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Molecular analysis of *odd-skipped*, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern

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odd-skipped (_odd_) is one of eight known pair-rule genes that establish portions of alternating segments during _Drosophila_ embryogenesis; _odd_ mutant embryos exhibit pattern defects in anterior regions of odd-numbered segments. P element transposon tagging was used to clone 25 kb of DNA from the _odd_ genomic region. Molecular analysis of phenotypic revertants confirmed that the P element used to tag the locus was responsible for the corresponding _odd_ mutation, and significant structural changes were identified in two additional _odd_ mutants. Several cDNA clones derived from a 2.2 kb embryonic transcript were isolated and the longest was sequenced. The predicted _odd_ protein of 392 amino acids is highly basic and contains four tandem Cys-Cys/His-His zinc finger repeats, consistent with a presumed function for _odd_ as a DNA binding protein and transcriptional regulator. _In situ_ hybridization analysis indicated that _odd_ transcripts accumulate in a dynamic pattern during early embryogenesis, with two temporally distinct modes of expression. The first mode results in a 'pair-rule' pattern of seven stripes at the blastoderm stage, representing the expected double segment periodicity. During gastrulation, the seven primary stripes are supplemented by secondary stripes which appear in alternate segments, resulting in the equivalent labeling of every segment in the extended germ band. Similar double to single segment transitions have now been reported for four of the six pair-rule genes analyzed.

Key words: Drosophila/embryogenesis/*odd-skipped*/pair-rule gene

Introduction

Early *Drosophila* embryogenesis is characterized by the rapid subdivision of the embryo into segments. This process is controlled by a discrete set of genes, the segmentation genes. Loss-of-function mutations in these genes result in the locus-specific deletion of particular regions of the metameric pattern, with three classes of phenotype recognized depending on the size and periodicity of the pattern deletion: segment-polarity and pair-rule mutants lack homologous regions within every segment or every other segment, respectively, while the gap mutants exhibit larger, non-periodic deletions of several contiguous segments (Nüsslein-Volhard and Wieschaus, 1980).

Molecular studies (reviewed by Ingham, 1988) have indicated that many of these genes are expressed during early embryogenesis in spatial patterns which roughly match the patterns of requirement inferred from the mutant phenotypes. Thus, gap gene products accumulate in broad bands comprising several segmental primordia, while pair-rule genes and certain segment-polarity genes are expressed periodically along the anterior–posterior axis, marking every other segment (seven stripes) or every segment (fourteen stripes), respectively. Furthermore, considerable evidence indicates that the three classes function in a regulatory hierarchy which progressively subdivides the embryo into transient blocks (gap genes), transient double segment units (pair-rule genes), and stable segments (segment-polarity genes). In this scheme, the overall role of the pair-rule class is to respond to positional cues generated by the gap gene patterns and in turn to activate segment-polarity genes in appropriate patterns. While the functions of individual pair-rule genes during this process have not been thoroughly defined and are probably often complex, it appears that some function primarily to regulate other genes within the pair-rule class, whereas others are likely to regulate genes in the segment polarity class directly.

The *odd-skipped* (*odd*) locus differs from most other pair-rule genes in that the pattern deletions in mutant embryos are relatively small and are consistently associated with mirror image duplications (Nüsslein-Volhard and Wieschaus, 1980; Coulter and Wieschaus, 1988). In *odd-skipped* mutants, cells in the anterior region of each odd-numbered segment fail to form the pattern elements characteristic of that region. Instead, these cells give rise to a nearly perfect mirror image duplication of the pattern immediately to the anterior, namely the posterior of the even-numbered segments; the duplicated regions therefore correspond to even-numbered domains of the segment-polarity gene *engrailed* (*en*). Altered programming in this region is already evident following the cellular blastoderm stage as an ectopic expression of *engrailed* transcripts (Martinez-Arias and White, 1988) and protein (DiNardo and O'Farrell, 1987) in cells which normally would form the anterior margins of the odd-numbered segments. Genetic studies (Coulter and Weischaus, 1988) suggest that this ectopic *en* expression is in fact responsible for the *odd-skipped* pattern deletions and associated transformations. In *odd* embryos which are also mutant for *en*, a normal pattern is restored to the anterior regions of the odd-numbered segments; the defects observed in the double mutant are confined to other regions, those associated with loss of *en* function alone. Thus, the phenotypic effects associated with loss of *odd* function might be largely explained by the failure of a single step, i.e., the inability of *odd* mutant embryos to restrict *en* expression from the anterior margins of the odd segments.

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The results outlined above indicate that *odd-skipped* may play a simple and discrete role in the transition from the pair-rule level of organization to the establishment of the segment-polarity pattern during early embryogenesis. A more detailed description of that role requires a molecular handle on the *odd-skipped* gene product. In this report we describe the molecular cloning and sequencing of the *odd* gene and the spatial distribution of *odd* transcripts during embryogenesis.

Results

Isolation of odd-skipped genomic DNA

We used the method of P element transposon tagging in conjunction with a hybrid dysgenesis induced allele, odd^{hd1}, to obtain DNA clones from the *odd-skipped* locus. Embryos homozygous for the *hd1* mutation exhibit a segmentation phenotype characteristic of strong *odd* mutations, suggesting a complete loss of *odd* function. Hybridization *in situ* of a P element probe to polytene chromosomes derived from the *hd1* strain indicated the presence of P element homologous sequences at band 24A (not shown), within the cytogenetic interval (23E–24B) to which *odd* gene had been localized (Nüsslein-Volhard *et al.*, 1984). These results indicated that the insertion of a P element within the *odd* gene is probably responsible for the odd^{hd1} mutation.

To isolate DNA containing the P element insertion and the surrounding genomic sequences from polytene band 24A, a lambda bacteriophage library was constructed from a balanced odd^{hd1} stock and screened for P-homologous clones. The subline used had been extensively backcrossed to an M strain to eliminate nearly all unrelated P elements from the stock (see Materials and methods), with the result that the first P-homologous clone isolated contained sequences from the desired chromosomal region (polytene band 24A). A fragment from this phage which contained the P element and surrounding genomic sequences was subcloned and used to screen a wild-type *Drosophila* genomic library (Maniatis *et al.*, 1978). Four independent wild-type bateriophage clones were obtained. These overlap in the region of the probe fragment and represent a 25 kb span of genomic DNA. A restriction map of this region is shown in Figure 2 (top).

Analysis of DNA alterations in odd-skipped mutants

Comparison of wild-type and *hd1* DNA clones indicated that the P element present in the latter consists of 1.1 kb of DNA. Both its size and restriction map (not shown) indicated that this is a degenerate element related to the full length (2.9 kb) autonomous P factor (O'Hare and Rubin, 1983) by the deletion of internal sequences. Southern analysis indicated that this element was present in the original *hd1* stock and at least four derived sublines, but absent from all wild-type strains tested.

