd*, the pair-rule gene *slp* is negatively regulated by Ftz: ectopic expression of Ftz results in the differential repression of secondary *slp* stripes (Fig. 6H). Again, the penetrance of repression at the 20 minute recovery time was about 60%, as has been measured for the other genes exhibiting direct responses. As might be expected, *slp* stripes expand in *ftz* mutant embryos, filling the regions where Ftz is normally expressed (Fig. 6I). Thus, as with *wg*, Ftz appears to act as a direct repressor of *slp*. This effect is likely exerted through the response elements or *trans*-acting factors that regulate secondary stripe expression.

Gooseberry (*gsb*) is an indirect target of Ftz

Genetic studies have suggested that the segment polarity gene *gooseberry* (*gsb*), like *wg*, may be repressed by Ftz. In *ftz* mutant embryos, *gsb* stripes expand into the regions where Ftz is normally expressed, fusing to form seven wide stripes (Fig. 7B). To test whether this interaction is direct, we examined *gsb* expression in *hsf2* embryos fixed 20 and 35 minutes after a 4 minute heat shock. As shown in Fig. 7C, no response was observed 20 minutes post heat shock. However, changes were detected in embryos fixed 35 minutes post heat shock; stripes expanded into the ventral regions of odd-numbered parasegments (Fig. 7D). Interestingly, this response is a positive one, in contrast to the negative response predicted from expression in *ftz*⁻ embryos. This apparent contradiction can be reconciled by postulating that *gsb* is indirectly regulated by Ftz and that different intermediary factors are involved in each case. The delayed nature of the response in HSFtz embryos is consistent with this interpretation, and a likely intermediary factor is Prd, since *gsb*, like *wg*, appears to be activated by Prd (Cai et al., 1994). In *ftz*⁻ embryos, a likely intermediary activator is *wg*, since *wg* also expands in *ftz*⁻ embryos (Ingham et al., 1988) and appears to function as an activator of *gsb* (Li and Noll, 1993). An alternative explanation is that Ftz has the ability to repress *gsb* directly, but that this effect is spatially limited to regions where *ftz* is normally expressed. An exception would have to be made, however, in the anterior-most cell of each *ftz* stripe, where Ftz and *gsb* normally overlap.

DISCUSSION

Using kinetics to distinguish between direct and indirect gene interactions

Distinguishing between direct and indirect gene interactions is necessary prior to studying the complex molecular interactions that control them. In this study, we used a kinetic approach to identify direct and indirect targets of ectopically expressed Ftz. An advantage of this approach is that it can be carried out *in vivo* at the time(s) that the protein of interest is normally expressed. This is crucial for Ftz as, like many other transcription factors, it is dependent upon interactions with specific cofactors in order to recognize and regulate specific targets (Copeland et al., 1996; Guichet et al., 1997; Yu and

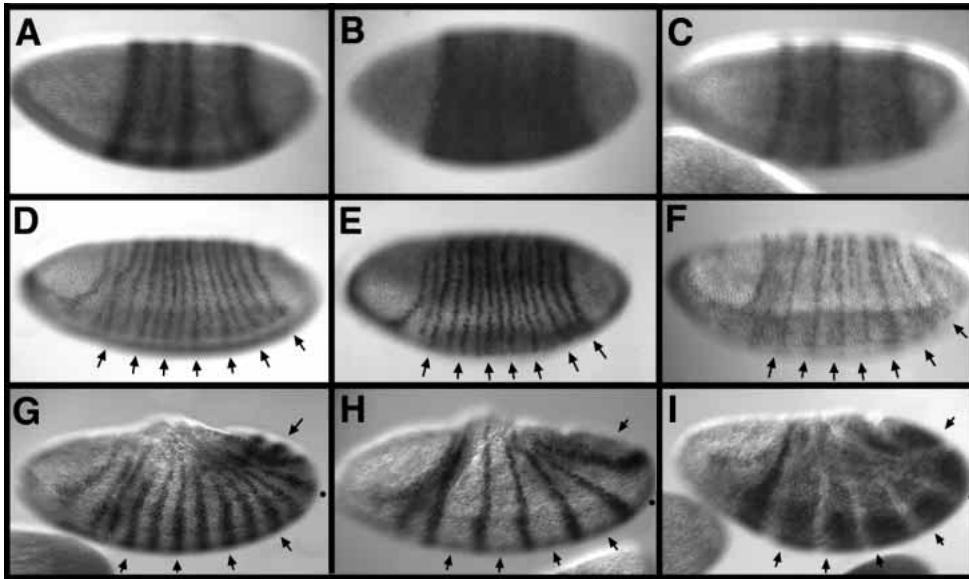


Fig. 6. Ftz activates *odd* and represses *slp*. Transcript patterns are shown, from top to bottom, for the pair-rule genes: early *odd* (stage 5: A-C), late *odd* (stage 6: D-F), and *slp* (stage 7: G-I). Embryos on the left (A,D,G) are wild-type, embryos in the middle (B,E,H) are HSFtz embryos heat-treated for 8 minutes and recovered for 20 minutes, and embryos on the right (C,F,I) are *ftz*⁻ embryos. Note that the effects of ectopic Ftz are opposite to those of loss of *ftz*. Arrows point to the affected stripes.

