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Dynamic changes in the functions of Odd-skipped during early *Drosophila* embryogenesis

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SUMMARY

Although many of the genes that pattern the segmented body plan of the *Drosophila* embryo are known, there remains much to learn in terms of how these genes and their products interact with one another. Like many of these gene products, the protein encoded by the pair-rule gene *odd-skipped* (Odd) is a DNA-binding transcription factor. Genetic experiments have suggested several candidate target genes for Odd, all of which appear to be negatively regulated. Here we use pulses of ectopic Odd expression to test the response of these and other segmentation genes. The results are complex, indicating that Odd is capable of repressing some genes wherever and whenever Odd is expressed, while the ability to repress others is temporally or spatially restricted. Moreover, one target gene, *fushi tarazu*, is both repressed and activated by Odd, the outcome depending upon the stage of development. These results indicate that the activity of Odd is highly dependent upon the presence of cofactors and/or overriding inhibitors. Based on these results, and the segmental phenotypes generated by ectopic Odd, we suggest a number of new roles for Odd in the patterning of embryonic segments. These include gap-, pair-rule- and segment polarity-type functions.

Key words: *odd-skipped*, Pair-rule, *Drosophila*, Segmentation, *fushi tarazu*

INTRODUCTION

Early *Drosophila* embryogenesis is optimized for rapid subdivision of the embryo into segmentally repeated units. This process is controlled by a hierarchy of maternally and zygotically expressed segmentation genes. The zygotic segmentation genes have been classified into three groups based on their mutant phenotypes, gap genes, pair-rule genes and segment polarity genes (Nusslein-Volhard and Wieschaus, 1980). Gap genes define regions that span several contiguous segments, pair-rule genes control homologous regions within every other segment and segment polarity genes control homologous regions within every segment (for a review, see Ingham, 1988).

The majority of these genes encode transcription factors. They interact combinatorially (Gergen and Wieschuas, 1985; DiNardo and O'Farrell, 1987; Ingham et al., 1988a; Manoukian and Krause, 1992, 1993), generating expression patterns of increasing complexity. The outcome is a segmental blueprint in the form of segment polarity gene expression patterns. Shortly thereafter, the first physical signs of segmentation, epidermal indentations referred to as parasegmental grooves, are observed (Martinez-Arias and Lawrence, 1985). These subdivide the embryo along the anterior-posterior axis into segment-wide metameres called parasegments (Martinez-Arias and Lawrence, 1985; Lawrence et al., 1987). Parasegments are shifted anteriorly relative to the segmental units that will form later.

odd-skipped (*odd*) is a member of the pair-rule class of segmentation genes. It encodes a zinc-finger-containing transcription factor (Odd), which is expressed first in stripes that span the even-numbered parasegments, and later in narrow stripes in the middle of both even- and odd-numbered parasegments (Coulter et al., 1990; Manoukian and Krause, 1993). Mutations in the *odd* gene cause loss of portions of even-numbered parasegments and partial replacement by mirror-image duplications of flanking regions (Coulter and Wieschaus, 1988). These patterning defects have been explained, in part, by alterations in the expression patterns of three putative target genes, *fushi tarazu* (*ftz*), *engrailed* (*en*) and *wingless* (*wg*) (DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995). Similar pattern rearrangements have been noted in HSeve and HSrun embryos (Manoukian and Krause, 1992, 1993), where ectopic expression of *eve* and *run* led to repression of *odd*. Taken together, these studies suggest that the main function of Odd may be to repress expression of the *ftz*, *en* and *wg* genes in the middle regions of even-numbered parasegments. The *paired* gene may also be negatively regulated by *odd* in these regions (Baumgartner and Noll, 1990).

Genetic analyses such as those just described are important as they determine the positive or negative relationships between genes. However, they do not distinguish between direct and indirect interactions. For example, Odd may bind to the promoters or promoter-binding proteins of the *ftz* and *en*

genes to directly repress their transcription. Alternatively, Odd may act indirectly by regulating the expression of intermediary genes whose products then act on the *ftz* and *en* promoters. In the case of *en*, for example, Odd could act as a direct repressor of the *en* promoter, or alternatively, could act indirectly by repressing *ftz*, since *ftz* is required for *en* activation (Mullen and DiNardo, 1995).

A further complication in the interpretation of these studies is that segmentation gene expression patterns are extremely dynamic, resulting in protein combinations that change rapidly within a particular cell. Because the segmentation proteins are highly dependent upon combinatorial interactions, their effects on target genes may change within the span of a few minutes. At one time, a protein could function as an activator of a particular target gene and then, with the addition or removal of another protein, could be neutralized, or could even become a repressor. An example of this is the pair-rule protein Even-skipped, which changes from an activator to a repressor of the *ftz* gene within a period of 10-20 minutes (Manoukian and Krause, 1992). Such changes in activity are difficult to elucidate solely by the observation of mutant embryos, since these would exhibit the cumulative effects of both regulatory interactions.

For these reasons, we have been using an alternative approach to help distinguish between direct and indirect gene interactions, and to dissect changing gene interactions that are temporally or spatially regulated (Manoukian and Krause, 1992, 1993). These studies make use of heat-inducible segmentation gene constructs to enable activation of the genes in developing embryos. Expression is induced at the desired stage using short (2-6 minutes) heat pulses and changes in putative target gene expression patterns are monitored at 5 minute intervals thereafter. Typically, we find that there are two temporal windows of response within 40 minutes of the heat pulse. Genes that we presume to be direct targets begin to respond within 15 minutes of the heat pulse, while those that require the expression and function of intermediary gene products show delayed responses beginning at 30 and then 45 minutes after *trans*-gene induction. Our definition of direct is that the interaction occurs in the absence of intermediary gene regulation and the subsequent synthesis or degradation of these intermediary gene products. However, they need not involve direct molecular contact between the induced regulator and target gene promoters.

Using this kinetic approach, we confirm interactions suggested by previous genetic analyses as well as identifying several new targets of Odd. The regulation of these targets changes rapidly in both a temporal and spatial fashion. For one target, Odd switches from an activator to a repressor. Taken together, our data show that Odd plays major roles in determining both the size and polarity of even-numbered as well as odd-numbered parasegments. Studies such as this indicate that current models of the segmentation gene hierarchy are somewhat oversimplified and need further investigation.

MATERIALS AND METHODS

pHSodd construction and fly transformation

The vector pHSodd was constructed as follows: the *odd* full-length cDNA (1.95 kb) was excised as a *Not*I-*Eco*RV fragment from the clone podd 7.4 II-F (Coulter et al., 1990), obtained from D. Coulter. This fragment was inserted between the *Not*I and *Stu*I cloning sites of the P-element transformation vector, pCaSpeR-HS (Thummel and Pirrotta, 1992). This vector has a *white* selectable marker and allows expression of the cloned cDNA under control of the *hsp70* promoter.

HSodd transgenic flies were obtained by P-element-mediated germline transformation (Spradling and Rubin 1982). The pHSodd vector was injected into w^-; P[Δ2-3]/TM6 embryos, which contain a stable integrated source of transposase (Robertson et al., 1988). Two independent transformant lines were obtained, HSodd1 and HSodd2, both carrying a single copy of the pHSodd vector on the second chromosome. HSodd1 is homozygous lethal and balanced over SM5. HSodd2 is homozygous viable. Two more lines, HSodd3 and HSodd9, each with a single P-element insertion on the third chromosome, were obtained from the HSodd1/SM5 line by jump-start transposition (Engels et al., 1987). HSodd3 is homozygous viable and HSodd9 is kept as a balanced stock over TM3. HSodd; odd^- lines were generated using the third chromosome HSodd3 line crossed to the *odd* mutant lines odd^{IIId} and odd^{7L} (Coulter and Wieschaus, 1988). *odd* mutant chromosomes were balanced over a *CyO* balancer marked with a *hb-lacZ* reporter gene.