The assumption that the *hd1* associated P element was the direct cause of the *odd* mutation was central to our cloning strategy. To verify that the isolated clones were indeed derived from the *odd* locus, a second round of hybrid dysgenesis was carried out to induce revertants of the odd^{hd1} allele (see Materials and methods). These revertants were identified by testing individual chromosomes for viability over odd^{IIC}, a hypomorphic allele which causes embryonic lethality but shows a reduced severity of segmentation defects (Coulter and Wieschaus, 1988). Southern blot analysis of genomic DNA obtained from eleven independent revertant

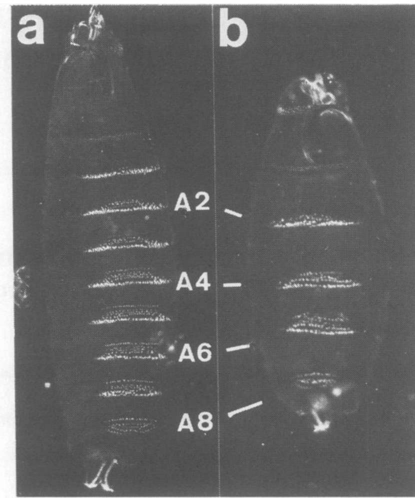

Fig. 1. The *odd-skipped* segmentation phenotype. Cuticle patterns of a wild-type first instar larva (**A**) and a homozygous odd^{hd1} mutant at the end of embryogenesis (**B**); anterior is shown up and the plane of focus is ventral. The mutant is characterized by pattern deletions which are confined to anterior portions of the odd-numbered segments; these regions correspond to the posterior of the even-numbered parasegments and are prominently marked in the wild-type pattern by the ventral denticle belts in the abdominal segments A1, A3, A5, and A7. These and other elements which are deleted in the mutant are partially replaced by mirror image duplications of adjacent elements (Coulter and Wieschaus, 1988).

lines (Figure 3A) indicated the loss of sequences from the 1.1 kb P element in every case, thereby confirming this element as a marker for the *odd* locus. In most lines (9/11), the relevant restriction fragment exhibited an electrophoretic mobility identical to wild-type, indicating the complete excision of P element sequences. As expected, these alleles fully complement strong *odd* alleles and so appear to restore wild-type levels of function.

In contrast to the apparent precise excisions of the P element observed in the majority of the revertants, we isolated two revertants in which excision was incomplete. These partial revertants, $odd^{hd1\text{-}6R}$ and $odd^{hd1\text{-}8R}$, exhibited restriction fragments which were ~500 bp and 50 bp larger than wild-type, respectively. Further Southern analyses (not shown) indicated that these resulted from internal deletion of P element sequences, as each retained a subset of the restriction sites present in the parental *hd1* element. Although both alleles were isolated as viable revertants based upon their ability to complement a hypomorphic *odd* allele, only the smaller element (*hd1-8R*) restored wild-type viability over a strong mutant allele. The larger element (*hd1-6R*), which retains roughly half of the original insertion, was associated with both a significantly reduced viability *in trans* with other *odd* alleles and frequent larval cuticle defects characteristic of the *odd* segmentation phenotype. This association between an intermediate level of *odd* function and an intermediate sized P element provides further proof that insertion at this site is responsible for the parental *hd1* mutation.

To define the *odd* locus further, we analyzed genomic DNA from seven additional *odd* mutants. Two of these showed significantly altered restriction patterns within the region covered by genomic *odd* clones (Figure 3B,C). A second hybrid dysgenesis-induced allele, odd^{hd3}, contains a P element inserted at a position indistinguishable by Southern blotting from that of the element in *hd1*. The size (2.9 kb)

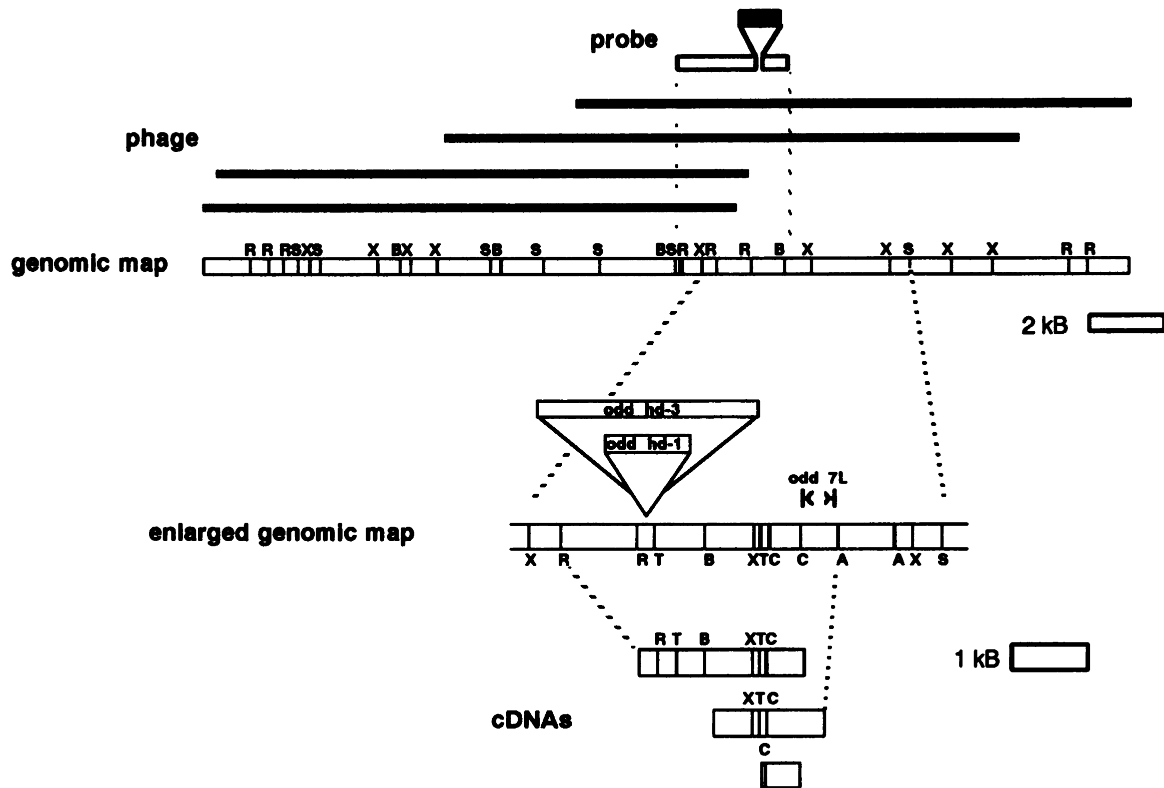

Fig. 2. Structure of the *odd-skipped* locus. Cloned wild-type genomic sequences are contained in four overlapping phage recombinants (ODD 1.16, 1.1, 1.14 and 1.13 from top to bottom) indicated above the genomic map. These were obtained using the indicated P element containing fragment ('probe') which was isolated from the *hdl* mutant. The enlarged genomic map shows the central transcribed region and the sites of identified mutant lesions, including the 1.1 and 2.9 kb P elements in *hdl* and *hd3* and the 0.07 kb deletion in *7L*. Restriction maps of representative cDNA clones are aligned with the genomic pattern; these include those extending furthest 5' (clone 7.4; top) and 3' (clone 6.10, middle), as well as the small clone (clone A, bottom) used for *in situ* hybridization (Figure 4). Connecting dotted lines indicate genomic restriction sites (*Eco*RI to the left and *Acc*I to the right) which define outer limits of homology between the indicated cDNA probes and genomic fragments detected by Southern blotting. The direction of transcription was determined by probing Northern blots and tissue sections with single stranded probes and is from left to right. Restriction sites: A = *Acc*I, B = *Bam*HI, C = *Cla*I, R = *Eco*RI, S = *Sal*I, T = *Sac*I, X = *Xho*I.