Pick, 1997). Another advantage of the kinetic approach is that analysis is relatively fast and can be applied to a large number of putative targets with no prior knowledge of their genetic relationships. When combined with other techniques, such as expression pattern analyses in mutant backgrounds and promoter deletion analyses, results can be confirmed and mechanisms determined.

Primary and secondary response windows

The results of our analysis with Ftz show that target genes respond to pulses of Ftz expression within two distinct temporal windows. Direct responses are 50% complete within about 18 minutes post heat shock. Indirect responses do not reach the same level of response until 26 minutes post heat shock. Thus, the time between successive gene interactions, for the genes studied here, is just under 10 minutes. This

time interval is shorter than the 10-15 minute approximation of previous studies (Manoukian and Krause, 1992, 1993; Saulier-Le Drean et al, 1998). Our analysis of protein and RNA levels indicates that the rate-limiting process in this <10 minute interval, for activated intermediates, is the time required for protein synthesis and translocation to the nucleus. Secondary responses are detected immediately thereafter. The products of repressed target genes are degraded within the same time frame. Hence, secondary responses occur within the same time frame regardless of whether the intermediary gene is activated or repressed. Although this generalization appears to hold for the genes tested in this and previous studies, other genes in the pathway should be tested for further verification.

The ~18 minute response time observed for primary responses following heat shock is consistent with the response times observed for targets of other ectopically expressed segmentation proteins (Ish-Horowicz and Pinchin, 1987; Manoukian and Krause, 1992, 1993; John et al., 1995; Saulier-Le Drean et al, 1998). The relatively long time period required for these primary responses (18 minutes, versus 8 minutes for subsequent responses) is likely due to the use of heat-inducible transgenes. Time is required for the heat shock effect itself, and then synthesis, processing and translation of the induced mRNAs are further delayed by the heat shock response (Yost and Lindquist, 1986; Saavedra et al., 1996). Secondary and tertiary response times do not appear to be appreciably affected by the prior heat shock, since these occur well after the embryos have returned to normal temperatures and their response times are approximately equal (≤ 10 minutes each).

Factors determining primary and secondary response times

Within a transcriptional cascade, the time required between successive gene interactions will depend upon a multitude of

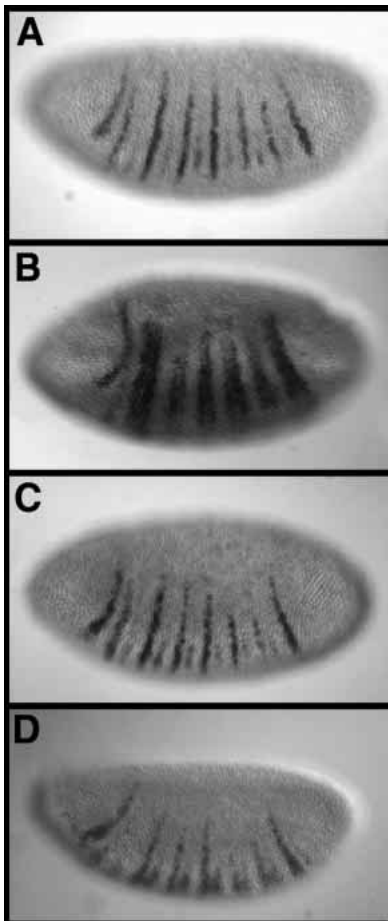


Fig. 7. Indirect regulation of *gsb* by Ftz. Expression of *gsb* transcripts is shown in WT (A), *ftz*⁻ (B) and *hsf2* embryos heat-treated for 4 minutes and recovered for 20 minutes (C) or 35 minutes (D). In *ftz*⁻ embryos, *gsb* stripes expand into even-numbered parasegments (C), suggesting negative regulation by *ftz*. However, no change in *gsb* expression was observed 20 minutes post-heat shock (C), and the secondary response (D) was positive.

