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The *Drosophila fish-hook* gene encodes a HMG domain protein essential for segmentation and CNS development

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SUMMARY

We describe the isolation and analysis of the *Drosophila fish-hook* (*fish*) gene, which encodes a novel member of the SOX subgroup of High Mobility Group (HMG) domain proteins that exhibit similarity to the mammalian testis determining factor, SRY. The *fish* gene is initially expressed in a pair-rule-like pattern which is rapidly replaced by strong neuroectoderm expression. *fish* null mutants exhibit severe segmentation defects, including loss and/or fusion of abdominal denticle belts and stripe-specific defects in pair-rule and segment polarity gene expression. *fish* mutant embryos also exhibit loss of specific neurons, fusion of adjacent ventral nerve cord ganglia and aberrant axon scaffold organization. These results indicate an essential role for *fish* in anterior/posterior pattern formation and nervous system development, and suggest a potential function in modulating the activities of gap and pair-rule proteins.

Key words: *Drosophila*, *fish-hook*, HMG domain, SOX protein, segmentation, CNS

INTRODUCTION

The process of segmentation in *Drosophila* has served as a powerful paradigm for understanding early embryonic pattern formation and specification of cell fates. This process is mediated by a complex cascade of gene regulatory and cell signaling events (reviewed in Martinez Arias, 1993; Pankratz and Jäckle 1993), which ultimately results in establishment of distinct anterior/posterior polarity both along the entire length of the embryo and within each segmental unit. Transcription regulation plays a major role in the generation of segmental periodicity, which is first indicated by stripes of pair-rule gene expression. In this regard, the maternal effect gene, *bicoid*, as well as nearly all of the gap and pair-rule genes encode transcription factors, such as homeodomain, zinc finger, basic-helix-loop-helix or basic-leucine zipper proteins (reviewed in Pankratz and Jäckle, 1993). Recent studies suggest that the stripes of most or all pair-rule genes are generated through distinct mechanisms in each segment, with direct positive and negative transcriptional regulatory input from maternal effect and gap genes (Gutjahr et al., 1993; Yu and Pick 1995). Subsequent refinement and stabilization of stripes of pair-rule gene expression involves both auto- and cross-regulatory interactions among pair-rule genes (reviewed in Pankratz and Jäckle, 1993). These complex regulatory networks are reflected in the organization of pair-rule gene regulatory regions, which typically contain multiple copies of distinct transcription factor binding sites dispersed over large DNA regions.

A key issue in understanding the segmentation process is how these many regulatory interactions are coordinated. One important mechanism is likely to be the regulation of chromatin structure to facilitate the assembly and stabilization of multiple protein/DNA and protein/protein complexes. Although this process is in general not well characterized, two clear examples are the members of the *polycomb* and *trithorax* gene families, which encode disparate types of proteins that form complexes capable of altering chromatin structure (reviewed in Simon, 1995). These ubiquitously expressed genes act as positive and negative regulators essential for proper maintenance of homeotic gene expression (e.g. Peifer and Wieschaus, 1990) and mutations in some of these genes also influence the expression of other classes of segmentation genes (e.g. McKeon et al., 1994).

Members of the High Mobility Group (HMG) protein family also regulate gene transcription through modulating chromatin structure (see Landsman and Bustin, 1993; Grosschedl et al., 1994). These proteins contain one or more HMG domains, a discrete ~80 amino acid DNA-binding structure (Jantzen et al., 1990). One major subgroup of HMG proteins includes the mammalian testis determining factor, SRY (Koopman et al., 1991) and a large number of related SOX proteins that share at least 60% amino acid identity to the HMG domain of SRY (Laudet et al., 1993). SRY and all SOX proteins have a single HMG domain and exhibit similar sequence-specific DNA-binding properties, and diverse tissue specific expression patterns (e.g. Harley et al., 1992; Denny et al., 1992; Hosking et al., 1995). The developmental functions and downstream target genes of these SOX proteins are just beginning to be defined. SOX-9 has been shown to function in mammalian bone formation and sex determination, as SOX-9 mutations underlie the genetic disorder, campomelic dysplasia (Foster et al., 1994). SOX-4 mediates activation of the T cell receptor

and CD-2 genes in lymphoid cells (van de Wetering et al., 1993; Wotton et al., 1995), while SOX-2 is strongly expressed in the developing neuroepithelium of vertebrate embryos (Uwanogho et al., 1995) and regulates gene expression in developing lens cells (Kamachi et al., 1995). The strong DNA bending properties of SRY and SOX proteins (Giese et al., 1992; Harley et al., 1992; Connor et al., 1994), as well as the inability of several SOX proteins to directly transactivate transcription of target genes (Kamachi et al., 1995; Yuan et al., 1995), suggest that they may provide important architectural functions in assembly and stabilization of transcription factor/DNA complexes.

In this study, we describe the isolation and analysis of a *Drosophila* Sox gene that is required for segmentation and nervous system formation. We have named this gene *fish-hook* (*fish*), based on the appearance of its expression pattern in sagittal views of early germ-band-extended embryos. The *fish* gene is dynamically expressed, with early expression in the entire trunk of syncitial blastoderm embryos that is rapidly refined into a series of 7 irregular ectodermal stripes in the cellular blastoderm. During germ-band extension, *fish* expression becomes largely restricted to the developing ventral and cephalic neuroectoderm. Generation and analysis of *fish* null mutant strains indicated loss and/or fusion of abdominal segments coupled with stripe-specific defects in pair-rule and segment polarity gene expression. *fish* mutant embryos also exhibited CNS defects which included fusion of adjacent ganglia, loss of specific neurons and aberrant axon scaffold organization. The data suggest the FISH protein may modulate the actions of other transcription factors, including gap and pair-rule proteins.