Heat-shock protocol and cuticle preparation

For cuticle preparations, embryos were collected on apple juice/agar plates for 20 minutes. After aging at 25°C for the indicated times (see figure legends), embryos were washed off the plates into small plastic cylinders containing a nylon mesh fastened to the bottom. Heat shocks were performed by immersion of the cylinder in a 36.5°C water bath. For the precise timing of different cuticular phenotypes, five collections of 20 minutes each were made and aged prior to heat shock such that each collection represented a different 20 minute interval, each separated by 10 minutes, between 2:10 (2 hours, 10 minutes) and 3:10 AEL (i.e. 2:10-2:30 AEL; 2:20-2:40; 2:30-2:50; etc.). After heat shock, embryos were rinsed with 25°C water, blotted dry, transferred to a microscope slide and covered with a thin layer of halocarbon oil. Properly staged embryos (approximately 100) were then selected under a dissecting microscope and transferred to an apple juice plate for further aging. After 24 to 30 hours at 25°C, hatched larvae were transferred into methanol. Unhatched larvae were dechorionated in bleach, the vitelline membranes removed by vigorous agitation in methanol/heptane (1:1) and then washed once with methanol. Hatched and unhatched larvae were pooled and washed several times in methanol prior to being deposited on a slide. After evaporation of the methanol, larvae were cleared in Hoyer's medium/lactic acid (4:1) for 24 hours at 65°C (Weischaus and Nusslein-Volhard, 1986).

In situ hybridization

For in situ hybridization, embryos were collected on apple juice/agar plates for 30 minutes or 1 hour as indicated. After appropriate aging at 25°C, the embryos were heat shocked as described above. After heat shock, embryos were allowed to recover for different periods of time and then fixed in 4% formaldehyde as described by Lehmann and Tautz (1994) in protocol 1A.

The detection of transcripts in embryos was achieved by whole-mount in situ hybridization using digoxigenin-11-dUTP-labeled probes (Tautz and Pfeifle, 1989), essentially as described in protocol 2 by Lehmann and Tautz (1994), except that the hybridization was performed at 48°C. Embryos were mounted in 70% glycerol, 1× PBS. In experiments involving *odd* mutant embryos, homozygous mutants were identified by double labeling (Manoukian and Krause, 1992) with *odd* mRNA probes and anti-β-galactosidase antibodies. Expression of β-galactosidase indicates the presence of the wild-type chromosome (marked with *hb-lacZ*).

Images were captured using a Sony XC275 CCD camera and Northern Exposure software. Figures were compiled using Adobe Photoshop.

RESULTS

Ectopic expression of Odd in transgenic embryos

A heat-inducible *odd* vector (pHSodd) was generated by placing the *odd* cDNA under control of the *hsp 70* heat-shock gene promoter in the P-element vector pCaSpeR (Thummel and Pirrotta, 1992). Two transformant lines harboring this construct were isolated, HSodd1 and HSodd2, each with single insertions mapped to the second chromosome. Additional lines were generated by jumping the P-element construct in HSodd1 to new chromosomal locations (see Materials and Methods). All lines gave similar results. The majority of results presented here were obtained with the HSodd2 line.

HSodd2 embryos were heat pulsed at 36°C for different lengths of time (2-10 minutes) in order to find conditions that would induce physiological levels of *odd* expression. As shown in Fig. 1B, a 4 minute heat shock was sufficient to induce ectopic transcript levels similar to those normally expressed in *odd* stripes. Note that the underlying pattern of six stripes normally seen at this stage is still visible (although darker) and that the intensity of ectopic expression is similar to or lower than the levels seen in wild-type embryo stripes (Fig. 1A). With a 6 minute heat shock (Fig. 1C), levels of transgene expression were substantially higher, making the endogenous striped expression pattern difficult to make out. Since these levels are probably higher than endogenous levels of expression, the remaining data were obtained using 4 minute inductions. Oregon R embryos heat shocked for the same duration were used as controls.

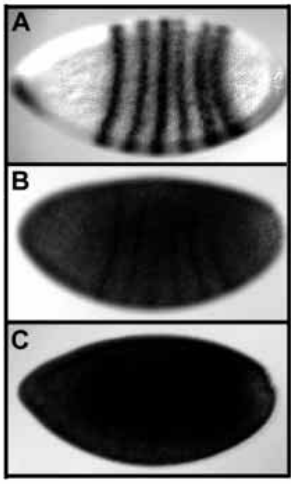

Fig. 1. *odd-skipped* expression in HSodd embryos. *odd-skipped* transcripts were detected by in situ hybridization in wild-type (A) and HSodd embryos heat shocked for 4 minutes (B) or 6 minutes (C) between 2:30 and 2:50 AEL. Embryos in B and C were fixed 15 minutes after the beginning of heat shock. The wild-type embryo (A) was fixed at a similar stage. Note that endogenous *odd* stripes are still visible in B and that the intensity of interstripe staining in B is similar to or less than the intensity of normal *odd* stripes in the WT embryo shown in A.

Ubiquitous expression of *odd* causes three cuticular phenotypes

HSodd embryos were heat pulsed at different stages of embryogenesis in order to determine when segmental patterning was most susceptible to ectopic Odd expression and to determine how segmental patterning would be affected. Segmental patterning was assessed by examination of larval cuticles prepared at the completion of embryogenesis (Fig. 2). Segmental defects were found to occur at the highest penetrance when heat pulses were administered between 2:10 and 3:10 (stages 5-7) after egg laying (AEL). Factoring in the 10-20 minute delay required for heat-inducible transcripts to accumulate, this interval closely approximates the window of endogenous *odd* expression. Three major segmental phenotypes were observed. These include a phenotype with head defects only (Fig. 2B), a pair-rule phenotype (Fig. 2C) and a pair-rule phenotype restricted to the dorsal half of the embryo (Fig. 2D). The stage at which each phenotype was induced was determined by administering heat shocks to collections of embryos that were collected over 20 minute intervals and aged appropriately (i.e., aged 2:10-2:30, 2:20-2:40 etc at the time of heat shock). The correlation between when heat shocks were administered and the frequency with which each phenotype was observed is summarized in Table 1.

The earliest phenotype observed ('Head') is a specific loss of head regions beginning just anterior to the T1 'beard' up to the maxillary sense organs (Fig. 2B). A cone-shaped indentation replaces the missing structures. Central portions of the mouth hooks such as the ectostomal sclerite are also malformed or missing. This phenotype prevails when Odd is induced between 2:10 and 2:30 AEL. Head defects, usually more severe, are also observed following later Odd inductions, but are not indicated in Table 1 since they are also associated with other defects. Also seen at the 2:10-2:30 interval, but at very low frequency (6%: grouped within 'Other' category), is a deletion of even-numbered thoracic and odd-numbered abdominal denticle belts, as seen in *ftz* mutant cuticles. However, this phenotype was also observed in heat-shocked Oregon R controls, albeit at a lower frequency (~2%).

The second phenotype observed is a classic pair-rule phenotype, with alternate segmental regions deleted (Fig. 2C).