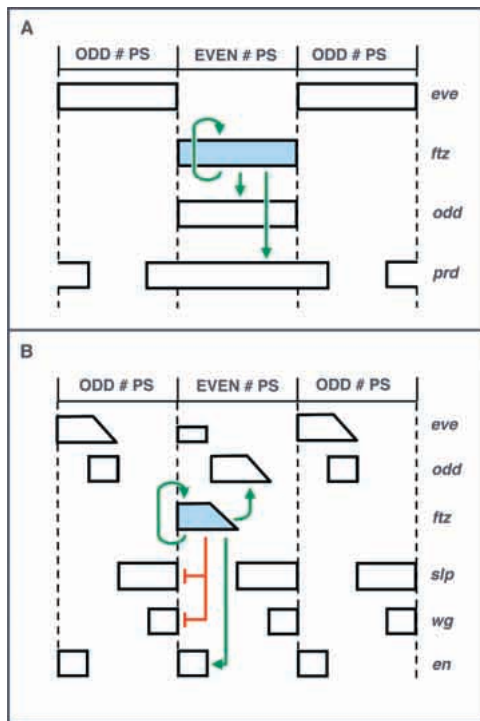


Fig. 8. Direct gene-regulatory interactions triggered by Ftz. A schematic representation of three consecutive parasegmental intervals is shown. Each parasegment is designated by text and solid black lines at the top of each panel as well as vertical dashed lines. Spatial gene expression is presented in the form of boxes. Odd-numbered parasegments are distinguished by expression of *even-skipped* (*eve*) whereas even-numbered ones express *fushi tarazu* (*ftz*) (blue box). Positive interactions triggered by Ftz are shown in green and the negative ones are shown in red. During early stage 5 (A), Ftz autoregulates its own expression as well as positively regulates *odd* and *prd* (green solid arrows). These interactions are believed to be direct. Later, during stage 6 (B), Ftz continues to autoregulate and begins to activate even-numbered stripes of *en* (green solid arrows). At the same time, it represses (red blunt-ended lines) odd-numbered stripes of *wg* and secondary stripes of *slp*, preventing their expression in even-numbered parasegments.

factors. These include the time required for transcription, RNA processing, transport, translation and protein localization. The duration of these events will depend, in turn, on the size of the transcription unit and its products. The stability of each gene product is also a factor. If there is significant variability amongst genes with regard to each of these parameters, then target gene responses will fall into a variety of overlapping temporal windows. This appears not to be the case with the genes investigated in this study.

All of the genes investigated here encode transcripts that are ~2 kb in length, have few or no introns (e.g. Kuroiwa et al., 1984; Ish-Horowitz et al., 1985; Poole et al., 1985; Kilchherr et al., 1986; Macdonald et al., 1986; Baumgartner et al., 1987; Drees et al., 1987; Gergen and Butler, 1988) and have similar half-lives (between 5 and 10 minutes) (Edgar et al., 1986; Manoukian and Krause, 1992, 1993). In addition, the protein products of each gene are on average ~50 kDa, and also have similar half-lives (between 5 and 10 minutes) (Edgar et al., 1987; Kellerman et al., 1990; Manoukian and Krause, 1992).

This means that the time required for synthesis of each gene product should be very similar, consistent with the results of this and previous studies. Importantly, the times required for protein import (activated targets) and removal (repressed targets) from the nucleus appear to be virtually identical, indicating that protein synthesis and degradation rates are also closely matched. This matching of synthesis and degradation rates may have evolved to ensure that activation and repression events can proceed in parallel. In pathways such as this, where progression down the hierarchy from one class of genes to the next must be kept in synchrony, and at the same time progress as rapidly as possible, this optimization and matching of rates may be crucial. Further support for matched rates of activation and repression comes from a study in which the Hairy protein was converted from a repressor into an activator and expressed ectopically (Jimenez et al., 1996). Activation of target genes that are normally repressed by Hairy was achieved within the same 20 minute recovery period.

Although these results show that target gene responses can be readily separated into distinct temporal windows, variations in the curves within these windows may occur, not just because of synthesis and degradation rate differences, but also due to differences in the molecular mechanisms underlying each interaction. For example, the curve representing the response of *prd* differed from those of the other direct responses, most likely because it is the only response that occurs where *ftz* autoregulation does not. Where autoregulation occurs, the responses are enhanced and maintained by the endogenous *ftz* gene product. Another important point to keep in mind is that direct interactions, as defined here, need not involve direct contact between Ftz and its target regulatory elements. Effects could also be relayed via a series of protein interactions, the complexity and nature of which could vary the speed or magnitude of the response. However, from the data obtained here, it seems that these types of molecular variations, if they are occurring, cause temporal shifts that are minor in comparison to the significant intervals of time required for the transcription, translation and localization of intermediary gene products.