MATERIALS AND METHODS

Generation of *fish-hook* mutants

The *fish* gene was initially identified via a viable P element (the PZ a.k.a. P[*rosy*+, lacZ] vector of Y. Hiromi, see Klämbt et al., 1991) enhancer trap insertion, rJ375, on the third chromosome. rJ375 was generated in a large screen by C. Goodman and colleagues (Klämbt et al., 1991). In situ hybridization of a biotinylated P element DNA probe to rJ375 larval polytene chromosomes identified a single site of hybridization at position 70D on the left arm of the third chromosome. Imprecise excision alleles were generated by crossing rJ375 flies to a P[Δ2-3] transposase strain (Robertson et al., 1988) and screening for a *rosy* eye phenotype due to loss of *rosy*+ gene function on the P element. From 100 unique excision events, 6 recessive lethal chromosomes were identified where lethality mapped to the 70D region. The 6 excision strains, $fish^{8}$, $fish^{55}$, $fish^{65}$, $fish^{70}$, $fish^{87}$ and $fish^{96}$, all failed to complement each other as well as Df(3L)*fz*-D21 (70D1-2;70E7), Df(3L)*fz*-GF-3b (70C1-2;70D4-5) and Df(3L)*fz*-GS1a (70C6-15;70E4-5). The excisions were all viable over Df(3L)*fz*-M21 (70D2-3;71E4-5). $fish^{87}$ and $fish^{96}$ are null alleles that do not express any detectable *fish* mRNA transcripts. Genomic Southern blot assays indicated that these alleles both possess small deletions of DNA in the *fish* locus (data not shown).

In situ hybridization, immunocytochemistry and larval cuticle preparations

In situ hybridizations were performed by generating digoxigenin-labeled antisense RNA probes from *fish*, *even-skipped*, *wingless* and β-galactosidase DNA clones using reagents from the Genius Labeling Kit (Boehringer Mannheim) and T3 and T7 RNA polymerases. Whole-mount in situ hybridizations to embryos collected from wild-type and *fish* mutant strains were performed as described by Tautz (1992). The stained embryos were cleared in 80% glycerol and viewed and photographed using Nomarski optics.

Immunocytochemistry was performed as described in Zhou et al. (1995) using: a mouse anti-β-gal monoclonal antibody (Promega) at 1:800 dilution, mAb BP102 (provided by Karen Jensen, Developmental Studies Hybridoma Bank) at a 1:10 dilution and a rabbit anti-FTZ antiserum (provided by Y. Hiromi) at 1:1000 dilution. Visualization of antibody binding was performed using biotinylated secondary antibodies, streptavidin-horseradish peroxidase and diaminobenzidine reactions using the Vectastain ABC kit (Vector Labs). Stained embryos were dehydrated in an ethanol series, cleared in methyl salicylate and mounted in Permount (Fisher). Stained embryos were viewed and photographed using Nomarski optics.

1st instar larval cuticles were prepared from non-hatching *fish* mutant embryos essentially as described in Wieschaus and Nüsslein-Volhard (1985). The cuticles were observed and photographed under dark-field optics.

Molecular techniques

Plasmid rescue of *fish* DNA was accomplished by digesting genomic DNA from the rJ375 strain with *Xba*I and *Nhe*I, ligating under dilute conditions, and transforming into *E. coli* DH5α cells (GIBCO/BRL). A 1.4 kb *Hin*dIII/*Sac*I DNA fragment was isolated from the rescued DNA, labeled with ^{32}P and used to screen 100,000 recombinant phage from a *Drosophila* genomic DNA library (provided by L. Schwartz). 10 clones were identified that contain overlapping DNA from the 70D region.

fish cDNA clones were isolated by screening a 9-12 hour embryonic λgt-11 library (provided by K. Zinn) using a ^{32}P-labeled 6.0 kb *Sac*I *fish* genomic DNA probe. Approximately 300,000 phage were screened and 20 clones were identified that contain cDNA insertions ranging from 0.5 kb to 2.0 kb. These cDNA insertions were all found to derive from a 2.6 kb *Bam*HI/*Eco*RI fragment located within the 6.0 kb *Sac*I *fish* genomic DNA fragment. The cDNA inserts were subcloned into pBS plasmid vectors (Stratagene) and purified DNA from the longest clone, 2-5, was sent to Retrogen Inc. for double-stranded DNA sequence analysis using primer walking techniques.

Northern blots were performed using poly(A)$^+$ mRNA isolated via oligo(dT) columns from 0-5 and 0-24 hour collections of Canton-S embryos. The RNA was electrophoresed on a formaldehyde/agarose gel, transferred to Nytran filters (Schleicher and Schuell), and hybridized to a ^{32}P-labeled *fish* 2.0 kb cDNA probe and, subsequently, to a ^{32}P-labeled ribosomal protein 49 (RP49) 600 bp DNA probe (clone provided by M. Rosbash).

RESULTS

Drosophila fish-hook mutants exhibit segmentation defects

In an attempt to identify genes important for embryonic CNS development, in situ hybridization was used to detect the earliest transcription of β-galactosidase (β-gal) in several P[lacZ] enhancer trap strains. One strain, rJ375 (obtained from C. Goodman), exhibited prominent early β-gal transcription in a series of seven irregular ectodermal stripes and the procephalic region in stage 5 cellular blastoderm embryos (Fig. 1A; stages defined in Campos-Ortega and Hartenstein, 1985). At this point, insufficient β-gal protein was present to be detected via anti-β-gal immunocytochemistry (data not shown). During germ-band extension, β-gal transcripts became largely restricted to the developing neuroectoderm (Fig. 1B) and the expression of β-gal protein could first be detected. This