Table 1. Correlation between cuticular phenotypes and times of *odd* induction

	Percentage of embryos exhibiting phenotype at times indicated				
Cuticular phenotype	2:10-2:30 (*n*=82)	2:20-2:40 (*n*=93)	2:30-2:50 (*n*=92)	2:40-3:00 (*n*=83)	2:50-3:10 (*n*=135)
Wild-type	12	9	5	3	37
Head	72	23	0	0	0
Intermediate pair-rule	6	45	31	32	5
Pair-rule	0	16	49	48	0
Bean-shape	0	0	6	9	56
Other	10	7	9	8	2

Numbers shown in each column refer to the percentages of embryonic cuticles exhibiting the phenotype indicated to the left and at the developmental interval (hours:minutes after egg laying) indicated above (*n*=number of cuticles scored). 'Head' refers to cuticles exhibiting defects anterior to T1 only. Intermediate pair-rules exhibit 1-4 fused or partially fused segment pairs (usually A2/A3, A4/A5 and/or A6/A7). Those classed as pair-rule show complete or very near complete fusion of every segment pair (T1/T2, T3/A1, A2/A3, A4/A5 and A6/A7). 'Bean-shaped' cuticles exhibit pair-rule fusions on their dorsal surface only. 'Others' refers to phenotypes that were seen at similar frequencies in heat-shocked Oregon R control embryos.

This phenotype prevails when heat pulses are provided between 2:30 and 2:50 AEL. Closer examination of the fused and partially fused segments reveals a phasing of the deleted regions that is neither segmental nor parasegmental (Fig. 2C,F,G). On the ventral side, composite denticle belts are observed (T1/2, T3/A1, A2/3, A4/5 and A6/7), with the regions in between deleted. Most of the remaining regions are composed of even-numbered parasegments, which is where *odd* is normally expressed. This phenotype is complementary to the *odd* mutant phenotype, in which the regions remaining are primarily derived from odd-numbered parasegments (Coulter et al., 1990). Also complementary to the *odd* mutant phenotype (Coulter and Wieschaus, 1988) is the frequency at which the different regions are deleted. The most frequently fused segments in HSodd embryos are A4 and A5, which are fused in 100% of the intermediate pair-rule cuticles examined. Sensitivity then decreases away from A4/A5 towards the extremities of the embryo (A6/A7≧A3/A4>T3/A1>T1/T2).

The third phenotype observed ('bean-shape') is a pair-wise deletion of segmental regions identical to the pair-rule phenotype just described, but limited to the dorsal half of the embryo (Fig. 2D). This phenotype prevails when heat shocks are administered between 2:50 and 3:10 AEL.

Effects of ectopic Odd on late *en* and *wg* expression

Expression patterns of the segment polarity genes *en* and *wg* were monitored about a third of the way through embryogenesis (stage 11) in order to help elucidate the mechanisms underlying the HSodd pair-rule phenotypes (Fig. 3). These two genes are normally expressed in 14 equally spaced stripes flanking either the posterior (*en*, Fig. 3A) or anterior (*wg*, Fig. 3D) edges of parasegmental grooves. In HSodd embryos, the spacing and/or intensity of these stripes are altered. Two different patterns that show pair-wise alterations in stripe spacing or intensity are seen for each gene. These expression patterns (described below) correlate with the pair-rule and bean-shape cuticular phenotypes based upon the timing of the heat shocks that produced them.

The most frequently observed *en* and *wg* patterns originating from the heat-shock interval that yields the pair-rule phenotype are shown in Fig. 3B (*en*) and E (*wg*). For both genes, the spacing between adjacent stripes is no longer uniform. For example, *en* stripes 1 and 2, 3 and 4, and so on are closer together than normal, giving a coupled appearance. The same is true for *wg* stripes 0 and 1, 2 and 3, and so on. In approximately 30% of these embryos, the even-numbered *en* stripes and odd-numbered *wg* stripes are partially missing. The most severely affected are *en* stripe 10 and *wg* stripe 9. This is consistent with the observation that denticle belt fusions occur with the highest frequency between A4 and A5 (parasegments 9 and 10). In embryos fixed at progressively later stages, these stripes were missing at increasingly higher frequencies. Loss of *wg* expression appeared to precede that of *en*. Based on these observations and the cuticular phenotypes, we surmise that the deleted segmental regions include each set of unstable *en* and *wg* stripes, and extend approximately half way to the *en*

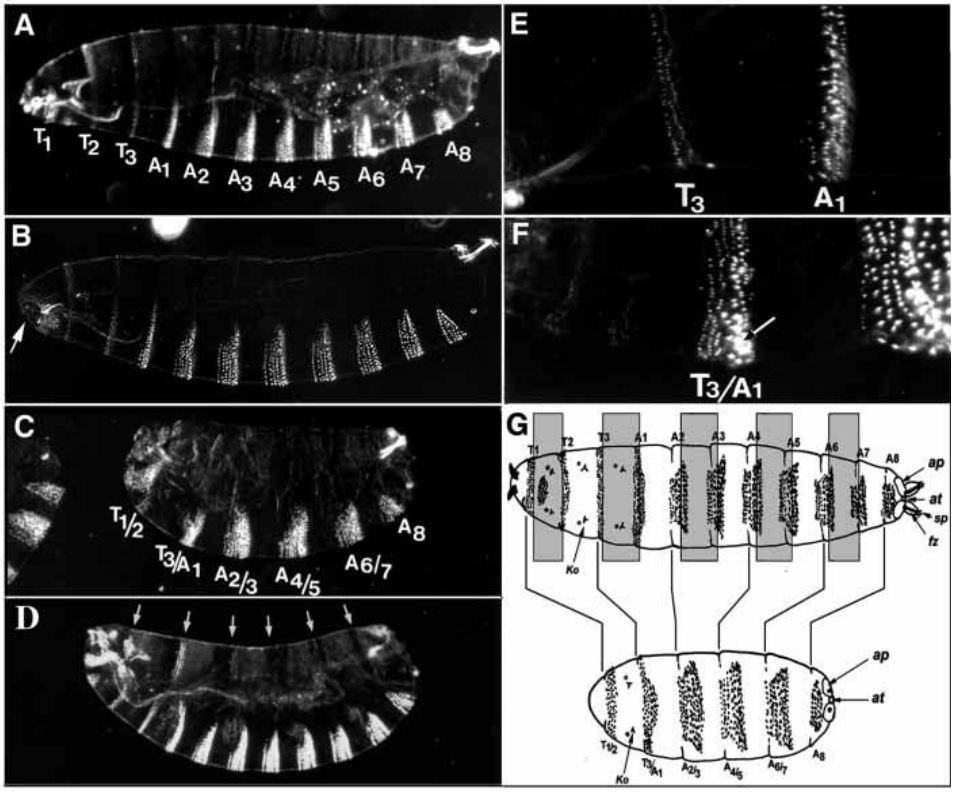

Fig. 2. Cuticular phenotypes caused by ectopic odd. Dark-field photomicrographs show cuticular phenotypes that arise from HSodd2 embryos that received a 4 minute heat shock between 2:10 and 3:10 AEL. (A) Wild-type cuticle: thoracic segments are numbered T1 to T3, and abdominal segments A1 to A8. (B) 'Head' phenotype: this phenotype is most prevalent when Odd is induced between 2:10 and 2:30 AEL. Defects are limited to a region (indicated with arrow) just anterior to the 'beard' in T1. Portions of the mouth hooks are missing or malformed. Missing regions are often replaced with a funnel-shaped hole. (C) Pair-rule phenotype:this phenotype is most frequently observed when HSodd embryos are heat shocked between 2:40 and 2:50 AEL. Posteriorly, the filzkorper (fz) and spiracles (sp) are present, although not always well formed, but the stigmatophore is not. Fused denticle belts are indicated. (D) Bean-shape cuticle: this phenotype arises most frequently when heat shocks are administered between 2:50 and 3:10 AEL. Pair-rule deletions (indicated with arrows) are observed in the dorsal half of the embryo. (E) Higher magnification of a wild-type cuticle showing T3 and A1 ventral denticle belts. Note that the T3 denticle belt contains two to three rows of tiny denticles, while the denticles in A1 are much larger. (F) The same region as in E in a HSodd cuticle. The denticle belts of T2 and A1 are fused, with the intervening regions deleted. (G) Schematic representation of a wild-type larval cuticle (top) showing regions missing in HSodd pair-rule mutants as gray boxes. The resulting phenotype is shown below. Also indicated are posterior defects. Head defects are not shown.