Direct targets of Ftz

Our results suggest that genes directly activated by Ftz include the endogenous *ftz* gene, the pair-rule genes *prd* and *odd*, and the segment polarity gene *en*. Genes that appear to be directly repressed by Ftz include the pair-rule gene *slp* and the segment polarity gene *wg*. All six loci responded with similar kinetics. The spatial and regulatory relationships amongst these genes are summarized in Fig. 8.

The importance of *ftz* gene autoregulation and *en* activation is well described in the literature (Morata and Lawrence, 1975; Kornberg, 1981; Hiromi and Gehring, 1987), and the data arguing that both interactions are direct were already quite persuasive (Hiromi et al., 1985; Hiromi and Gehring, 1987; Desplan et al., 1988; Han et al., 1989; Pick et al., 1990; Schier and Gehring, 1993; Florence et al., 1997). There is less support for the activation of *prd* and the repression of *wg* (Ingham et al., 1988; Ish-Horowitz et al., 1989; Baumgartner and Noll, 1990; Gutjahr et al., 1994). Regulation of the *odd* and *slp* genes by Ftz has not been previously noted. Support for the less-well-characterized interactions will be provided below, as well as their likely relevance to the segmentation process.

Previous observations suggesting a positive regulatory relationship between Ftz and *prd* include the overlapping expression patterns of the two genes, the presence of Ftz-binding sites within the *prd* promoter, and inappropriate narrowing of *prd* and *prd* reporter gene stripes in *ftz* mutant embryos (Kilchherr et al., 1986; Baumgartner and Noll, 1990; Gutjahr et al., 1994). Our data support the occurrence of a direct interaction between the two genes and suggest that the most important function of this interaction is to maintain *prd* expression in the posterior regions of even-numbered parasegments, where *prd* is required for the activation of *wg* (this study and Ingham et al., 1988; Copeland et al., 1996). Interestingly, *prd* was the only gene that responded to ectopic Ftz in the anterior regions of odd-numbered parasegments. This suggests that the molecular interactions governing activation of *prd* may differ from those involved in other Ftz target gene responses.

Our data also indicate that Ftz is an effective activator of *odd*. This is consistent with the coinciding spatial expression patterns of the two genes during stages 5 and 6 (Coulter et al., 1990; Manoukian and Krause, 1992), and the reduction in levels of *odd* expression in *ftz* mutant embryos (Fig. 6C,F). The requirement of Ftz as an activator of *odd* beyond stage 5 also suggests a unique mechanism for the regulation and kinetics of *odd* stripe narrowing. During stage 6 and 7, stripes of *odd* and *ftz* become narrower as expression is lost at their posterior edges (see Fig. 8B). The narrowing of *ftz* stripes occurs first as a result of repression by Odd (Saulier-Le Drean et al., 1998). The resolution of *odd* stripes, which follows immediately thereafter, may be due to prior loss of Ftz. Thus, Odd appears to be instigating its own removal by repressing its own activator. The participation of other genes must be invoked, however, to explain why Ftz fails to maintain *odd* expression in the anterior-most Ftz-expressing cells of each Ftz stripe, and why *odd* continues to be expressed just behind each Ftz stripe, well after Ftz has been removed from those cells.

The broadening of *wg* stripes in *ftz* mutant embryos (Ingham et al., 1988) and repression of *wg* in HSFTz embryos (Ish-Horowicz et al., 1989) had previously suggested that *wg* may be a target of Ftz. However, because of the very different nature of this interaction, opposite to that of the well-characterized activation functions, further confirmation of a direct interaction was sought. The near identical kinetics of *wg* repression and *ftz* autoregulation are consistent with the ability of Ftz to function as both an activator and a repressor of transcription. Our ectopic expression studies suggest that Ftz acts on *wg* via regulatory elements that specifically regulate odd-numbered stripes, and that it is these stripes that expand in *ftz* mutant embryos.

The repression by Ftz of *slp* stripes, as well as *wg* stripes, indicates that repression is an important aspect of Ftz function. Negative regulation of *slp* by Ftz is consistent with the non-overlapping expression patterns of the two genes (see Fig. 8B) and with the broadening of *slp* stripes that we observed in *ftz* mutant embryos (Fig. 6I). Our data suggest that the normal role of Ftz is to prevent posterior expansion of secondary *slp* stripes into even-numbered parasegments.