and restriction pattern of the *hd3* element suggest that it is a full length P factor (O'Hare and Rubin, 1983) with an orientation opposite to that of the degenerate element present in *hdl*. An ethylmethanesulfonate induced allele, odd^{7L}, is associated with a small deletion (70−80 bp) at least 2 kb from the site of the P element insertions in the two dysgenic alleles (see Figure 2). Because the *7L* allele was fortuitously isolated during a screen for lesions on the third chromosome (Jürgens *et al.*, 1984), an isogenized stock of the parental second chromosome was not available. However, we did not detect this deletion in the stock in which this allele was induced or in any other wild-type strain analyzed by Southern blotting. In view of the proximity to the *odd-skipped* transcription unit (see below), it is likely that this lesion is the cause of the associated mutation.

Isolation of cDNAs and sequence analysis

The structural changes identified in the *hdl*, *hd3* and *7L* mutants indicated that the cloned sequences correspond to the *odd* locus and suggested a likely proximity to the *odd* transcription unit. Northern blot analysis of embryonic RNA using genomic DNA fragments from this region as probes indicated that a poly(A)$^+$ transcript of 2.1−2.2 kb is derived from this region (data not shown). However, the presence of repetitive sequences in certain fragments resulted in the labeling of an uninterpretable diversity of RNA species; Southern blot analysis (not shown) and DNA sequencing (below) indicated that at least some of this repetitive DNA is homologous to the previously characterized opa or M-repeat (McGinnis *et al.*, 1984; Wharton *et al.*, 1985). We therefore relied on the analysis of cDNA clones to define the *odd* transcription unit better (Figure 2).

cDNA clones were isolated by probing early embryonic libraries (Poole *et al.*, 1985; Kilcherr *et al.*, 1986) with a genomic fragment which lacked the opa homology noted above. A total of 12 *odd* cDNA clones was obtained, with the longest (2.05 and 2.1 kb) approaching the length of the *odd* transcript determined by Northern analysis. Restriction map comparisons and blot hybridization analyses indicated that essentially all of the sequences present in the longest cDNA clone fall within a 4 kb region of genomic DNA; this transcribed region appears to span the positions of the three identified mutant lesions. The 5' end of the transcript clearly falls upstream of the position of the P element insertions in the *hdl* and *hd3* mutations. These mutations appear to disrupt an exon, as the genomic restriction fragment to which both P elements map and the corresponding fragment in the longer cDNAs exhibited identical electrophoretic mobilities (not shown). The deletion associated with the *7L* allele mapped within the smallest genomic fragment known to contain the 3' end of the cDNAs. The previously noted repetitive sequences are confined to 5' regions of the *odd* transcript; smaller cDNA clones derived from the 3' end of the gene

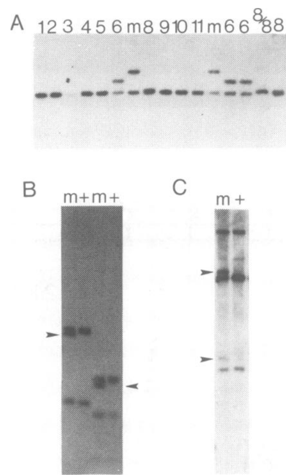

Fig. 3. Southern analysis of DNA rearrangements in *odd* mutants and revertants. Genomic DNA samples (2–4 fly equivalents/lane) were digested with the indicated restriction endonucleases and probed with ^{32}P-labeled, nick-translated DNA. Unless otherwise indicated, DNA was isolated from heterozygous flies bearing the CyO balancer. m indicates DNA prepared from the indicated mutant; + indicates DNA prepared from wild-type (odd^{+}) flies. **A**, odd^{hd1} and revertants. *Eco*RI–*Sal*I digested DNA probed with a 2.1 kb *Xho*I fragment shown on the enlarged genomic map of Figure 2 (the rightward of the two *Xho*I fragments). Equivalent results were obtained when *Bam*HI digested DNA was probed with the *Bam*HI fragment spanning the P element site, except that numerous background bands were observed. Note that no *Sal*I or *Eco*RI sites are present in the parental *hd1* P element. m refers to the parental *hd1* strain; numerals refer to various *hd1* revertants. 8/8 indicates DNA from *hd1-8R* homozygotes. **B**, odd^{7L}. *Acc*I–*Bam*HI (left lanes) or *Ava*II (right lanes) digested DNA probed as in A. Note that each mutant lane contains one additional band (arrow) that is 0.06–0.08 kb smaller than the corresponding wild-type fragment. **C**, odd^{hd3}. *Sal*I digested DNA probed with genomic bacteriophage clone ODD1.16. The mutant lane shows two fragments of 6.5 and 2.4 kb (arrows) which are not present in wild-type; these are derived from a single fragment which is 2.9 kb larger than the 6.0 kb wild-type *Sal*I fragment, and result from cleavage at a site within the full-length P element inserted in the hd3 mutant.

failed to cross-react with other sequences on Northern and Southern blots and so appear to be non-repetitive.

The DNA sequence obtained from the large (1.95 kb) *Eco*RI fragment of the longest cDNA contains a single long open reading frame encoding an apparent odd protein of 392 amino acids (Figure 4A). This protein has a predicted mol. wt of 44.6 kd and is highly basic (77 basic versus 33 acidic residues; predicted isoelectric point of 10.2). The carboxy-terminal half of this protein includes four tandem zinc finger repeats, each with two cysteines and two histidines (Figure 4B). *odd* is the only example to date of a zinc finger-encoding pair-rule gene, although this putative DNA binding motif has been identified in other *Drosophila* segmentation genes in the gap (Rosenberg *et al.*, 1986; Tautz *et al.*, 1987; Nauber *et al.*, 1988) and segment-polarity (Orenic *et al.*, 1990) classes.

Additional features of the predicted primary structure of the *odd* protein include several homopolymeric stretches, including polyserine (residues 369–375), three polyglutamine runs (residues 82–93, 101–106, 142–149) corresponding to the opa sequences noted above, and a histidine rich region (residues 152–169). Despite the overall excess of positively charged residues, the amino-terminal region of the protein is notably more acidic. Among the 25 side chains from residues 15–39, twelve are acidic versus only three basic, which might indicate a function analogous to acidic transcriptional activator sequences present in many DNA binding proteins (e.g., Hope and Struhl, 1986; Hollenberg and Evans, 1988).