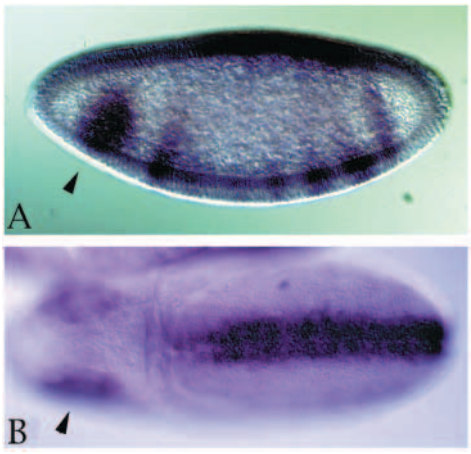


Fig. 1. β-galactosidase transcription pattern of the rJ375 enhancer trap strain. (A) Embryonic in situ hybridization of a β-gal antisense RNA probe to a stage 5 cellular blastoderm rJ375 embryo, showing β-gal transcripts in 7 irregular ectodermal stripes and procephalic region (arrowhead). Sagittal view with anterior to left. (B) Similarly stained stage 9 germ-band-extended rJ375 embryo, showing prominent β-gal expression in the ventral and cephalic neuroectoderm (arrowhead). Dorsal view with anterior to left.

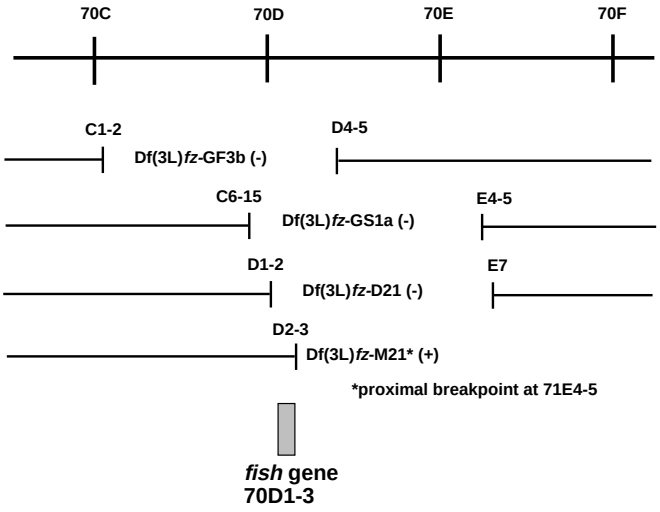


Fig. 2. *fish* mutations map to 70D1-3. Chromosomal breakpoints and deleted regions in several deficiency strains are denoted. The ability of *fish* excision mutants to complement these deficiencies is shown, as is the region where the *fish* mutants map.

expression pattern hinted that the corresponding gene, which we have named *fish-hook* (*fish*), might play a role in segmentation and nervous system development, and led us to pursue further analyses. The rJ375 strain was found to contain a single P element insertion that mapped to 70D on the third chromosome. Genetic analyses were initiated via generation of P element excision alleles and six embryonic lethal excision strains were isolated that all fail to complement each other (see Materials and Methods). Additional complementation tests to four deficiency strains, Df(3L)*fz*-D21, Df(3L)*fz*-GF-3b, Df(3L)*fz*-GS1a and Df(3L)*fz*M21, localized the site of lethality to 70D1-3 (Fig. 2).

The potential role of *fish* in segmentation was assayed by larval cuticle preparations from two *fish* null mutant alleles, $fish^{87}$ and $fish^{96}$ (see Materials and Methods and below). Strikingly, both alleles exhibited severe segmentation defects, including loss or fusion of abdominal denticle belts (Fig. 3A-C). Although the defects were variable, in strongly affected mutant embryos only 4 abdominal denticle belts were present. Unlike pair-rule mutants, however, this loss of denticle belts does not correspond to a segmentally repeated pattern. Typically, the A2 and A8 denticle belts were missing, the A3 denticle belt was narrowed and there were fusions between the A4-A5 and A6-A7 denticle belts. Many of the *fish* mutants also exhibited defects in the organization of head structures. Alternately, the thoracic segments appear largely unaffected. Similar phenotypes were seen in $fish^{87}$/Df(3L)*fz*-D21 *trans*-heterozygotes (Fig. 3D). These experiments indicated that the *fish* gene plays an important role in segmentation.

fish regulates specific stripes of pair-rule and segment polarity gene expression

In order to understand the basis of segmentation defects in *fish*

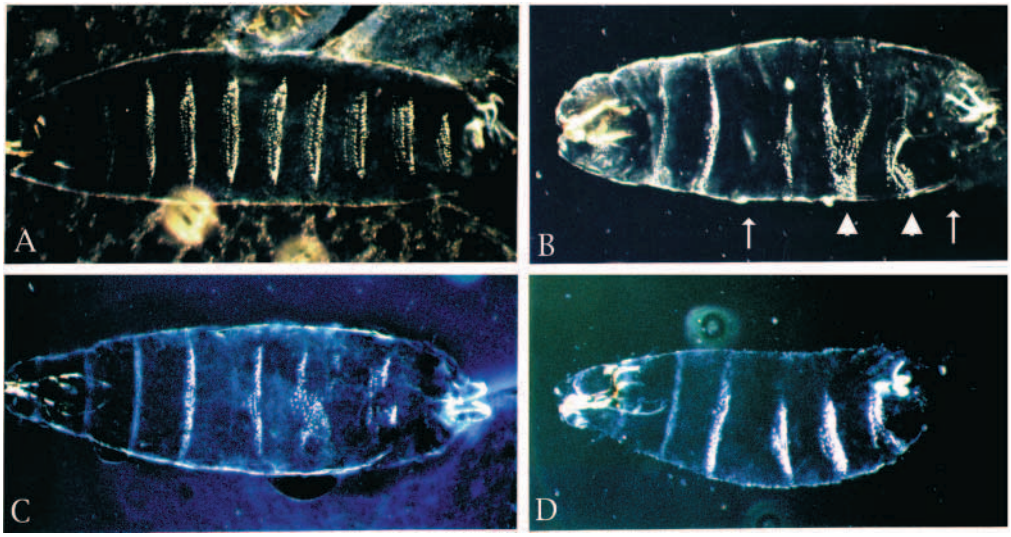


Fig. 3. *fish* is essential for segmentation. Larval cuticle preparations from wild-type and *fish* mutant strains. (A) Wild-type 1st instar larval cuticle. Note 8 abdominal denticle belts. (B) Unhatched $fish^{87}$ mutant cuticle exhibiting severe segmentation defects in abdominal denticle belts. The A2 and A8 denticle belts are deleted (arrows), while A3 is narrowed, and A4,5 and A6,7 are fused (arrowheads). (C) Unhatched $fish^{96}$ mutant cuticle exhibiting similar segmentation defects. (D) Unhatched $fish^{87}$/Df(3L)*fz*-GF-3b mutant embryo showing similar segmentation defects. All views ventral with anterior to right.