In summary, Ftz controls even-numbered parasegment formation and polarity by a number of direct and indirect interactions that span a time interval of at least 30–60 minutes (summarized in Fig. 8). Ftz activates *en* expression at the

anterior edges of each parasegment directly. It also activates *en* indirectly by repressing the *en* repressor *slp*. At the same time, Ftz excludes expression of *wg* throughout all but the posterior edge of each even-numbered parasegment. Facilitation of *wg* expression in these cells is brought about indirectly by the activation of *prd*. Exclusion of *en* and *wg* expression within the middle of these parasegments is controlled indirectly by activation of *odd*, which encodes a repressor of both genes (Manoukian and Krause, 1993; Mullen and DiNardo, 1995; Saulier-Le Drean et al., 1998). The activation of *odd* also results in *ftz* stripe narrowing and further polarization of each even-numbered parasegment. Thus, Ftz has many functions throughout each even-numbered parasegment. This conclusion differs from that of an earlier study in which it was suggested that the only real requirement of Ftz may be to activate *en* at the anterior edges of even-numbered parasegments (Lawrence et al., 1987).

Five of the eleven genes tested in this study did not respond to ectopic Ftz with the kinetics of direct target genes. These include the pair-rule genes *eve*, *h*, *run* and *opa*, and the segment polarity gene *gsb*. Except for *gsb*, secondary responses were also absent. This suggests that the effects observed in previous genetic studies suggesting that *eve* (Kellerman et al., 1990) and *run* (Klingler and Gergen, 1993) may be regulated by Ftz, are most likely due to indirect effects. We cannot exclude the possibility, however, that such interactions do occur and that they were missed here due to an inability of Ftz to regulate these genes outside the normal domains of Ftz expression.

Kinetic assessment of genetic hierarchies

A major goal of the post genome sequencing era will be to organize all newly identified genes into comprehensive genetic circuits. Powerful new methods will be required to perform this task efficiently and accurately. One new approach that shows a great deal of potential is the use of high-density oligonucleotide arrays (DeRisi et al., 1997; Wodicka et al., 1997). Like the kinetic approach described here, this is a functional assay in that it has the potential to identify genes that respond directly to specific cellular stimuli or programmed steps in differentiation. As currently employed, however, changes in gene expression may not represent direct interactions. For example, a recent analysis of genes affected by mutations in components of the general transcription machinery used a 45 minute recovery time following inactivation of temperature-sensitive alleles (Holstege et al., 1998). Based on our results, 45 minutes is enough time for several successive gene interactions to take place. To identify direct targets, a kinetic approach, much as described here, would have to be employed. Genes could be turned on or off using a variety of techniques, and then the responses monitored at intervals thereafter. Genome-wide analyses of this type can then be used to comprehensively solve complex hierarchies such as the segmentation gene hierarchy studied here. Once direct circuitry is established, the more time-consuming business of establishing the relevance and molecular mechanisms underlying each interaction can follow.