Patterns of odd transcript accumulation

We used the method of *in situ* hybridization to tissue sections to determine the spatial and temporal pattern of *odd-skipped* expression during early embryogenesis (Figure 5). These experiments utilized an antisense RNA probe synthesized from a small (0.4 kb) cDNA clone derived from the 3′ end of the *odd-skipped* transcript; this clone was selected in preference to longer cDNAs to avoid cross-hybridization with heterologous transcripts.

The *odd-skipped* transcript is first detected in sectioned syncytial blastoderms as a single stripe at ~62% egg length (% EL) (Figure 5a). This stripe arises at the end of nuclear division cycle 13 and covers a width of ~5 nuclei. As the nuclei begin to elongate and cellularization commences, this initial anterior domain is supplemented by newly accumulated transcripts which extend posteriorly to ~24% EL (Figure 5b). Within this extended domain, transcripts are distributed non-uniformly, initially consisting of 3–4 broad domains separated by narrow regions with little or no accumulation.

As cellularization proceeds, the pattern evolves into six regular stripes due to the progressive loss of transcripts from intervening gaps (Figure 5c). These six stripes narrow to ~3–4 cell widths by the time cellularization is half complete. In addition to these six stripes, a non-periodic domain appears at the extreme anterior end of the embryo during early cellularization and persists through subsequent stages. Both the initial appearance of transcripts and subsequent generation of gaps within the striped region occur with a slight but noticeable posterior and dorsal lag, indicating a temporal gradient from anterior to posterior and ventral to dorsal. Labeling of alternate sections with probes for the pair-rule gene *fushi tarazu* (*ftz*; Hafen *et al.*, 1984; Weir and Kornberg, 1985) and *odd* indicated that the resolution of the *odd* pattern into six distinct stripes is coincident with the appearance of a distinct seven-stripe *ftz* pattern (data not shown).

A seventh, posteriormost stripe appears at 15% EL after nuclear elongation but prior to the completion of cellularization (Figure 5d). Consistent with the ventral to dorsal progression noted above, this stripe initially arises in ventral cells and spreads dorsally. The resulting seven stripe or 'pair-rule' pattern persists until the onset of gastrulation. The width and spacing of these stripes indicates a double-segment repeat unit with expression 'on' in domains which are ~3 cells wide and 'off' in intervening regions 4–5 cells wide. This pattern is somewhat irregular; the spacing between the sixth and seventh (most posterior) stripes is consistently wider than that observed for the more anterior interstripe regions.

The pair-rule pattern of seven stripes seen at the blastoderm stage evolves rapidly during gastrulation to a pattern in which the *odd* gene is expressed in homologous portions of every segment (Figure 5e–h). At least two distinct processes are responsible for this transition. First, transcripts are eliminated from some of the cells in each of the seven primary domains, resulting in the narrowing of these stripes.

A.

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GAATTCAGTTTGTGTCGAGACGTTGTCGGTGGCACGTCGCATCCAAAAGAACGCTCAGTCAGCTGGGAAATATACTGAGAAAAGATACAT   90
TCGTAGATACAAGTGCTAAGCCAAAGTGAACCGTGCAGTTCGCGCAACTAAACAATTTTAAGCCAAATAAAACTACACAAGGCCAACAAA  180

          M  S  S  T  S  A  S  P  I  S  N  I  T  V  D  D  E  L  N  L  S  R  E  Q  D  F  A    27
GACAGTATAATGTCTTCCACATCGGCCTCACCCATCAGCAACATAACCGTGGATGACGAGCTCAACTTAAGCAGAGAACAAGACTTTGCT  270

 E  D  D  F  I  V  E  K  E  E  R  E  T  S  L  S  P  M  L  T  P  P  H  T  P  T  E  E  P  L    57
GAAGACGATTTCATAGTGATCAAGGAGGAGCGCGAGACAAGTCTCTCCCCCATGCTGACGCCCCCGCACACGCCCACCGAGGAGCCGCTG  360

 R  R  V  H  P  A  I  S  E  E  A  V  A  T  Q  L  H  M  R  H  M  A  H  Y  Q  Q  Q  Q  Q  Q    87
AGGAGAGTGCATCCGGCGATAAGCGAGGAGGCGGTGGCCACCCAGCTGCACATGCGACACATGGCCCACTACCAGCAGCAGCAACAACAG  450

 Q  Q  Q  Q  Q  Q  H  R  L  W  L  Q  M  Q  Q  Q  Q  Q  Q  H  Q  A  P  Q  Q  Y  P  V  Y  P   117
CAGCAACAGCAGCAGCAGCACCGCCTGTGGCTGCAGATGCAGCAGCAGCAGCAACAACATCAGGCTCCACAGCAGTATCCAGTTTATCCC  540

 T  A  S  A  D  P  V  A  V  H  Q  Q  L  M  N  H  W  I  R  N  A  A  I  Y  Q  Q  Q  Q  Q  Q   147
ACAGCCAGCGCCGATCCCGTGGCCGTGCACCAGCAGTTGATGAACCACTGGATCCGCAACGCAGCCATCTACCAGCAGCAACAGCAGCAG  630

 Q  Q  H  P  H  H  H  H  H  H  G  H  P  H  H  P  H  P  H  P  H  H  V  R  P  Y  P  A  G  L   177
CAGCAACATCCGCACCATCATCACCACCACGGCCATCCGCACCACCCTCACCCACATCCGCATCATGTGCGTCCCTATCCCGCTGGCCTC  720

 H  S  L  H  A  A  V  V  G  R  H  F  G  A  M  P  T  L  K  L  G  G  A  G  G  A  S  G  V  P   207
CATAGTCTGCATGCCGCGGTCGTGGGTCGCCACTTCGGAGCCATGCCCACCCTGAAACTGGGTGGTGCCGGTGGAGCGAGTGGTGTACCC  810

 S  C  A  T  G  S  S  R  P  K  K  Q  F  I  C  K  Y  C  N  R  Q  F  T  K  S  Y  N  L  L  I   237
AGCTGCGCAACTGGCAGCAGTCGGCCAAAGAAGCAGTTCATCTGCAAGTACTGCAACCGGCAGTTCACCAAGTCGTACAATCTGCTCATC  900

 H  E  R  T  H  T  D  E  R  P  Y  S  C  D  I  C  G  K  A  F  R  R  Q  D  H  L  R  D  H  R   267
CATGAGAGAACCCACACGGACGAGAGGCCTTACTCCTGCGACATCTGCGGCAAGGCCTTCCGGCGACAGGATCATCTGCGGGACCACCGT  990

 Y  I  H  S  K  D  K  P  F  K  C  S  D  C  G  K  G  F  C  Q  S  R  T  L  A  V  H  K  V  T   297
TACATCCACTCCAAGGACAAGCCCTTCAAGTGCAGCGATTGCGGCAAGGGTTTCTGCCAGTCGCGCACCCTGGCCGTGCACAAGGTCACC 1080