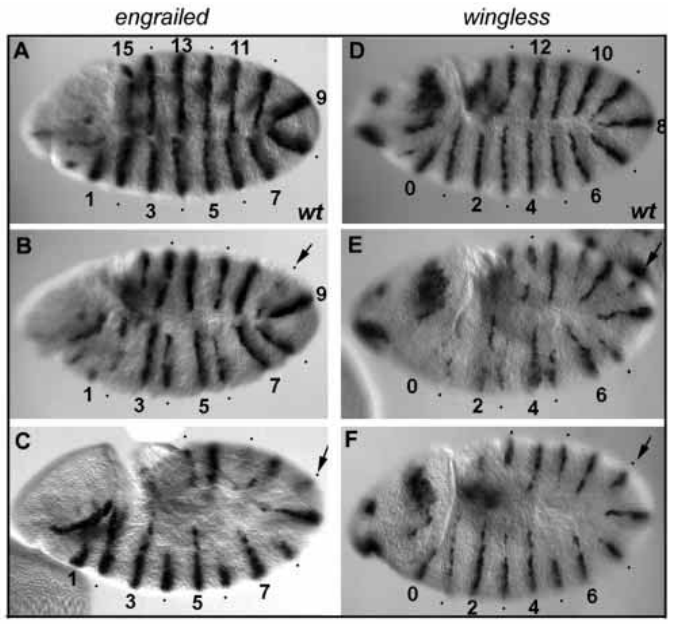

Fig. 3. Late expression of the segment polarity genes *engrailed* and *wingless*. Embryos were stained for *en* (A-C) or *wg* (D-F) transcripts by in situ hybridization. (A,D) Wild-type embryos fixed at 5:00-5:30 AEL (stage 10). (B,C,E,F) HSodd embryos, heat shocked for 4 minutes, and fixed at the same stage as the embryos in A and D. (B,E) Embryos heat shocked at 2:40-2:50 AEL: even-numbered *en* stripes and odd-numbered *wg* stripes appear to be shifted anteriorly and are often weaker or partially missing. (C,F) Embryos heat shocked at 2:50-3:10: spacing of stripes is more normal, but even-numbered *en* stripes and odd-numbered *wg* stripes are weak or missing, particularly dorsolaterally. The arrows highlight *en* stripe 10 and the adjacent *wg* stripe 9, which are generally the first to be lost.

and *wg* stripes in front and behind. These deletions are out of register with both segmental and parasegmental boundaries.

The temporal window giving rise to the bean-shaped phenotype generates the *en* and *wg* patterns shown in Fig. 3C,F. In these embryos, the spacing between *en* and *wg* stripes is usually normal. However, even-numbered stripes of *en* and odd-numbered stripes of *wg* are weaker or missing at their dorsal edges, consistent with the dorsal deletions seen in the bean-shaped cuticles. As with the pair-rule patterns, *en* stripe 10 and *wg* stripe 9 often show defects that are more severe than the others.

Effects of Odd on pair-rule gene expression

To address the nature of the genetic alterations underlying the HSodd phenotype, we analyzed the expression of segmentation genes expressed during the Odd-sensitive period (2:10-3:10 AEL). In previous studies, we found that genes responding directly to ectopic gene expression show peak changes in expression patterns within 25 minutes of the start of heat shock (Manoukian and Krause, 1992, 1993). Genes that require an intermediary gene product to be transcribed and translated, or alternatively repressed and degraded, in order to respond to the induced protein only begin to respond 30-35 minutes post-heat shock, and peak at 40-45 minutes post-heat shock. Fig. 4 shows pair-rule gene expression patterns in embryos fixed 25 minutes after the beginning of a 4 minute heat shock. Hence, these effects likely reflect direct transcriptional responses to ectopic Odd expression. These Odd-induced patterns (center column) are compared to the corresponding pair-rule gene expression patterns in wild-type (left) and *odd*$^{-}$ (right) embryos. The changes in expression patterns shown in HSodd embryos were not observed in Oregon R control embryos heat shocked under identical conditions (not shown).

Two of the seven pair-rule genes tested do not show significant changes in expression at the stages shown. These include the genes *odd-paired* (*opa*, Fig. 4B) and, to our surprise, *ftz* (Fig. 4E). In *odd*$^{-}$ embryos, *ftz* stripes do not resolve properly (Fig. 4F), remaining about 3 cells wide until well into the process of germ band extension (Mullen and DiNardo, 1995). This suggested that Odd may be a repressor of *ftz*. However, as shown in Fig. 4E, ectopic Odd does not repress *ftz* expression. Also unexpected was the fact that ectopic Odd has effects on all three of the 'primary' pair-rule genes. These were previously thought not to be regulated by Odd (Carroll and Scott, 1986; Carroll et al., 1988; Klingler and Gergen, 1988). In stage 5 embryos, stripe 1 of *h* is efficiently repressed by ectopic Odd (Fig. 4H). The first stripe of *eve* was also repressed at this stage (not shown). Repression of *h* stripe 1 continues in older embryos and is accompanied by weaker repression of stripes 2-6. These effects of Odd on *h* correlate with what appears to be a modest broadening of *h* stripes in *odd*$^{-}$ embryos, particularly stripe 1 (Fig. 4I). Early repression of the first stripes of *h* and *eve* likely accounts for the cuticular head defects that arise from early pulses of ectopic Odd expression (Fig. 2B). Interestingly, in *odd*$^{-}$ embryos, the entire 7-stripe pattern of *h* appears to expand, both anteriorly and posteriorly. This is also true of *eve* and *run* stripes (see below). Our data provide no explanation for this, but it may explain the fairly consistent spacing of *h* stripes, despite their apparent broadening.

In stage 6 embryos, all seven stripes of *run* are moderately repressed by ectopic Odd (Fig. 4K). This correlates with what appears to be a slight broadening and strengthening of *run* stripes in *odd*$^{-}$ embryos (Fig. 4L). *eve* is the most dramatically affected of the so called primary pair-rule genes at this stage. All 7 *eve* stripes are strongly repressed by ectopic Odd (Fig. 4N). In *odd*$^{-}$ embryos, however, *eve* stripes are only marginally wider (Fig. 4O), but at later stages (not shown), widening is more obvious, with both primary and secondary *eve* stripes widening from one to two or three cells in width. The response of *prd* is unusual in that only the anterior portions of each stripe are repressed (Fig. 4Q). This is the stage at which each *prd* stripe normally begins to split into two narrower stripes, beginning at the anterior end of the embryo (Fig. 4P). In *odd*$^{-}$ embryos, stripe splitting is substantially delayed (Fig. 4R).