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REFERENCES

- Baumgartner, S., Bopp, D., Burri, M. and Noll, M. (1987). Structure of two genes at the gooseberry locus related to the paired gene and their spatial expression during *Drosophila* embryogenesis. *Genes Dev.* **1**, 1247-67.
- Baumgartner, S. and Noll, M. (1990). Network of interactions among pair-rule genes regulating paired expression during primordial segmentation of *Drosophila*. *Mech. Dev.* **33**, 1-18.
- Cai, J., Lan, Y., Appel, L. F. and Weir, M. (1994). Dissection of the *Drosophila* paired protein: functional requirements for conserved motifs. *Mech. Dev.* **47**, 139-50.
- Capovilla, M., Brandt, M. and Botas, J. (1994). Direct regulation of decapentaplegic by Ultrabithorax and its role in *Drosophila* midgut morphogenesis. *Cell* **76**, 461-75.
- 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**, 113-26.
- Chan, S. K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. S. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell* **78**, 603-15.
- Copeland, J. W., Nasiadka, A., Dietrich, B. H. and Krause, H. M. (1996). Patterning of the *Drosophila* embryo by a homeodomain-deleted Ftz polypeptide. *Nature* **379**, 162-5.
- Coutler, D. E., Swaykus, E. A., Beran-Koehn, M. A., Goldberg, D., Wieschaus, E. and Schedl, P. (1990). Molecular analysis of odd-skipped, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. *EMBO J* **9**, 3795-804.
- DeRisi, J. L., Iyer, V. R. and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**, 680-6.
- Desplan, C., Theis, J. and O'Farrell, P. H. (1988). The sequence specificity of homeodomain-DNA interaction. *Cell* **54**, 1081-90.
- DiNardo, S. and O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of engrailed expression by pair-rule genes. *Genes Dev.* **1**, 1212-25.
- Drees, B., Ali, Z., Soeller, W. C., Coleman, K. G., Poole, S. J. and Kornberg, T. (1987). The transcription unit of the *Drosophila* engrailed locus: an unusually small portion of a 70,000 bp gene. *EMBO J* **6**, 2803-9.
- Driever, W., Thoma, G. and Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**, 363-7.
- Edgar, B. A., Weir, M. P., Schubiger, G. and Kornberg, T. (1986). Repression and turnover pattern fushi tarazu RNA in the early *Drosophila* embryo. *Cell* **47**, 747-54.
- Edgar, B. A., Odell, G. M. and Schubiger, G. (1987). Cytoarchitecture and the patterning of fushi tarazu expression in the *Drosophila* blastoderm. *Genes Dev.* **1**, 1226-37.
- Fajas, L., Fruchart, J. C. and Auwerx, J. (1998). Transcriptional control of adipogenesis. *Curr. Opin. Cell Biol.* **10**, 165-73.
- Fitzpatrick, V. D., Percival-Smith, A., Ingles, C. J. and Krause, H. M. (1992). Homeodomain-independent activity of the fushi tarazu polypeptide in *Drosophila* embryos. *Nature* **356**, 610-2.
- Florence, B., Guichet, A., Ephrussi, A. and Laughon, A. (1997). Ftz-F1 is a cofactor in Ftz activation of the *Drosophila* engrailed gene. *Development* **124**, 839-47.
- Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation gene paired and the *Drosophila* PRD gene set as part of a gene network. *Cell* **47**, 735-46.
- Gergen, J. P. and Butler, B. A. (1988). Isolation of the *Drosophila* segmentation gene runt and analysis of its expression during embryogenesis. *Genes Dev.* **2**, 1179-93.
- Gilmour, D. S. and Lis, J. T. (1986). RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in *Drosophila melanogaster* cells. *Mol. Cell Biol.* **6**, 3984-9.
- Gould, A. P., Brookman, J. J., Strutt, D. I. and White, R. A. (1990). Targets of homeotic gene control in *Drosophila* [published erratum appears in *Nature* 1990 Dec 6;348(6301):560]. *Nature* **348**, 308-12.
- Guichet, A., Copeland, J. W., Erdelyi, M., Hlousek, D., Zavorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H. M. and Ephrussi, A. (1997). The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* **385**, 548-52.
- Gutjahr, T., Vanario-Alonso, C. E., Pick, L. and Noll, M. (1994). Multiple regulatory elements direct the complex expression pattern of the *Drosophila* segmentation gene paired. *Mech. Dev.* **48**, 119-28.
- Han, K., Levine, M. S. and Manley, J. L. (1989). Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* **56**, 573-83.