 H  L  E  E  G  P  H  K  C  P  I  C  Q  R  S  F  N  Q  R  A  N  L  K  S  H  L  Q  S  H  S   327
CATCTGGAGGAGGGTCCGCACAAGTGTCCCATCTGCCAGCGCAGCTTCAACCAGCGGGCCAACCTCAAGAGTCACCTCCAGAGCCACAGC 1170

 E  Q  S  T  K  E  V  V  V  T  T  S  P  A  T  S  H  S  V  P  N  Q  A  L  S  S  P  Q  P  E   357
GAGCAGAGCACCAAGGAGGTGGTGGTGACCACCTCACCAGCCACTTCACATTCCGTGCCAAACCAGGCATTGAGTTCGCCTCAACCTGAG 1260

 N  L  V  Q  H  L  P  V  L  D  L  S  S  S  S  S  S  S  E  K  P  K  R  M  L  G  F  T  I  D   387
AATTTGGTACAGCATCTGCCCGTCCTGGATCTATCCTCGTCATCCTCGAGCTCAGAGAAACCCAAGCGGATGCTGGGCTTCACCATCGAT 1350

 E  I  M  S  R  -                                                                             392
GAGATCATGAGCAGATAGATTGAAGGTCCACCGAAACCCGGTATCCGGTTTCTTTTGAGACATTCTAAAAGAGATTCAGAGAGCTGGACG 1440

TCCGCCAGGCATGGGAAATGCCTGGAATGGAAAACCAAGCGAGTCCAGCCCGCATTCTGGCCAAAGGTGCAAGTGGAAAATCCTCGCCAG 1530
CAACTCGTAATCGCAACCCAGTGAGCAGAAGAGTCATCCTCCGTGCAATCGGTTCTATGTAGCAGATCCCAATTGCGTTAAACAAATGCC 1620
AAAGTCAAATAGCAGAGGAAAACTGATGAAAAATCCAATGAAAAGTATTGCTAGCACAAGCCTCCTACAAATCAATTCCAAAACACACAC 1710
ACACACAACTGTAAACTATTAACATAAATAAATAGCACATAATTTATATTGTAATCTAACTATTATTGTATGTACCTTATAGTCGTAGTA 1800
GCCCATTCAGTTCGTAGTGCTTATGGAGTAAACCAAATGTTATGTTACAATAGCTCAATCAAACAAACCTCAAACCCGCTTTGGTTATTG 1890
AAAGACTGTTTTAATTATTATATTTAAATAGTACTTGAATAAATCAATCGATCGATTGTGTAATGTTAAAAAAAAAAAAAA    1971
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B.

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219         Q F I C K Y C N R Q F T K S Y N L L I H E R T H T D E R
247         P Y S C D I C G K A F R R Q D H L R D H R Y I H S K D K
275         P F K C S D C G K G F C Q S R T L A V H K V T H L E E G
303         P H K C P I C Q R T F N Q R A N L K S H L Q S H S E Q S

consensus   _ Y _ C _ _ C _ _ _ F _ _ _ _ _ L _ _ H _ _ _ H _ _ _ _
              F
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Fig. 4. **A**, nucleotide sequence of an *odd* cDNA and predicted amino acid sequence of the *odd* protein. The complete nucleotide sequence of the large *Eco*RI fragment of cDNA clone 7.4 (see Figure 2) is shown. The amino acid sequence encoded by the single long open reading frame is given above the corresponding nucleotide sequence. The nucleotide sequence is numbered starting from the 5′ *Eco*RI site. Amino acids are numbered from the first in frame initiation (ATG) codon at base 190; although the sequence shown does not extend to the transcription start, this codon is preceded by upstream stop codons in all three reading frames. **B**, *odd* zinc fingers. Amino acids 219–330 are aligned to indicate the four zinc finger repeats (28 amino acids each) present in the C-terminal portion of the predicted *odd* protein. Residues which are highly conserved or invariant in other members of the Cyc-Cys/His-His class of finger proteins are indicated by the consensus sequence (Evans and Hollenberg, 1988).

Because of the morphological changes involved in gastrulation and the absence of any fixed reference point, we were unable to determine whether transcripts are eliminated from anterior or posterior cells within each stripe.

Coincident with the narrowing of the seven primary stripes, eight new stripes arise as transcripts appear in rows of cells which had no detectable accumulation at the previous stage. As with the primary stripes, these secondary stripes are formed in an overall anterior to posterior sequence, though the lag is short enough that differences between adjacent secondary stripes were not obvious. The first two secondary stripes appear in the two segments anterior to the primary stripe, while the remaining six stripes bisect the regions between the primary stripes. These intensify rapidly as gastrulation proceeds, becoming equivalent to the primary stripes by the time the germ band has elongated by one-third. The resulting pattern in which every segment is equivalently labeled persists through the remainder of germ band elongation, although the intensity of each stripe gradually decreases.

Relationship between odd-skipped and other segmentation gene patterns

The width and double segment spacing of the seven blastoderm stripes fit the pattern expected for *odd-skipped* based upon the cuticle phenotypes of mutant embryos (e.g., Figure 1). To determine the registration of this pattern relative to other segmentation genes, alternate sections of wild-type embryos were probed with clones for *odd* and *engrailed* (*en*; Kornberg *et al.*, 1985; Weir and Kornberg, 1986). The fourteen *en* stripes mark the posterior of each segment (or the anterior of each parasegment) and appear in a complex sequence in which the second stripe from the anterior is established first. This stripe arises at ~62% EL