mutants, we examined the expression of pair-rule and segment polarity genes. In situ hybridizations using a *fushi-tarazu* (*ftz*) antisense RNA probe and immunocytochemistry using an anti-FTZ serum indicated that *ftz* expression is initiated normally in *fish*^{96} mutant syncitial blastoderm embryos (data not shown), however, by the onset of cellularization specific stripes of *ftz* expression became altered (Fig. 4A,B). Typically, *fish* mutant embryos exhibited complete or partial fusions between *ftz* stripes 3 and 4, and a weakening of stripes 5 and 6. These results indicate that *fish* function is essential for either repressing or maintaining *ftz* expression in different segmental domains. These distinct roles are consistent with proposed stripe-specific mechanisms of *ftz* gene regulation (Yu and Pick, 1995). The effects of *fish* mutations on *even-skipped* (*eve*) expression were also examined. *eve* transcription is also initiated normally in *fish*^{96} mutant syncitial blastoderm embryos but, by the onset of cellularization, *eve* stripe 4 is severely weakened or lost (Fig. 4C,D), and *eve* stripes 5, 6 and 7 exhibit variable weakening and/or partial fusion.

Expression of the segment polarity gene *wingless* (*wg*) was initiated normally in the *fish*^{96} mutant blastoderm embryos; however, during gastrulation, alterations in the intensity of several stripes became evident and, in fully germ-band-extended *fish* mutant embryos, there were clear segment-specific defects in *wg* expression (Fig. 4E-H). These defects typically included a loss of *wg* stripes in ventral regions of the maxillary and labial segments, fusions between the A1 and A2 stripes, and fusion or reduction of other abdominal stripes. Interestingly, the loss of *wg* expression in head segments was independent of corresponding defects in *ftz* or *eve* expression. Several segments also exhibited aberrant spacing between *wg* stripes at the ventral midline (see Xiao et al., 1996), and there is an enlargement of the expression domain at posterior tip of the germ band at the site of hindgut invagination. Similar segment-specific defects were also detected in *engrailed* expression, as immunocytochemistry using the mAb 4D9 revealed loss and/or fusion of specific stripes in germ-band-extended embryos (data not shown).

fish-hook mutants exhibit defects in CNS development

The strong neuroectodermal expression of *fish* suggested a potential role in nervous system development. This was assayed via immunostaining with mAb BP102, which labels all CNS axons (Elkins et al., 1990). *fish*^{87} mutant embryos exhibited severe and variable defects in CNS organization (Fig. 5A,B). Typically, there were fusions between several adjacent