Effects of Odd on late pair-rule gene and early segment polarity gene expression

Odd induction affects segmentation when induced as late as 3:10 AEL, suggesting that Odd may also be affecting late pair-rule and early segment polarity gene expression. Therefore, we heat pulsed embryos at these later stages and fixed them 25 minutes after heat shock to test for direct effects on these genes in stage 6/7 embryos. Except for *prd* and *slp*, the effects of

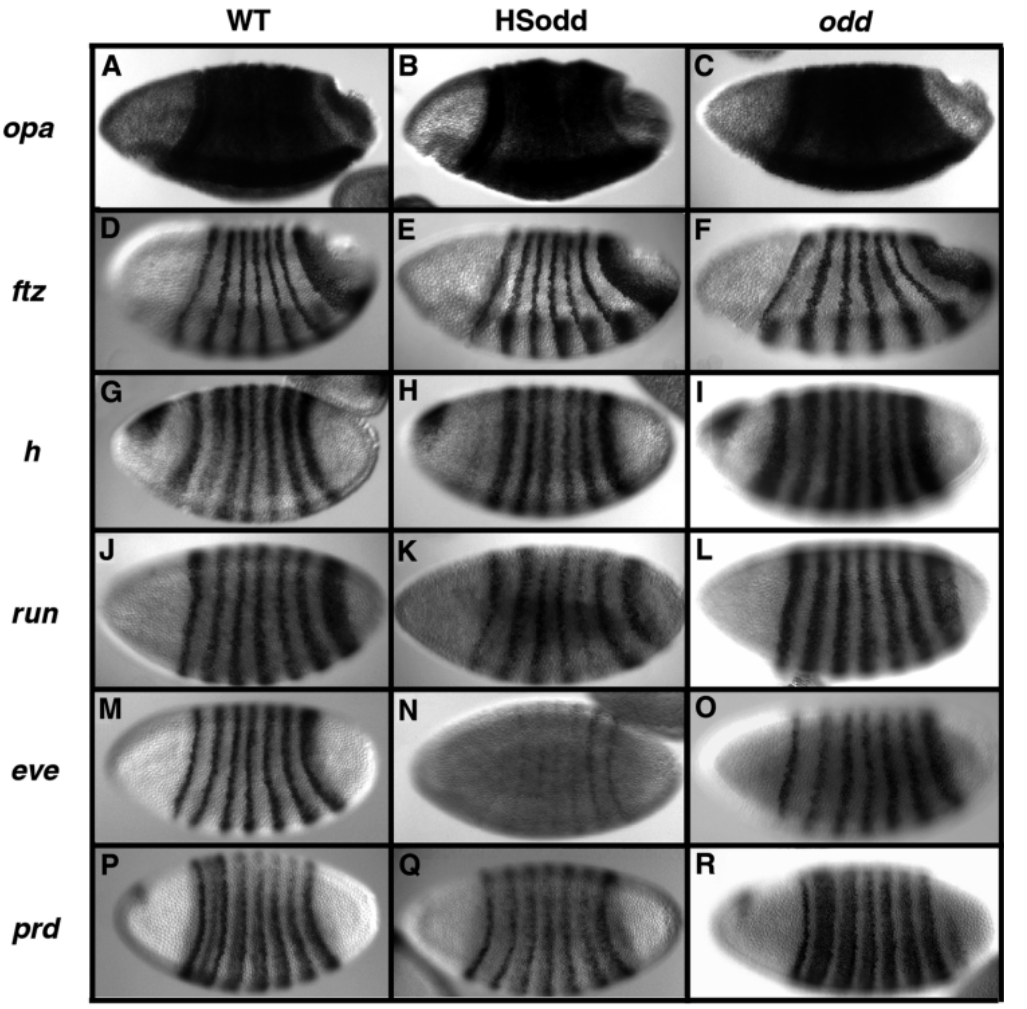

Fig. 4. Effect of ectopic *odd-skipped* on pair-rule genes. RNA expression patterns are shown, from top to bottom, for the pair-rule genes *odd-paired* (*opa*; A-C), *fushi tarazu* (*ftz*; D-F), *hairy* (*h*; G-I), *runt* (*run*; J-L), *even-skipped* (*eve*; M-O), and *paired* (*prd; P-R*) in stage 5-7 embryos. Embryos on the left (A,D,G,J,M,P) are wild-type, embryos in the middle (B,E,H,K,N,Q) are HSodd embryos fixed 25 minutes after a 4 minute heat shock and embryos on the right (C,F,I,L,O,R) are similarly staged *odd*$^{-}$ embryos. Changes in expression patterns are described in the text.

ectopic Odd on the pair-rule genes are similar to those described in the previous section. For *prd* and *sloppy-paired* (*slp*), expression normally changes at gastrulation from 7-stripe to 14-stripe patterns of expression (Fig. 5A,C). Following induction of ectopic Odd, expression of every second *prd* and *slp* stripe is repressed (Fig. 5B,D). The repressed stripes are the secondary stripes, which lie in the posterior regions of odd-numbered (*eve*-expressing) parasegments and, in the case of *prd*, also overlap with the anterior edges of even-numbered (*ftz*-expressing) parasegments. In *odd*$^{-}$ embryos, these same stripes of *prd* (Baumgartner and Noll, 1990) and *slp* (data not shown) become wider.

The effects of ectopic Odd were also examined on expression of the segment polarity genes *engrailed* (*en*) and

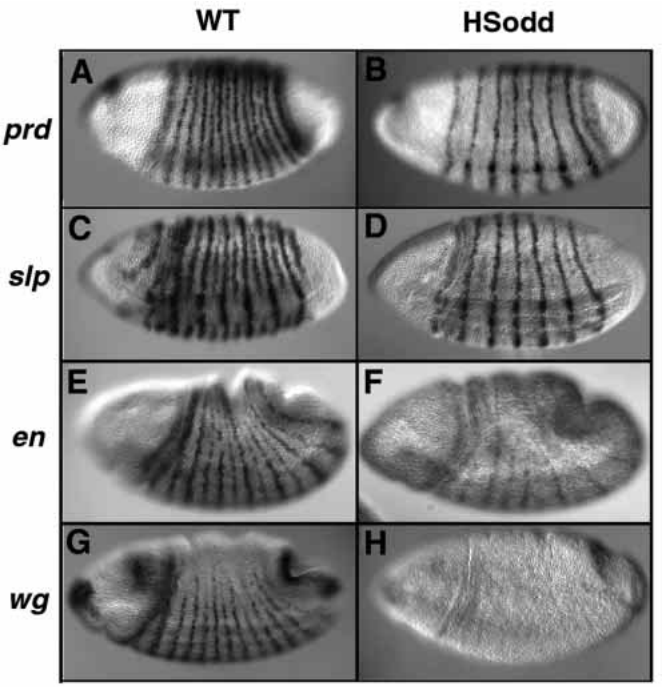

Fig. 5. Effects of ectopic *odd* on late pair-rule and early segment polarity gene expression. RNA expression patterns are shown, from top to bottom, for *paired* (*prd*; A,B), *sloppy-paired* (*slp*; C,D) *engrailed* (*en*; E,F) and *wingless* (*wg*; G,H) in stage 6 (gastrulating) embryos. Embryos on the left are wild-type (A,C,E,G) and those on the right are HSodd embryos (B,D,F,H) fixed 25 minutes after a 4 minute heat shock. Secondary stripes of *paired* and *sloppy-paired* are differentially repressed by ectopic Odd, whereas all 14 *en* and *wingless* stripes are repressed.

wingless (*wg*), since previous studies suggested that Odd may be a repressor of both genes (Manoukian and Krause, 1992, 1993; Mullen and DiNardo, 1995). Consistent with these studies, all 14 stripes of *en* and *wg* were rapidly repressed in HSodd embryos (Fig. 5F,H). This repression is short-lived, as stripes of both *en* and *wg* were observed once again in stage 11 embryos (Fig. 3). Indeed, expression returned about 45-60 minutes post-heat shock (data not shown).