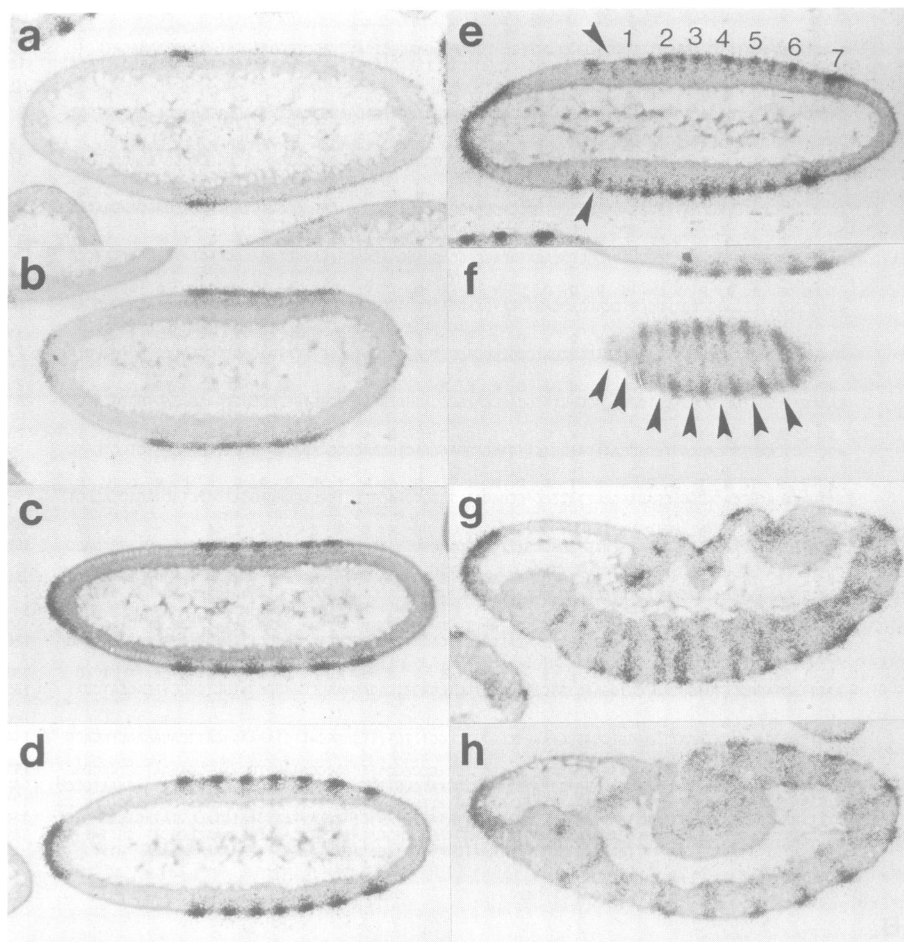

Fig. 5. Accumulation of *odd* transcripts during early embryogenesis. The distribution of *odd* transcripts in paraffin-sectioned wild-type embryos was detected using single-stranded probes labeled with ^{35}S. Panels a−d show the evolution of a seven stripe pattern as the blastoderm cellularizes; e−h depict the appearance of eight new secondary stripes during gastrulation. **a**, a syncytial blastoderm at late cycle 13/early cycle 14, showing the initial accumulation of *odd* transcripts in a single anterior stripe. **b**, an early cellular blastoderm. *odd* expression has expanded into more posterior cells, exhibiting irregular pattern which has begun to resolve into stripes; labeling of the anterior pole is also apparent. **c**, a blastoderm with cellularization half complete. Transcripts are present in six distinct stripes with a double-segment periodicity. **d**, a late cellular blastoderm (horizontal section). The seventh posterior *odd* stripe has been established, completing the pair-rule pattern. **e**, early gastrulation; numbers indicate the positions of the seven primary stripes. At this stage, two new anterior stripes and six intervening stripes have appeared. The second stripe from the anterior marks partially invaginated cells at the cephalic furrow (arrow). **f**, superficial section of an early gastrula; arrows indicate newly appearing intervening stripes. Note that the most posterior primary stripe is not present in this section. **g**, early germ band elongation. The secondary stripes are now equivalent to the primary stripes. **h**, extended germ band. The single segment pattern persists through this stage, although the intensity of each stripe has diminished. Autoradiographic exposure was for 12 days, and all sections shown were present on the same slide. All sections are oriented with anterior to the left; sagittal sections (a−c, f−h) are shown with the dorsal side up.

during cellularization and marks one to two cell rows in the maxillary segment primordium. We compared the *odd* and *en* patterns in adjacent sections from three different embryos in which the second stripe was the only *en* band visible (data not shown). In each case, the seven *odd* pair-rule stripes were fully established, but the secondary stripes were barely detectable. By aligning images of adjacent sections, we determined that the first primary *odd* stripe and the second *en* stripe occupied the same general region of the embryo. In each case, it appeared that the *en* band was adjacent and immediately anterior to the *odd* stripe, although the possibility that the two stripes overlap could not be excluded by this method. By extrapolation, this result suggests that each of the seven *odd* stripes lies posterior to an even-numbered *en* stripe.

Discussion

Spatial patterning of odd-skipped

The early *Drosophila* embryo is subdivided into a metameric pattern through a cascade of gene regulation which is mediated primarily by transcriptional control. The *odd-skipped* gene, in common with most other segmentation genes, exhibits two features which are consistent with a function in this cascade; the *odd* transcript is differentially expressed, and the *odd* locus encodes a putative DNA binding protein which is likely to function in transcriptional regulation.

odd transcripts accumulate in a dynamic pattern during early embryogenesis, with two temporally distinct modes. The first mode, which occurs as the blastoderm cellularizes

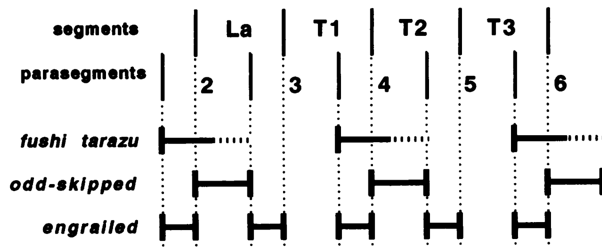

Fig. 6. Proposed relationship between *odd-skipped*, *engrailed*, and *fushi tarazu*. The primordia of four segments (labial and thoracic)/five parasegments at the blastoderm/early gastrula stage are represented schematically; the bars aligned below represent the spatial patterns of expression for the indicated genes. The anterior boundaries of the *ftz* stripes and the even-numbered *engrailed* stripes coincide cell-by-cell at the boundaries of alternate parasegments (Lawrence *et al.*, 1987; Carroll *et al.*, 1988), reflecting cell-specific positive regulation of *engrailed* by *ftz*. The posterior boundary of the *ftz* stripes is not precisely delineated due to the progressive loss of expression in this region. The *odd* pattern shown represents only the primary seven-stripe pattern. The boundaries indicated for *odd-skipped* should be considered approximate based upon the data presented here. We propose that the *ftz* product is unable to activate *engrailed* in more posterior cells due to the presence of the *odd-skipped* product in this region. Loss of *odd* function results in derepression (ectopic expression) of *engrailed* in some of these cells. Repression in wild-type embryos could result from direct interactions between *odd* and the *engrailed* gene and/or ftz protein. Alternatively, *odd* function might be mediated via repression of a second factor which acts in combination with *ftz* to activate *engrailed*; one likely candidate for such a factor is the *odd-paired* gene product.

(early division cycle 14), culminates in a pattern of seven periodic stripes. This represents the expected double-segment pattern common to the six pair-rule genes analyzed to date, although the various genes exhibit differences in the width and registration of blastoderm stripes. In addition, *odd-skipped* shares certain dynamic features with other pair-rule genes, including the refinement of broader patterns into discrete stripes and an anterior-to-posterior and ventral-to-dorsal temporal gradient as the pattern evolves during cellularization.