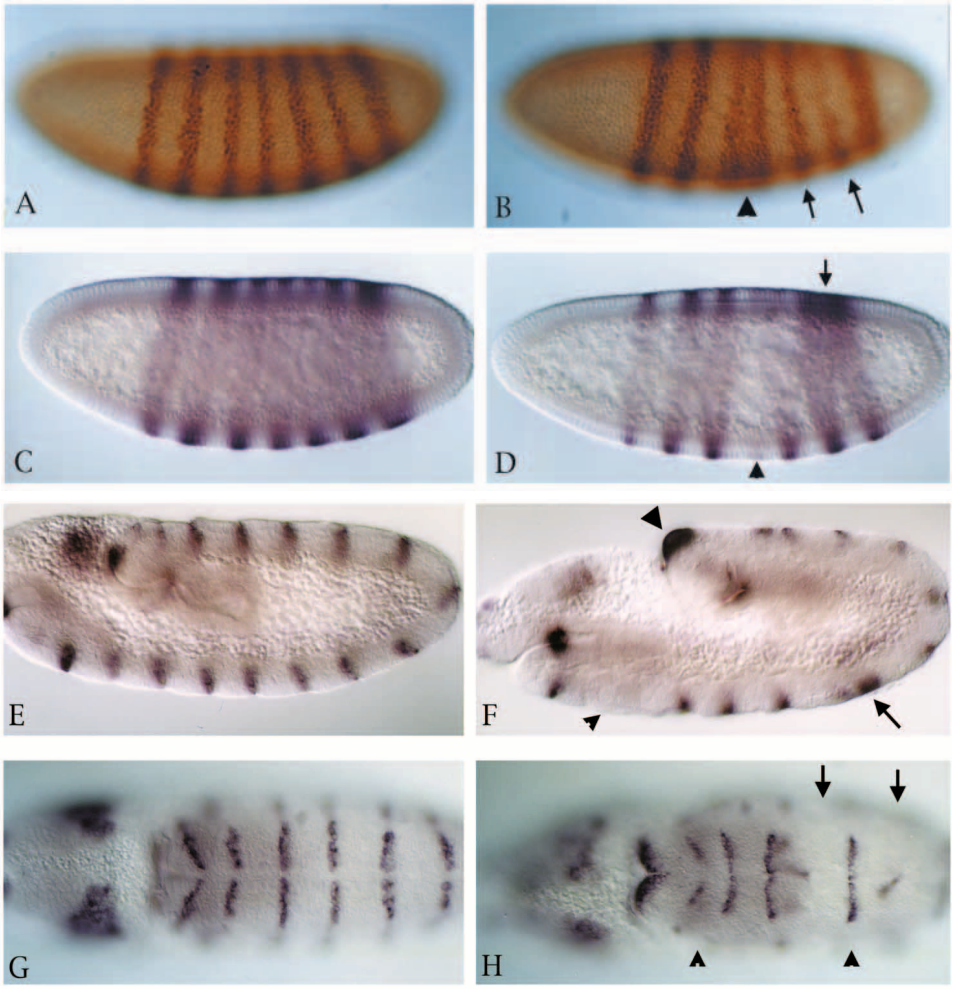


Fig. 4. *fish* mutants exhibit stripe-specific defects in pair-rule and segment polarity gene expression patterns. (A) Expression of FTZ protein in a stage 5 wild-type embryo as detected via anti-FTZ immunostaining. Note regular array of 7 pair-rule stripes in even parasegments. (B) Similarly stained stage 5 *fish*^{96} mutant embryo. Note fusion of FTZ stripes 3 and 4 (arrowhead) and weakened stripes 5 and 6 (arrows). (C) Expression of *eve* mRNA in stage 5 wild-type embryo as detected via in situ hybridization using an *eve* antisense RNA probe. Note regular array of pair-rule stripes in odd parasegments. (D) Similarly stained stage 5 *fish*^{96} mutant embryo. Note weakened *eve* stripe 4 (arrowhead) and partial fusions between stripes 5-7 (arrow). (E) Expression of *wingless* mRNA in stage 11 wild-type embryo as detected via in situ hybridization using a *wingless* antisense RNA probe. Note regularly spaced segment polarity stripes at the anterior border of each segment. (F) Similarly stained stage 11 *fish*^{96} mutant embryo. Note loss of expression in *wingless* stripes 2 and 3 in head segments (small arrowhead) as well as fusions of stripes 7,8 (arrow) and 13,14. There is also ectopic *wingless* expression in cells at the end of the germ band near the site of hindgut invagination (large arrowhead). (G) Dorsal view of a stage 11 wild-type embryo showing regular array of *wingless* stripes in the ventral and lateral neuroectoderm, but not in CNS midline cells. Anterior is to left. (H) Dorsal view of a similarly stained stage 11 *fish*^{96} mutant embryo. Note loss of stripes 10 and 12 in this specimen (arrows) and defects in spacing of some *wingless* stripes at the midline (arrowheads). (A-F) Sagittal views with anterior to left.

neuromeres resulting in 3-4 fewer ganglia as compared to wild type. In some segments, often including the thoracic segments, there was a moderate to severe narrowing of the longitudinal axon connectives and fusion of the anterior and posterior axon commissures. This phenotype is similar to that of mutations in *slit*, as well as *single-minded* and other *spitz* class genes, where there are defects in differentiation or migration of CNS midline cells (e.g. Klämbt et al., 1991). The CNS defects were further analyzed via immunostaining with mAb 4D9 to identify subsets of CNS cells that express the *engrailed* gene (Patel et al., 1989). There did not appear to be defects in the formation of *engrailed*-expressing cells in the CNS, which were normal in number in germ-band-extended embryos (data not shown). However, during germ-band retraction nearly all segments began to exhibit loss and/or fusion of midline and lateral *engrailed*-expressing CNS cells (Fig. 5C,D). Loss of CNS midline cells, including the median neuroblast and VUM neurons, is likely responsible for at least some of the axon scaffold defects observed. These results suggest that *fish* is essential for proper differentiation and/or survival of specific CNS cells.

The *fish* gene encodes a novel SOX protein

Molecular analysis of the *fish* gene was initiated via plasmid rescue techniques to isolate genomic DNA flanking the P element insertion. A 1.4 kb *Hin*dIII/*Sac*I fragment was recovered and used to screen a *Drosophila* genomic DNA library, resulting in the isolation of 34 kb of genomic DNA from the *fish* locus at 70D (Fig. 6A). In situ hybridization experiments identified a 6.0 kb *Sac*I genomic DNA fragment adjacent to the site of P element insertion that yielded a hybridization pattern closely mimicking the β-gal expression pattern from the rJ375 strain. Significantly, no other DNA fragment in this region detected any embryonic expression. In Northern blots, the 6.0 *Sac*I fragment detected a single 2 kb mRNA transcript in poly(A)$^+$ mRNA from both 0-5 hour and 0-24 hour wild-type embryo collections (Fig. 6B). This genomic fragment was then used to isolate several *fish* cDNA clones from an embryonic cDNA library. These clones all exhibited embryonic expression patterns identical to each other (see below) and to the 6.0 kb genomic DNA fragment.

DNA sequence analysis was performed on the longest *fish* cDNA clone, 2-5, to define the structure of the *fish* gene product. The sequenced region of the cDNA insert is 1914 bp in length, not including an approximately 40 nucleotide poly(A) tail, the presence of which permitted unambiguous assignment of the direction of translation. The sequence encodes a predicted polypeptide of 382 amino acids (Fig. 7A). This amino acid sequence was used for BLAST and FASTA searches of protein sequence databases, and a single ~80 amino acid region was identified that shares over 60% identity to the HMG domain of the mammalian SRY protein. Higher levels of identity were found with several SRY-related SOX proteins. In particular, the FISH protein is closely related to the vertebrate SOX-2 and SOX-3 proteins. BESTFIT analysis revealed that FISH and the human SOX-2 protein share 42% overall sequence identity with several introduced gaps (data not shown). Significantly, the HMG domains of FISH and SOX-2 are 88% identical with 94% similarity. Strong similarity is also seen with the human SOX-3 protein, which shares 35% overall sequence identity with FISH. The HMG domains of these two proteins are 83% identical with 91% similarity.