Early and late effects of Odd on *ftz*

Given the results of previous genetic studies, the lack of an effect of ectopic Odd on *ftz* was somewhat surprising. Hence, we looked more carefully for possible effects at other stages of *ftz* expression. Fig. 6 shows that ectopic Odd does indeed affect *ftz* expression in earlier stage 4/5 embryos. However, the result was once again unexpected. Rather than negatively regulating *ftz*, ectopic Odd causes a rapid expansion of all 7 *ftz* stripes (Fig. 6B). In some embryos, interstripe regions are difficult to discern (not shown). Since this activation is observed within 20-25 minutes of Odd induction, it likely reflects a direct interaction between the two genes. Consistent with this positive relationship, initiating *ftz* stripes are irregular in width and intensity in *odd* mutant embryos (Fig. 6C). Stripes 3-6 are the most strongly affected, particularly stripe 4.

In germband-extending (stage 7) embryos, ectopic Odd, once again, does not appear to have an effect on *ftz* expression (Fig. 7B). At this stage, endogenous *odd* is no longer expressed in the same cells as *ftz*, but rather immediately posterior to each narrowing *ftz* stripe (Manoukian and Krause, 1993). In *odd*$^-$ embryos, *ftz* continues to be expressed in the cells that normally would express *odd*, generating stripes that are 2 to 3 cells wide, rather than 1-2 cells wide, as normally observed at this stage (Mullen and DiNardo, 1995; Fig. 7C). Taken together, these observations suggest the possibility that Odd may function as a repressor of *ftz*, but only in the cells where Odd is normally expressed. To test this possibility, we induced Odd expression in *odd*$^-$ embryos. Fig. 7D shows that ectopic expression of Odd in *odd*$^-$ embryos does indeed repress the broadened 2- to 3-cell-wide *ftz* stripes, returning their width to a normal 1-2 cell width. Thus, Odd does appear to be able to repress the *ftz* gene, but only in the middle region of the *ftz* parasegment, and only after gastrulation.

This effect on *ftz* is paralleled by the response of *en*. When examined immediately after heat shock, all 14 *en* stripes are effectively repressed, as shown in Fig. 5. However, all 14 stripes reinitiate within an hour of heat shock, and are relatively normal in stage 9 embryos (Fig. 7F). In *odd*$^-$ embryos, ectopic *en* stripes appear along the posterior edges of the widened *ftz* stripes (Fig. 7G) due to activation by ectopic Ftz (DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995). Like the other 14 *en* stripes, these are also repressed by ectopic Odd when induced in stage

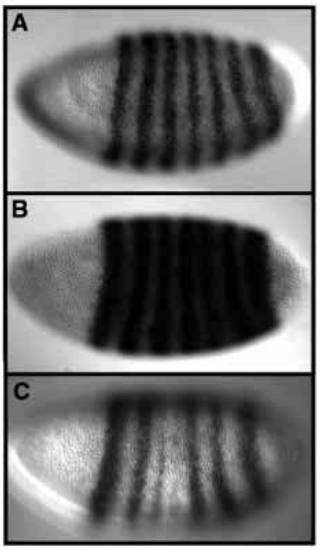

Fig. 6. Odd activates *ftz* in syncytial blastoderm embryos. Expression of *ftz* mRNA is shown in stage 5 (cellularization 50% complete) embryos. (A) Wild-type embryo: *ftz* stripes are on average 2-3 cells wide. (B) HSodd embryo fixed 25 minutes after a 4 minute heat shock: ectopic Odd causes a rapid broadening and intensification of all seven *ftz* stripes. (C) *odd*$^-$ embryo: stripes 2-6 are diminished in intensity and width.

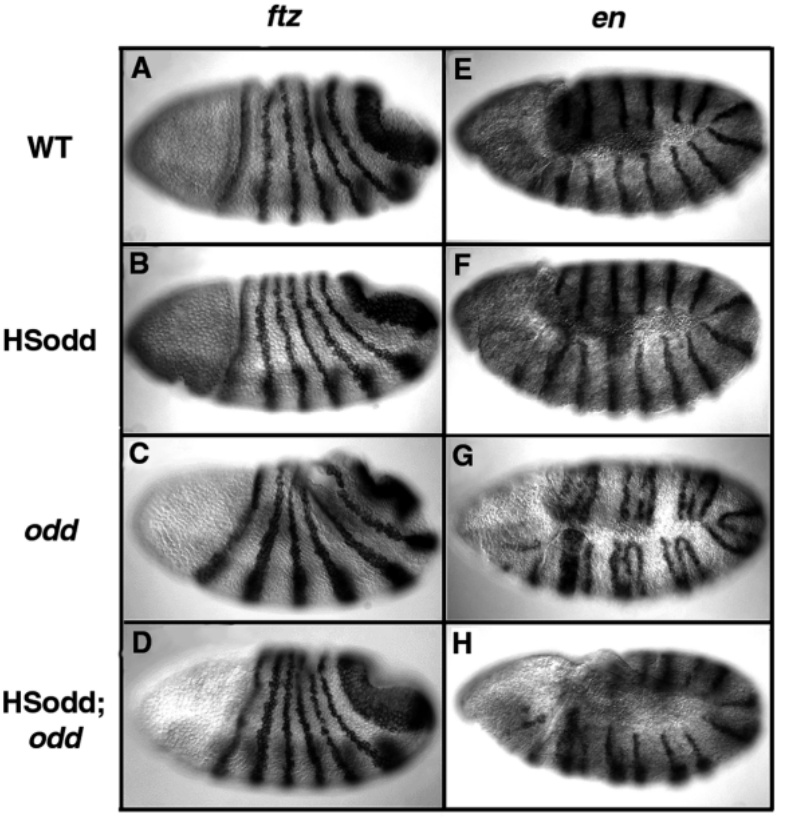

Fig. 7. Odd represses *ftz* in gastrulating embryos. In order to test whether Odd could repress *ftz* (A-D) within the normal domains of *odd* expression, ectopic Odd was expressed in *odd*$^-$ embryos. In a wild-type stage 7 embryo, stripes 1-6 of *ftz* are on average 1-2 cells wide (A). No effect is seen on these stripes when Odd is expressed ectopically (B). In *odd*$^-$ embryos, stripes 1-6 are 2-3 cells wide (C). These posteriorly expanded stripes are repressed by ectopic Odd, reverting the stripes to wild-type width (D). (E-H) Corresponding *en* patterns later on in stage 10 (5:00-5:30 AEL) embryos. (E) Wild-type embryo with stripes 1-14 indicated. (F) HSodd embryo: all 14 stripes are present. (G) *odd*$^-$ embryo: 7 additional *ftz*-dependent *en* stripes appear posterior to the normal even-numbered *en* stripes. (H) HSodd; *odd*$^-$ embryo: repression of the broadened *ftz* stripes in HSodd; *odd*$^-$ embryos (D) prevents ectopic *en* stripes from forming.