Despite the general similarities with other pair-rule patterns at the blastoderm stage, the *odd* pattern can be readily distinguished. First, *odd* is initially expressed in a narrow anterior band which resembles the early *prd* pattern (Kilcherr *et al.*, 1986); both patterns are distinct from the broad patterns of uniform (*ftz*, Weir and Kornberg, 1985; *hairy*, Ingham *et al.*, 1985; *runt*, Gergen and Butler, 1988) or graded (*eve*, Harding *et al.*, 1986; Macdonald *et al.*, 1986) expression seen in other cases. However, the *odd* and *prd* patterns diverge during the subsequent addition of more posterior stripes: here, *prd* transcripts are reported to accumulate as discrete stripes whereas the *odd* transcripts arise in a broader pattern and resolve into stripes as intervening gaps are created. Second, the seven stripe *odd* pattern is preceded by a distinct six stripe intermediate due to the delayed appearance of the seventh *odd* stripe. While such a six stripe pattern is unprecedented, it is notable that the *prd* pattern evolves from 7–8 stripes during late cellular blastoderm due to *de novo* accumulation in a posterior band of cells. This eighth *prd* stripe and the seventh *odd* stripe arise in a similar location at approximately the same time, raising the possibility of a common regulatory mechanism in this region. Finally, *odd* is expressed in a broad domain at the anterior pole of the embryo. While this is reminiscent of 'extraperiodic' anterior/dorsal domains of *paired* (Kilcherr *et al.*, 1986) or *hairy* (Ingham *et al.*, 1985), the *odd* patch is distinct in its dorsal/ventral symmetry and extreme anterior location. It is likely that expression in this region is required for proper development of the labrum, as certain labral derivatives are defective in *odd* mutant cuticles (Coulter and Wieschaus, 1988).

The second mode of *odd-skipped* expression commences at the onset of gastrulation with the appearance of eight secondary stripes. These result from *de novo* expression and arise in segments complementary to those which express the seven primary stripes. The intensity of these secondary stripes increases to the level of the primary stripes, such that homologous regions of every segment are equivalently labeled in the extending germ band.

Similar transitions from a double to a single segment mode have been reported for the pair-rule genes *prd* (Kilcherr *et al.*, 1986), *even-skipped* (*eve*; Macdonald *et al.*, 1986), and *runt* (Gergen and Butler, 1988), but the mechanism responsible is not the same in each case. With *prd*, the doubling occurs in the late cellular blastoderm via a complex process in which six of the broad primary stripes become split as transcripts are eliminated from cells in the middle. In contrast, *de novo* accumulation in interstripe regions generates the secondary stripes of *odd*, *eve*, and *runt*. With *eve*, the intensity of the intervening secondary stripes remains relatively low, resulting in a pattern similar to the earlier stages of the *odd-skipped* transition. On the other hand, the *runt* pattern more closely resembles that of *odd*: in both cases, transcripts in the secondary stripes accumulate to the level of the primary stripes during germ band elongation. Finally, a simple doubling to fourteen stripes is observed with *prd* and *eve*, where the *odd* pattern changes from seven to fifteen due to the labeling of the six intervening segments as well as two adjacent gnathal segments anterior to the first primary stripe.

Odd requirements and odd expression patterns

The coupling between localized requirements and corresponding patterns of gene expression is common to most zygotically active segmentation genes. In the case of the pair-rule genes, mutant phenotypes generally indicate requirements within homologous regions of every other segment. Moreover, both the temperature-sensitive periods (Nüsslein-Volhard and Wieschaus, 1980; Wakimoto *et al.*, 1984) and altered patterning of downstream genes in pair-rule mutants (e.g., DiNardo and O'Farrell, 1987; Ingham *et al.*, 1988) indicate that the blastoderm stage is a critical period for pair-rule gene function. The characteristic pair-rule pattern of seven blastoderm stripes presents an attractive simplifying theme for relating patterns of gene expression to domains of pair-rule gene function. However, the dynamic features of gene expression observed with several of these genes indicate that a less simplistic view might be necessary.

The regions of the segmental pattern known to be affected by *odd* mutations are confined to every other segment. Cuticle defects in mutant embryos are smaller than a segment and correspond roughly to posterior portions of the domains affected by *ftz* mutations (Coulter and Wieschaus, 1988; Wakimoto *et al.*, 1984). An indication that *odd* function is required at the blastoderm stage in this region is provided by studies of *engrailed* (*en*) expression in *odd* mutants (DiNardo and O'Farrell, 1987; Martinez-Arias and White, 1988). In the absence of *odd* function, *engrailed* arises in

an abnormal pattern in which the normal stripes are supplemented by ectopic expression in rows of cells posterior to the even-numbered *en* stripes. While the *en* pattern subsequently undergoes complex changes which apparently reflect a second-tier of *en* regulation (DiNardo and O'Farrell, 1987; S.DiNardo, personal communication), the initial pattern indicates a role for *odd* in defining the posterior boundaries of the even-numbered *en* stripes, possibly by preventing the activation of *en* by *ftz* (see Figure 6). Furthermore, the cuticle patterns of *odd en* double mutants (Coulter and Wieschaus, 1988) indicate that *en* is largely epistatic to *odd*, suggesting that ectopic *en* expression is responsible for the pattern deletions and duplications associated with the *odd* phenotype. Taken together, these studies suggest that a critical function of *odd* is to repress *en* in a portion of every other segment. The phasing, width and timing of the seven blastoderm *odd* stripes appear to be consistent with such a role (Figure 5). However, further evaluation of this role will require a precise definition of the registration between *odd* and *en* stripes on a cell-by-cell basis once an antibody to the *odd* protein becomes available.

While the seven-stripe *odd-skipped* pattern corresponds well with the pair-rule phenotype observed in *odd* mutants, it is not clear what role the later, single-segment pattern might play. The existence of similar modes with four of the six pair-rule genes analyzed indicates that this unexpected property is not an unusual aberration. However, among these four genes, only *eve* appears to have potentially analogous effects within every segment, namely the absence of both the even- and odd-numbered *en* stripes in *eve* null mutants (Harding *et al.*, 1986; Macdonald *et al.*, 1986; DiNardo and O'Farrell, 1987). The *prd* and *runt* phenotypes, like *odd-skipped*, give no indication that these genes are required to generate homologous patterns within every segment. The functional significance of the post-blastoderm patterns observed for these genes is obscure.

One possibility is that the expression of *odd-skipped* and similar pair-rule genes in alternate segments has no functional significance. The effects of many pair-rule genes appear to be modulated in response to local contexts; indeed, partial overlaps between pair-rule domains are probably critical for generating combinatorial signals which activate segment-polarity genes in relatively narrow stripes (DiNardo and O'Farrell, 1987; Ingham *et al.*, 1988). This raises the possibility that pair-rule gene products might be present but wholly inactive in particular cells where interacting gene products do not overlap. Similarly, temporally-limited factors might confine *odd-skipped* function to the blastoderm stage, such that the later expression in alternate segments (and the persistence of the primary stripes) is irrelevant. In principle, both possibilities can be tested by engineering a hybrid gene with expression of the *odd* product under control of an inducible promoter (e.g., Struhl, 1985; Ish-Horowicz and Pinchin, 1987). In either case, the single segment modes of expression might represent vestiges of control mechanisms with evolutionary significance, but would make the use of spatial patterns to infer extant developmental functions futile.