Based on homology within the HMG domains of SRY and SOX proteins, 6 distinct subgroups, A-F, have been proposed (Wright et al., 1993). The sequence of its HMG domain places FISH in the B subgroup, which includes SOX-1, SOX-2, SOX-3, SOX-11, SOX-14 and SOX-19. Comparison of the HMG domains from representatives of each of the 6 SOX subgroups is presented in Fig. 7B There are 25 positions at which an invariant residue is present in the HMG domains of SRY, FISH and the 13 SOX proteins analyzed. In addition, there are several other positions where there are strong consensus residues. Interestingly, the only position where FISH differs from an otherwise invariant residue is at position 18, which, except for the most variant F subgroup, is a lysine (it is an arginine in the F subgroup), but is a glutamine in the FISH HMG domain. Outside the HMG domain, the FISH protein possesses several short alanine-, glutamine- and serine-rich stretches that may serve as transcriptional activation domains, as well as 11 copies of a consensus G/S S Ø/S G S/M pentapeptide sequence (Ø= hydrophobic residue) (Fig. 7C).

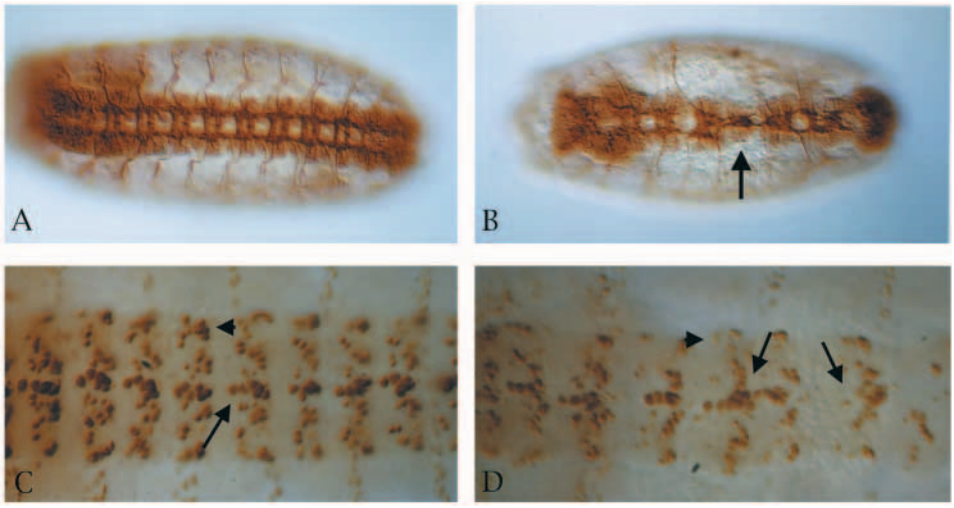
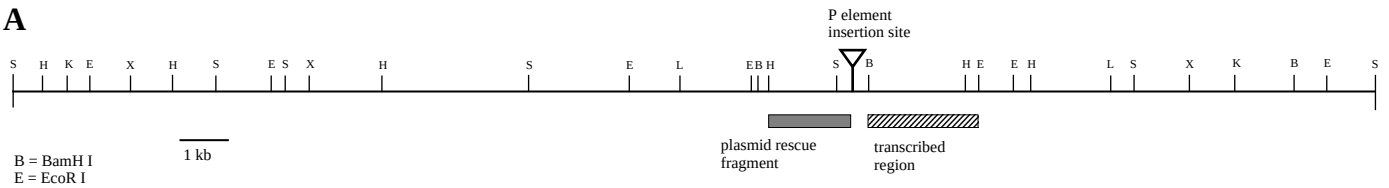


Fig. 5. *fish* mutants exhibit defects in CNS development. (A) Labeling of a stage 16 wild-type embryo via mAb BP102 immunocytochemistry. Note orderly arrangement of axon scaffold. (B) Similarly stained stage 16 *fish*^{87} mutant embryo. Note disorganization of axon scaffold, with some ganglia exhibiting narrowed longitudinal connectives and partial fusion of commissural axon tracts (arrow). In addition, several ganglia are lacking. (C) Stage 16 wild-type embryo stained via mAb 4D9 immunocytochemistry. Note regular reiterated clusters of *engrailed*-expressing neurons in the midline (arrow) and lateral CNS (arrowhead). (D) Similarly stained stage 16 *fish*^{96} mutant embryo. Note overall disorganization of *engrailed*-expressing neurons as well as loss and/or fusion of midline VUM (arrows) and lateral neurons (arrowhead). (A-D) All views ventral with anterior to left.



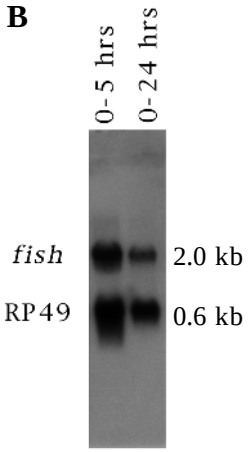


Fig. 6. Organization of the *fish* gene. (A) Restriction enzyme map of the cloned *fish* genomic DNA. The position of the rJ375 P element insertion, DNA recovered by plasmid rescue and transcribed region of the *fish* gene are denoted. (B) Northern blot of poly(A)$^+$ mRNA from 0-5 and 0-24 hour Canton-S embryo collections hybridized to a *fish* 6.0 kb *Sac*I genomic DNA probe and an RP49 cDNA probe. Note the expression of a single 2.0 kb *fish* mRNA transcript in both collections as well as the 0.6 kb RP49 mRNA.