- Han, W., Yu, Y., Su, K., Kohanski, R. A. and Pick, L. (1998). A binding site for multiple transcriptional activators in the fushi tarazu proximal enhancer is essential for gene expression in vivo. *Mol. Cell Biol.* **18**, 3384-94.
- Harding, K., Rushlow, C., Doyle, H. J., Hoey, T. and Levine, M. (1986). Cross-regulatory interactions among pair-rule genes in *Drosophila*. *Science* **233**, 953-9.
- Hiromi, Y., Kuroiwa, A. and Gehring, W. J. (1985). Control elements of the *Drosophila* segmentation gene fushi tarazu. *Cell* **43**, 603-13.
- Hiromi, Y. and Gehring, W. J. (1987). Regulation and function of the *Drosophila* segmentation gene fushi tarazu. *Cell* **50**, 963-74.
- Holstege, F. C. P., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, T. S. and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**, 717-728.
- Howard, K. and Ingham, P. (1986). Regulatory interactions between the segmentation genes fushi tarazu, hairy, and engrailed in the *Drosophila* blastoderm. *Cell* **44**, 949-57.
- Howard, K. R. and Struhl, G. (1990). Decoding positional information: regulation of the pair-rule gene hairy. *Development* **110**, 1223-31.
- Huibregtse, J. M. and Engelke, D. R. (1989). Genomic footprinting of a yeast tRNA gene reveals stable complexes over the 5'-flanking region. *Mol. Cell Biol.* **9**, 3244-52.
- Hulkamp, M., Pfeifle, C. and Tautz, D. (1990). A morphogenetic gradient of hunchback protein organizes the expression of the gap genes Kruppel and knirps in the early *Drosophila* embryo. *Nature* **346**, 577-80.
- Ingham, P. and Gergen, P. (1988). Interactions between the pair-rule genes runt, hairy, even-skipped and fushi tarazu and establishment of periodic pattern in the *Drosophila* embryo. *Development* **104 Supplement**, 51-60.
- Ingham, P. W., Baker, N. E. and Martinez-Arias, A. (1988). Regulation of segment polarity genes in the *Drosophila* blastoderm by fushi tarazu and even skipped. *Nature* **331**, 73-5.
- Ingham, P. W. and Martinez Arias, A. (1992). Boundaries and fields in early embryos. *Cell* **68**, 221-35.
- Ish-Horowitz, D., Howard, K. R., Pinchin, S. M. and Ingham, P. W. (1985). Molecular and genetic analysis of the hairy locus in *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol.* **50**, 135-44.
- Ish-Horowitz, 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**, 405-15.
- Ish-Horowitz, D., Pinchin, S. M., Ingham, P. W. and Gyurkovics, H. G. (1989). Autocatalytic ftz activation and metameric instability induced by ectopic ftz expression. *Cell* **57**, 223-32.
- Jackle, H., Tautz, D., Schuh, R., Seifert, E. and Lehmann, R. (1986). Cross-regulatory interactions among the the gap genes of *Drosophila*. *Nature* **324**, 668-670.
- Jiang, J., Hoey, T. and Levine, M. (1991). Autoregulation of a segmentation gene in *Drosophila*: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. *Genes Dev.* **5**, 265-77.
- Jimenez, G., Pinchin, S. M. and Ish-Horowitz, D. (1996). In vivo interactions of the *Drosophila* Hairy and Runt transcriptional repressors with target promoters. *EMBO J.* **15**, 7088-98.
- John, A., Smith, S. T. and Jaynes, J. B. (1995). Inserting the Ftz homeodomain into engrailed creates a dominant transcriptional repressor that specifically turns off Ftz target genes in vivo. *Development* **121**, 1801-13.
- Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. *Roux's Arch. Dev. Biol.* **193**, 283-295.
- Kellerman, K. A., Mattson, D. M. and Duncan, I. (1990). Mutations affecting the stability of the fushi tarazu protein of *Drosophila*. *Genes Dev.* **4**, 1936-50.
- Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E. and Noll, M. (1986). Isolation of the paired gene of *Drosophila* and its spatial expression during early embryogenesis. *Nature* **321**, 493-9.
- Klingler, M. and Gergen, J. P. (1993). Regulation of runt transcription by *Drosophila* segmentation genes. *Mech Dev* **43**, 3-19.
- Klingler, M., Soong, J., Butler, B. and Gergen, J. P. (1996). Disperse versus compact elements for the regulation of runt stripes in *Drosophila*. *Dev. Biol.* **177**, 73-84.
- Kornberg, T. (1981). Engrailed: a gene controlling compartment and segment formation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **78**, 1095-9.