6 embryos (not shown), but unlike the other stripes, these fail to reinitiate (Fig. 7H). The failure of *en* to reinitiate in these cells is likely due to lack of the *en* activator Ftz, which was repressed earlier by Odd (Fig. 7D).

DISCUSSION

Odd functions primarily as a repressor

Previous genetic studies suggested that the product of the *odd* gene would function as a repressor. Four putative target genes were identified; the pair-rule genes *ftz* (Manoukian and Krause 1992, 1993; Mullen and DiNardo, 1995) and *prd* (Baumgartner and Noll, 1990), and the segment polarity genes *en* (Manoukian and Krause, 1992, 1993; Mullen and DiNardo, 1995; Florence et al., 1997) and *wg* (Mullen and DiNardo, 1995). Our results are consistent with Odd acting predominantly as a repressor of target gene expression. All four genes, *ftz, prd, en* and *wg* were repressed in the same short time frame after ectopic Odd induction, suggesting that they are indeed direct targets of Odd. In addition, we identified four more genes that appear to be directly repressed by Odd; the genes *slp, eve, h* and *run*. Although we classify these interactions as direct, in that they most likely do not require the activation or repression of intermediary genes, they need not involve direct interactions between Odd and each of the target gene promoters. These regulatory relationships, summarized in Fig. 8, are consistent with the temporal and spatial expression patterns of these genes relative to *odd* in wild-type embryos, and with the changes observed in these patterns in odd^{-} embryos. They are also consistent with the segmental phenotypes produced by odd^{-} and HSodd embryos (discussed below).

Odd can also function as an activator

An advantage of using a heat-inducible transgene is that expression can be induced at virtually any stage of development. This allowed dissection of early, mid and late roles of Odd on other segmentation genes. A surprising discovery was that Odd functions not only as a repressor of the *ftz* gene, but also as an activator. Activation of *ftz* occurs when ectopic Odd is expressed prior to the completion of cellularization and is most pronounced at the beginning of cellularization. It occurs with the same rapid kinetics as observed for genes that are repressed by Odd, indicating that this also is likely to occur in the absence of intermediary gene regulation. This positive relationship between Odd and *ftz* is consistent with the expression patterns of the two genes at this stage: *odd* and *ftz* stripes overlap perfectly, except for stripe 7 of *odd* which is missing at this stage (Manoukian and Krause, 1993). Beginning at stage 6, *odd* and *ftz* stripes begin to resolve into non-overlapping patterns and it is at this later time that Odd becomes a repressor of *ftz*.

Since *ftz* is the only gene amongst those studied here that is activated by Odd, and is only activated within a short temporal window, the primary activity of Odd is most likely as a repressor. Its function as an activator probably requires the participation of a cofactor that is expressed early and exhibits specificity for the *ftz* promoter. A similar observation was made in a previous study (Manoukian and Krause, 1992), where it was noted that the *even-skipped* protein (Eve) could also function as both an activator and a repressor of *ftz*. The switch in Eve activities, from an activator to a repressor, occurs at about the same time as the switch in Odd activities. Moreover, the switch also occurs as the stripes of *eve* and *ftz* resolve into non-overlapping patterns. These similarities in Odd and Eve target gene specificity and regulation may be coincidental, or may be due to the participation of a common cofactor (or cofactor complex).

Odd activity is controlled temporally and spatially

Our ability to express Odd uniformly and at precise developmental stages also revealed a number of temporal and spatial limitations in Odd activities. For example, when Odd switches from an activator to a repressor of *ftz*, its ability to repress *ftz* is excluded from the anterior-most cells of each *ftz* stripe. The inability of Odd to repress *ftz* in these cells indicates that, either a necessary cofactor for Odd is missing in these cells, or that an overriding factor is present. Another possibility is that the levels of Odd required to repress *ftz* are higher than those that we induced.

Another example of spatial and temporal limitations in Odd activities is in the ability of Odd to repress *h* and *eve*. In early cellularizing embryos, repression of these genes is limited to their anterior-most stripes. This suggests a possible interaction at this stage with anteriorly localized gap or terminal group proteins. Shortly thereafter, Odd is capable of repressing *eve* throughout the embryo while *h* continues to be repressed predominantly in anterior regions. Yet another example of spatial and temporal limitations of Odd activity is the ability of Odd to repress only the secondary stripes of *prd* and *slp*, which are normally initiated in gastrulating embryos. This indicates differential interactions with the enhancers and proteins that regulate these stripes at this time.

A final example of spatially restricted Odd activity is its inability to alter segmentation in the ventral half of the embryo during later stages of the Odd-sensitive period. Induction of Odd between 2:50 and 3:10 AEL causes pair-rule fusions, but only in the dorsal half of the embryo. This suggests some form of interaction with components of the dorsoventral controlling pathways. Links between anteroposterior and dorsoventral controlling genes have been noted previously (see for example: DiNardo and O'Farrell, 1987; Manoukian and Krause, 1992; Akimura et al., 1997; Nibu et al., 1998; Fisher and Caudy, 1998), and likely occur at a variety of levels.

Revision of pair-rule gene paradigms

Three of the pair-rule genes that we show are repressed by Odd were previously classified as a subgroup of pair-rule genes called 'primary' pair-rule genes. These were thought to function as intermediary genes between the earlier functioning gap genes and the other pair-rule genes (Carroll and Scott, 1986; Howard and Ingham, 1986; Pankratz and Jackle, 1990). This deduction was based on three observations. First, primary pair-rule gene stripes were thought to form prior to those of most other pair-rule genes. Second, these patterns were thought to be unaffected by mutations in the other pair-rule genes. Third, all three genes were thought to be regulated by large, complex promoters that possess a unique ability to decode non-periodic gap gene cues using stripe-specific enhancers.

Our results here, as well as those of others (Gutjahr et al.,

1993; Tsai and Gergen, 1994; Yu and Pick, 1995; Klingler et al., 1996), suggest that the primary/secondary pair-rule gene paradigm requires revision. For example, *odd* expression initiates first in a single stripe and then in broad patterns that suggest direct responses to gap gene cues (Coulter et al., 1990). These patterns are initiated early enough (late stage 4/early stage 5) to regulate *eve, h, run* and *ftz*. Indeed, we show that ectopic Odd is capable of regulating all four genes and that the expression patterns of these genes are altered in *odd* mutant embryos. These changes may have been missed in earlier studies due to their subtle nature in stage 6 embryos. The full effect of these actions may also be masked by the redundant actions of other segmentation genes. Indeed, redundancy may be masking a number of important gene interactions that can only be revealed by approaches such as double mutant and ectopic expression analyses.

Gap-like functions of *odd*

The earliest observed effects of ectopic Odd (late stage 4/early stage 5) are on the anterior-most stripes of *eve* and *h*. Repression of these stripes results in larval head defects in structures primarily derived from maxillary segment primordia, which is where the first stripes of *h* and *eve* are centered. These defects are similar to those previously reported in h^- (Howard et al., 1988; Ingham et al., 1988b) and eve^- (Nusslein-Volhard et al., 1985) mutant cuticles. At this time, endogenous *odd* is expressed in a single stripe immediately posterior to the first stripes of *eve* and *h* (Coulter et al., 1990), suggesting that it acts at this time to prevent posterior expansion of the anterior-most *eve* and *h* stripes. Indeed, in *odd* mutant embryos, the first stripes of *eve* and *h* appear to expand (Fig. 4 and data not shown), and head defects occur in structures normally derived from adjacent regions (Nusslein-Volhard et al., 1985; Coulter and Wieschaus, 1988). Thus, *odd* appears to have an early gap-like function which is exerted on the primary pair-rule genes *h* and *eve*.