The *fish-hook* gene is transcribed in a dynamic pattern during embryogenesis

The embryonic transcription pattern of the *fish* gene was examined via in situ hybridization using a probe derived from *fish* cDNA clone 2-5. *fish* transcription was first detected in cycle 13 syncitial blastoderm embryos as a wide circumferential band, corresponding to the entire trunk region (15-65% egg length) (Fig. 8A). There does not appear to be any maternally deposited *fish* mRNA. This trunk expression rapidly split into two subdomains and, by early cycle 14, high levels of *fish* transcripts were present in a narrow stripe at approximately 50% egg length and a wider stripe from about 15-30% egg length (Fig. 8B). Lower levels of *fish* expression persist in the intervening regions. At this time, *fish* transcripts also became detectable in the procephalic region. During cellularization (stage 5), *fish* expression was quickly refined into a series of seven irregular stripes and a strong dorsal 'saddle' (Fig. 8C). The intensity of these stripes varies, with stripes 1, 5, 6 and 7 more intense than stripes 2, 3 and 4. These stripes do not exhibit even spacing, as they both overlap and flank specific *ftz* stripes. Thus, *fish* stripe 6 corresponds to parasegment 11, between *ftz* stripes 5 and 6, while *fish* stripe 7 is coincident with *ftz* stripe 7 in parasegment 14 (data not shown).

During gastrulation and early germ-band extension there is a rapid change in the *fish* transcription pattern, as the seven ectodermal stripes diminish and are replaced by two longitudinal columns of *fish* expression that are approximately 4 cells wide and flank the invaginating mesoderm (Fig. 8D). Expression is maintained in the developing cephalic neuroectoderm. In stage 10 germ-band-extended embryos, medial neuroectodermal cells exhibit high levels of *fish* transcripts, and this expression is maintained in stage 11 embryos (Fig. 8E). During germ-band retraction, there is an overall decrease in the level of *fish* transcripts. Expression is detected in subsets of cells in the brain and CNS midline, the hingdut and segmentally repeated stripes of cells along the ventral epidermis (Fig. 8F). Overall, the pattern of *fish* transcription corresponds well to the pattern of β-gal expression in the rJ375 strain. Analysis of *fish* transcription in the *fish* excision mutants indicated that the *fish*^{87} and *fish*^{96} alleles do not express any detectable *fish* mRNA transcripts (data not shown).

DISCUSSION

A novel segmentation gene

The elaboration of the *Drosophila* body pattern into discrete segments involves a series of transcriptional regulatory pathways that require complex yet precise interactions of regulatory proteins and target DNA sites. The *fish* gene appears to function parallel to or downstream of the gap genes, as defects in anterior/posterior patterning are first detected in the elaboration of pair-rule stripes. *fish* does not appear to be required for the initial activation of pair-rule or segment polarity genes, but is instead essential for their proper refinement by maintaining or repressing specific stripes. *fish* could function to modulate the activities of abdominal gap proteins, such as KRUPPEL, KNIRPS or GIANT, in their regulation of pair-rule gene expression. For example, because proper elaboration of *eve* stripes 4 through 6 requires the gap gene *Krüppel* (Frasch and Levine, 1987), the *eve* expression defects in *fish* mutants could be due to an alteration in *Krüppel* function. This does not appear to occur at the level of regulating early *Krüppel* transcription, which is normal in *fish* mutant blastoderm embryos (P. A. N. and J. R. N., unpublished data). *fish* might also modulate the function of pair-rule proteins in the refinement and stabilization of pair-rule stripes. For example, because the HAIRY protein represses *ftz* expression in odd parasegments (Ish-Horowicz and Pinchin 1987), the fusion of *ftz* stripes 3 and 4 in *fish* mutants could be due to a localized defect in *hairy* function. In this regard, it is notable that there are several *hairy* alleles that exhibit segment-specific defects in abdominal regions (Howard et al., 1988). Finally, because the defects in *wg* (and *en*) expression in *fish* mutants do not strictly correspond to the defects in pair-rule gene expression, *fish* may also directly modulate the function of regulatory complexes required for segment polarity gene expression. *fish* appears to be a member of an emerging class of novel segmentation genes that includes *hopscotch* and *marelle*, two components of the JAK/STAT signaling pathway, which act to regulate the generation of specific individual segments (Binari and Perrimon, 1994; Hou et al., 1996; Yan et al., 1996).

Role of *fish-hook* in CNS formation

Similar to many other segmentation genes (see Goodman and Doe, 1993), the *fish* gene exhibits prominent expression in cells of the developing embryonic nervous system and *fish* mutants exhibit severe alterations in CNS organization. *fish* likely plays a direct role in these processes as the CNS defects observed in *fish* mutants do not precisely correspond to the epidermal

defects. Clearly this issue will need to be further examined using conditional mutants. Because *fish* expression is activated in medial neuroectodermal cells shortly after gastrulation, it could play an early role in nerve cell development. However, because CNS defects in *fish* mutants were first detected during germ-band retraction, *fish* may instead function in nerve cell differentiation. One issue that will be important to address is whether *fish* has common functions in mediating gene expression during segmentation and nervous system development, for example, by interacting with the gap gene *Krüppel*, which is also widely expressed in many cells of the developing CNS (Hoch et al., 1990). In addition, because the vertebrate SOX-2 protein can interact with the POU domain protein, OCT-3 to regulate FGF-4 expression, perhaps *fish* interacts with POU domain proteins expressed within overlapping regions of the *Drosophila* nervous system, such as PDM-1, PDM-2/MITI-MERE and Cf1a/DRIFTER (Lloyd and Sakonju, 1991; Billin et al., 1991; Anderson et al., 1995). *pdm1*, *pdm-2*/*miti-mere* and *Cf1a*/*drifter* all appear to play important roles in specification or differentiation of specific CNS cell types (Yang et al., 1993; Bhat and Schedl, 1994; Anderson et al., 1995; Bhat et al., 1995; Yeo et al., 1995). Interestingly, the expression of the *pdm-1* and *pdm-2*/*miti-mere* genes also overlap with *fish* in abdominal segments of blastoderm embryos (e.g. Lloyd and Sakonju, 1991; Billin et al., 1991) and expression of a dominant negative *pdm-2*/*miti-mere* transgene results in segementation defects that include loss of A2 and A6 (Bhat and Schedl 1994).