Assuming the single segment modes of expression are functionally significant, the question arises of why no corresponding requirements have been detected in mutant phenotypes. Two possibilities exist: first, the effects in mutants could be quite subtle in comparison with the dramatic pair-rule defects, or might be limited to tissues which are not routinely examined (e.g., cuticle preparations only reveal defects in epidermal tissues). Alternatively, the secondary requirements might be 'buffered' by the presence of multiple gene products which play analogous roles. In this context, it is interesting to note that we have isolated several embryonic cDNA clones derived from other loci which cross-hybridize with the zinc finger-encoding regions of *odd-skipped* (D.Coulter and D.Kerr, unpublished observatons). It is possible that one or more of these structurally related genes could serve the same function as *odd* in the cells comprising the secondary *odd* domains. Any such overlapping functions are necessarily distinct from the primary pair-rule requirements, which must involve unique functions to give rise to obvious phenotypes; however, it is possible that such roles could be manifest in appropriate double mutant combinations.

Diversity of pair-rule patterns

Although the precise relationship between patterns of pair-rule gene expression and patterns of requirements remains uncertain, it is clear from genetic and molecular studies that each pair-rule gene is unique with respect to both. The diversity of expression patterns points to the complexities of pair-rule gene regulation. The differential spatial cues which regulate the pair-rule patterns appear to be largely provided by localized products of other segmentation genes, principally gap genes and/or other pair-rule genes, and in some cases the *cis*- and *trans*-acting elements are becoming well characterized at the molecular level (e.g., Hiromi and Gehring, 1987; Howard *et al.*, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989).

While the further identification and dissection of specific regulatory interactions between segmentation genes will be essential for a thorough description of the pattern forming mechanism, certain constraints on the structure of the regulatory network are suggested *a priori* by comparisons of expression patterns observed to date. For example, while *odd* expression is probably controlled by other pair-rule genes, the uniqueness of the *odd-skipped* pattern suggests that no other pair-rule gene is solely responsible for its differential regulation. Similarly, the diversity of pair-rule patterns indicates either that no two genes share an identical set of *trans*-regulators, or that quantitatively different responses must exist between any genes which do. An understanding of the dynamic process of segmentation will require the identification of the different sets of regulators and a determination of how they interact to control the patterned expression of each segmentation gene.

Materials and methods

Drosophila strains

The odd^{hd1} allele was fortuitously identified in a hybrid dysgenesis-induced X-linked lethal stock and subsequently recovered as a balanced second chromosome stock. odd^{hd3} was isolated by Howard Lipshitz during hybrid-dysgenic screens for maternal-effect mutations on the second chromosome, and provided as a generous gift. Other *odd* alleles are listed in Coulter and Wieschaus (1988). Unless otherwise indicated, all *odd* mutants were maintained as balanced heterozygotes over the CyO chromosome.

To eliminate unrelated P elements present in the odd^{hd1} stock, females were extensively backcrossed to M-strain males homozygous for a marked second chromosome (*al dp b pr c px sp*); at each generation, phenotypically dp^+ females were selected as probable odd^{hd1} heterozygotes. After 6–8 generations, balanced sublines were established from single dp^+ second chromosomes which had recombined both proximal (*b pr*, etc.) and distal (*al*) markers; 6 (of 11 total) of these sublines retained the *odd* lesion.

odd^{hd1} revertants

Hybrid dysgenesis induced reverants of the *hd1* allele were isolated by screening for loss of the recessive lethality characteristic of *odd* mutants. Dysgenesis was induced by mating females from an *hd1* subline (an M-strain) at 22°C to P-strain males bearing a second chromosome balancer (kindly provided by Claire Cronmiller). Dysgenic male progeny which carried the *hd1* bearing chromosome and the balancer were selected and mated to balanced heterozygotes from the hypomorphic odd^{IIIC} stock. *hd1*-derived chromosomes which complement the *IIIC* allele to adult viability were identified in surviving non-Cy adult progeny; these were individually mated to flies from a strong *odd* stock (odd^{IIID}) to retest and recover the revertant chromosomes. Overall, 30 revertants were identified (compared to 475 balanced siblings); only those known to represent independent events (i.e., from separate vials) were included in the Southern analysis reported in Results.

In addition to the revertants generated via the scheme above, several appeared spontaneously in odd^{hd1} stocks prior to the backcrossing scheme described above. These apparently resulted from an unstable cytotype in the initial stocks, as the allele remained stable in the backcrossed sublines. While not included among the revertants reported in Results, Southern analysis of chromosomes from seven such revertants (representing a minimum of four independent events) indicated precise excisions of the P element.

DNA isolation and analysis

Plasmid and lambda phage DNAs were prepared by standard methods (Maniatis *et al.*, 1982). Genomic *Drosophila* DNA was isolated from adult flies (50–100 per preparation) using the method of Ish-Horowicz *et al.* (1979). Southern blots were prepared and hybridized as described by Maine *et al.* (1985), with washes at 65°C in 0.2 × SSPE.

For DNA sequencing, fragments were subcloned into the Bluescript vector (Stratagene), and nested deletions for both strands were generated by ExoIII digestion (Henikoff, 1984). Single or double stranded DNA templates were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase 2.0 (US Biochemical).

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Isolation of odd-skipped clones

Prior to the construction of odd^{hd1} genomic libraries, the number of P elements present in four backcrossed odd^{hd1} sublines (see above) was esimated by probing Southern blots of *Bam*HI digested genomic DNA with pp25.1 (O'Hare and Rubin, 1983). A P-homologous fragment of 3.9 kb was identified in all four stocks; each contained one or two additional large (> 10 kb) fragments, but none of these were common to all four lines.

Two recombinant DNA libraries were constructed by inserting genomic DNA from a backcrossed odd^{hd1} subline into the *Bam*HI site of λ EMBL3, following either partial digestion of genomic DNA with *Sau*3A or complete digestion with *Bam*HI. Libraries were screened with pp25.1 as described (Maine *et al.*, 1985), and positives were counter-screened with a second P-homologous clone (RUD 5; Tsubota and Schedl, 1985) to eliminate recombinants derived from the p25.1 genomic site (band 17C). One positive, HD-5, contained the expected P-homologous *Bam*HI fragment of 3.9 kb and hybridized to polytene band 24A. The *Bam*HI fragment was subcloned and used to screen a wild-type *Drosophila* genomic library (Maniatis *et al.*, 1978). Seven positive clones representing four independent recombinant bacteriophage were obtained. Hybridization *in situ* to polytene chromosomes using one of these phage confirmed band 24A as its cytogenetic origin.

odd cDNA clones were isolated from embryonic cDNA libraries (the 0–3 and 3–12 h embryo libraries of Poole *et al.*, 1985, and 0–4 h library of Kilcherr *et al.*, 1986) using cloned genomic DNA fragments as probes. The initial probe used was a 6.4 kb *Bam*HI–*Eco*RI fragment (extending from the *Bam*HI site shown on the enlarged genomic map to the rightward end of the second phage shown); positive clones were rescreened with the 2.1 kb *Xho*I fragment described in Figure 3A to eliminate clones derived from other loci which cross-react with sequences to the left of this fragment.

In situ hybridization

In situ hybridization to embryo paraffin sections (6 μm) was performed as described by Ingham *et al.* (1985) using a ^{35}S-labeled *odd* antisense RNA transcribed by SP6 polymerase from a linearized 0.4 kb cDNA (clone A.4) subcloned into the *Eco*RI site of pGEM2.

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