Early pair-rule functions of *odd*

When ectopic Odd is induced just prior to gastrulation, all seven stripes of *eve* are repressed while *ftz* stripes are broadened. Later, *en* stripes that normally mark the boundaries of *eve*-dependent parasegments are closer together than in wild-type embryos, and conversely, those bordering *ftz*-dependent parasegments are farther apart (note, for example, the space between *en* stripes 5 and 6 in Fig. 3B). The same effect is seen in eve^{ID19} embryos grown at 25°C (Frasch et al., 1988) and, although not previously noted, the opposite is true in *odd* mutant embryos. Stripes of *en* that border the *eve*-dependent parasegments are farther apart and those that border *ftz*-dependent parasegments are closer together (Fig. 7G). These data suggest that Odd functions in early embryos to help position the parasegmental borders. This is achieved by activation of *ftz* and repression of *eve*.

Late pair-rule and segment polarity functions of *odd*

Previous studies could not establish unambiguously whether Odd acts as a direct or indirect repressor of the *en* and *wg* genes (Manoukian and Krause, 1992, 1993; Mullen and DiNardo, 1995; Florence et al., 1997). The data presented here show that, during gastrulation, Odd appears to regulate both genes, not only directly, but indirectly as well. Indirect repression is mediated by selective repression of the *en* and *wg* activators, *ftz, prd, eve* and *slp*. The result of these interactions in HSodd embryos is first the loss of all fourteen *en* and *wg* stripes due to direct repression and then failure of certain stripes to reinitiate.

Stripes of *en* reappear because their activators, *ftz, prd* and *opa* are not repressed where required for *en* activation. An exception is the set of ectopic *en* stripes that initiate later in *odd* mutant embryos. In HSodd; odd^- embryos, these stripes failed to initiate due to selective repression of Ftz in these cells. Interestingly, initiation of odd-numbered *en* stripes normally requires expression of *eve* as well as *prd*, and yet these stripes recover well despite near complete repression of *eve* by ectopic Odd. It is possible that low levels of *eve* are sufficient to activate *en*, or that *eve* acts earlier in an indirect

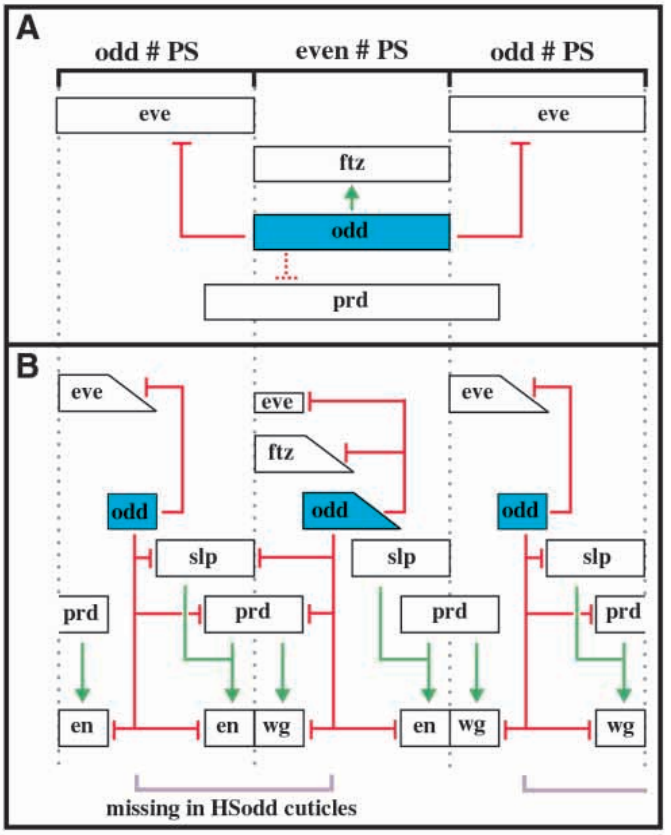

Fig. 8. Spatial and regulatory relationships between Odd and putative target genes. This schematic depicts segmentation gene expression patterns and relevant regulatory interactions within a 3-parasegment-wide interval (indicated by text and solid black lines at the top of A and vertical dashed gray lines in A and B). (A) Expression patterns and interactions that occur near the completion of cellularization (stage 5/6). (B) Patterns and interactions that occur in gastrulating embryos (stage 6/7). Segmentation gene stripes are indicated by boxes. The width of each box correlates with the relative width of the stripe indicated. Sloped sides indicate regions of stripes in the process of narrowing. Boxes indicating secondary stripes of *eve* in B are half the heights of others, indicating lower levels of expression. Positive regulatory interactions between genes are indicated as green arrows, while negative interactions are indicated as red, blunt-ended arrows. Dashed arrows indicate a weak interaction. Purple brackets at the bottom of B indicate the regions missing in HSodd cuticles. Earlier non-periodic interactions are not shown.

fashion, as has previously been suggested (Manoukian and Krause 1992, 1993).

Odd-numbered *wg* stripes fail to recover fully, most likely due to repression of the *prd* and *slp* stripes that are required for full *wg* expression. In turn, loss of these *wg* stripes eventually leads to loss of the adjacent (even-numbered) *en* stripes in older (stage 11) embryos. This is because *en* expression is dependent upon signaling from adjacent *wg*-expressing cells at this stage (Martinez-Arias et al., 1988; DiNardo and Heemskerk, 1990; Heemskerk et al., 1991). Loss of these *en* and *wg* stripes partially explains the HSodd pair-rule phenotype: the segmental regions that are missing include these cells (see Fig. 8). Thus, a major function of Odd in gastrulating embryos is to establish polarity within the middle of even-numbered parasegments by preventing expression of *en* and *wg*, and their respective activators. The expansion of *en* and *wg* stripes in *odd* mutant embryos (Mullen and DiNardo, 1995) is consistent with this function. The secondary stripes of *odd* likely play a similar role in odd-numbered parasegments, although such a role was not noted in the analysis of *odd* mutant embryos (Mullen and DiNardo, 1995) and may be functionally redundant. Odd may also regulate other segmentation genes at this stage, since the boundaries of regions deleted in HSodd embryos extend anteriorly and posteriorly beyond the missing *en*- and *wg*-expressing cells (see Fig. 8). Repression of odd-numbered *slp* stripes, which extend one cell anterior to *wg* stripes, may partially explain this observation.

Further elucidation of the segmentation gene hierarchy

The experimental approach described here, generating brief pulses of uniformly expressed protein, has confirmed findings of previous genetic studies, as well as revealing a number of unsuspected Odd activities. This approach is now well tested (Manoukian and Krause, 1992, 1993; A. N. and H. M. K., unpublished data), and has a great deal of potential for testing the regulatory relationships between other genes in an in vivo setting. Once these relationships are better understood, the intensive efforts required to identify and characterize relevant regulatory elements and *trans*-acting factors can be approached more confidently.

The results that we have obtained thus far emphasize the complexity and highly dynamic nature of the segmentation gene hierarchy. Previous genetic analyses of the circuitry involved have only provided a rough basis for the numerous and rapidly changing gene and protein interactions that occur. On occasions, interpretations have even been misleading. A great deal of work is still required to fully delineate and verify all of the interactions that occur between and within the various classes of segmentation genes. In the case of Odd, one of the next important steps will be to identify cofactors that modify its function in a temporally and spatially restricted fashion, permitting it to act as both an activator and a repressor of target gene expression.

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