- Kraut, R. and Levine, M.** (1991). Mutually repressive interactions between the gap genes giant and Kruppel define middle body regions of the *Drosophila* embryo. *Development* **111**, 611-21.
- Kuroiwa, A., Hafen, E. and Gehring, W. J.** (1984). Cloning and transcriptional analysis of the segmentation gene fushi tarazu of *Drosophila*. *Cell* **37**, 825-31.
- Lawrence, P. A., Johnston, P., Macdonald, P. and Struhl, G.** (1987). Borders of parasegments in *Drosophila* embryos are delimited by the fushi tarazu and even-skipped genes. *Nature* **328**, 440-2.
- Lewis, R. A., Kaufman, T. C., Denell, R. E. and Tallerico, P.** (1980a). Genetic analysis of the Antennapedia gene complex (ANT-C) and adjacent chromosomal regions of *Drosophila melanogaster*. I. Polytene chromosome segments 84B-D. *Genetics* **95**, 367-381.
- Lewis, R. A., Wakimoto, B. T., Denell, R. E. and Kaufman, T. C.** (1980b). Genetic analysis of the Antennapedia gene complex (ANT-C) and adjacent chromosomal regions of *Drosophila melanogaster*. II. Polytene chromosome segments 84A-84B1,2. *Genetics* **95**, 383-397.
- Li, X. and Noll, M.** (1993). Role of the gooseberry gene in *Drosophila* embryos: maintenance of wingless expression by a wingless—gooseberry autoregulatory loop. *EMBO J.* **12**, 4499-509.
- Macdonald, P. M., Ingham, P. and Struhl, G.** (1986). Isolation, structure, and expression of even-skipped: a second pair-rule gene of *Drosophila* containing a homeo box. *Cell* **47**, 721-34.
- Manoukian, A. S. and Krause, H. M.** (1992). Concentration-dependent activities of the even-skipped protein in *Drosophila* embryos. *Genes Dev.* **6**, 1740-51.
- Manoukian, A. S. and Krause, H. M.** (1993). Control of segmental asymmetry in *Drosophila* embryos. *Development* **118**, 785-96.
- Mirkovitch, J. and Darnell, J. E., Jr.** (1991). Rapid in vivo footprinting technique identifies proteins bound to the TTR gene in the mouse liver. *Genes Dev.* **5**, 83-93.
- Morata, G. and Lawrence, P. A.** (1975). Control of compartment development by the engrailed gene in *Drosophila*. *Nature* **255**, 614-7.
- Morrissey, D., Aske, D., Raj, L. and Weir, M.** (1991). Functional dissection of the paired segmentation gene in *Drosophila* embryos. *Genes Dev.* **5**, 1684-96.
- Mullen, J. R. and DiNardo, S.** (1995). Establishing parasegments in *Drosophila* embryos: roles of the odd-skipped and naked genes. *Dev. Biol.* **169**, 295-308.
- Nusslein-Volhard, C. and Wieschaus, E.** (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801.
- Pankratz, M. J., Seifert, E., Gerwin, N., Billi, B., Nauber, U. and Jackle, H.** (1990). Gradients of Kruppel and knirps gene products direct pair-rule gene stripe patterning in the posterior region of the *Drosophila* embryo. *Cell* **61**, 309-17.
- Perrimon, N., Engstrom, L. and Mahowald, A. P.** (1989). Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the X chromosome. *Genetics* **121**, 333-52.
- Pick, L., Schier, A., Affolter, M., Schmidt-Glenewinkel, T. and Gehring, W. J.** (1990). Analysis of the ftz upstream element: germ layer-specific enhancers are independently autoregulated. *Genes Dev.* **4**, 1224-39.
- Poole, S. J., Kauvar, L. M., Drees, B. and Kornberg, T.** (1985). The engrailed locus of *Drosophila*: structural analysis of an embryonic transcript. *Cell* **40**, 37-43.
- Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P. and Schutz, G.** (1998). DNA binding of the glucocorticoid receptor is not essential for survival [see comments]. *Cell* **93**, 531-41.
- Rivera-Pomar, R. and Jackle, H.** (1996). From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps. *Trends Genet.* **12**, 478-83.
- Saulier-Le Drean, B., Nasiadka, A., Dong J. and Krause, H.** (1998). Dynamic changes in the functions of *odd-skipped* during early *Drosophila* embryogenesis. *Development* **125**, 4851-4861.
- Saavedra, C., Tung, K. S., Amberg, D. C., Hopper, A. K. and Cole, C. N.** (1996). Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*. *Genes Dev.* **10**, 1608-20.
- Schier, A. F. and Gehring, W. J.** (1992). Direct homeodomain-DNA interaction in the autoregulation of the fushi tarazu gene. *Nature* **356**, 804-7.
- Schier, A. F. and Gehring, W. J.** (1993). Analysis of a fushi tarazu autoregulatory element: multiple sequence elements contribute to enhancer activity. *EMBO J.* **12**, 1111-9.
- Schupbach, T. and Wieschaus, E.** (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* **195**, 302-317.
- Shivdasani, R. A. and Orkin, S. H.** (1996). The transcriptional control of hematopoiesis [see comments]. *Blood* **87**, 4025-39.
- St Johnston, D. and Nusslein-Volhard, C.** (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-19.
- Struhl, G.** (1985). Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the *Drosophila* segmentation gene ftz. *Nature* **318**, 677-80.
- Tautz, D.** (1992). Redundancies, development and the flow of information. *BioEssays* **14**, 263-6.
- Thummel, C. S.** (1996). Files on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**, 306-10.
- Urness, L. D. and Thummel, C. S.** (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible E74A protein. *Cell* **63**, 47-61.
- Wakimoto, B. T. and Kaufman, T. C.** (1981). Analysis of larval segmentation in lethal genotypes associated with the antennapedia gene complex in *Drosophila melanogaster*. *Dev. Biol.* **81**, 51-64.
- Wakimoto, B. T., Turner, F. R. and Kaufman, T. C.** (1984). Defects in embryogenesis in mutants associated with the antennapedia gene complex of *Drosophila melanogaster*. *Dev. Biol.* **102**, 147-72.
- Walter, J., Dever, C. A. and Biggin, M. D.** (1994). Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos. *Genes Dev.* **8**, 1678-92.
- Wodicka, L., Dong, H., Mittmann, M., Ho, M. H. and Lockhart, D. J.** (1997). Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **15**, 1359-67.
- Yost, H. J. and Lindquist, S.** (1986). RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* **45**, 185-93.
- Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N. and Pick, L.** (1997). The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. *Nature* **385**, 552-5.
- Zink, B. and Paro, R.** (1989). In vivo binding pattern of a trans-regulator of homeotic genes in *Drosophila melanogaster*. *Nature* **337**, 468-71.