A *Drosophila* Sox gene

This study provides the first functional analysis of an invertebrate member of the Sox gene family and may provide a useful paradigm for analyzing the functional roles of Sox genes in specific developmental contexts. SRY and related SOX proteins regulate gene expression by acting as architectural proteins and/or classical transcription factors. Thus a crucial aspect to understanding their functions will be to characterize the mechanisms by which these proteins act and the identities of downstream target genes. SRY and SOX proteins bind to A/T A/T CAAAG or AACAAT consensus sequences (Harley et al., 1992; van de Wetering et al., 1993; Connor et al., 1994; Wotton et al., 1995; Yuan et al., 1995), and the binding of SRY and SOX-5 to target DNA sites induces strong DNA bending, at angles of 85° for SRY and 73-90° for SOX-5 (Giese et al., 1992; Harley et al., 1992; Connor et al., 1994). The strong similarity between the HMG domain of FISH and other SOX proteins suggests similar DNA-binding and

Fig. 7. The *fish* gene encodes a novel HMG domain protein. (A) Amino acid sequence of the predicted FISH protein. The HMG domain (bold and underlined), pentapeptide repeats (1-11), and glycine/serine- [G/S], alanine- [A], glutamine- [Q], and serine- [S] rich stretches are denoted. (B) Pentapeptide repeats in FISH protein. (C) Comparison of HMG domains from FISH, SRY, and representative SOX proteins. Subgroupings of SRY and SOX proteins (Wright et al., 1993) are denoted by letters (A-F). INV = positions at which there are invariant residues. Note that FISH has a glutamine (**Q**) at position 18 which is a lysine (K) in all the other HMG domains except those of the divergent F subgroup.

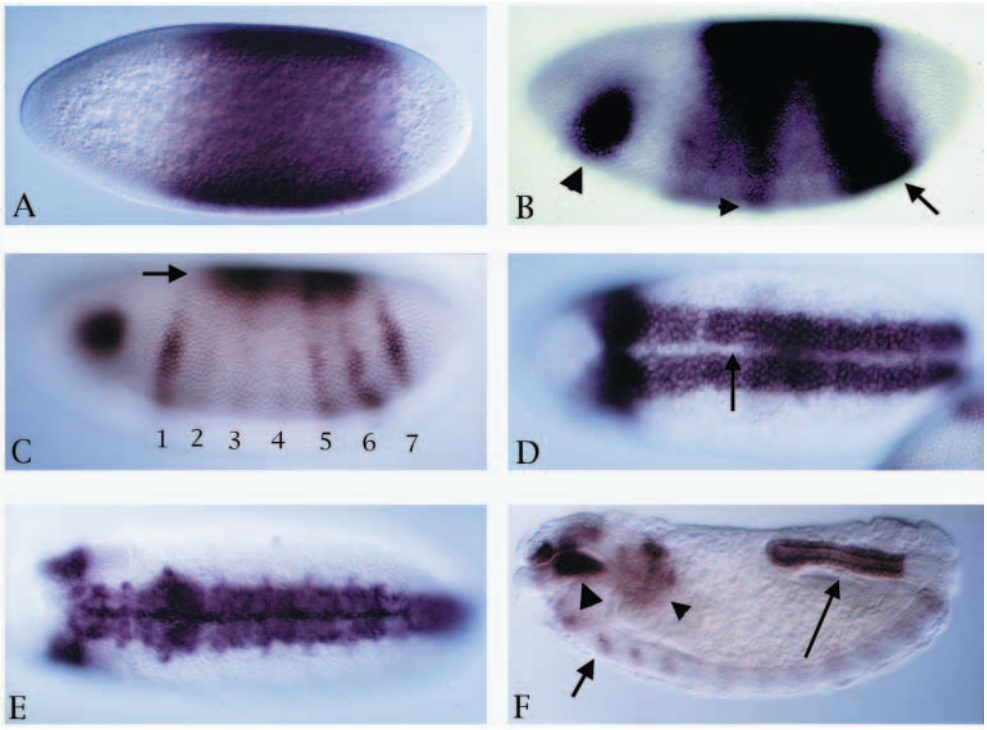


Fig. 8. *fish* mRNA transcripts are dynamically expressed during embryogenesis. Embryonic in situ hybridization to wild-type embryos with an antisense RNA probe derived from *fish* cDNA clone 2-5. (A) Stage 5 cycle 13 blastoderm embryo. Note uniform expression of *fish* mRNA throughout entire trunk region of embryo. (B) Stage 5 early cycle 14 blastoderm embryo. Note splitting of trunk expression domain into 2 subdomains at approximately 50% (small arrowhead) and 15-30% egg length (arrow). Lower levels of *fish* mRNA are still detected in intervening regions. *fish* expression is also detected in the procephalic region (large arrowhead). (C) Stage 5 cellular blastoderm embryo. *fish* mRNA is now present in a dorsal saddle (arrow) and 7 irregular stripes that display variable spacing and intensity. (D) Stage 7 gastrulating embryo. The *fish* stripes have been replaced by two columns of *fish* expression in neuroectodermal cells flanking the invaginating mesoderm (arrowhead). (E) Stage 11 fully germ-band-extended embryo. High levels of *fish* mRNA are present in the medial neuroectoderm. (F) Stage 15 germ-band-retracted embryo. *fish* mRNA is present in subsets of cells within the ventral nerve cord (short arrow), brain (small arrowhead), pharynx (large arrowhead) and hindgut (long arrow). (A-C,F) Sagittal views with anterior to left. (D,E) Ventral views with anterior to left.

-bending capabilities. However, it is notable that the FISH HMG domain has a glutamine substitution in an otherwise invariant basic residue (generally a lysine). A similar glutamine versus lysine difference in the recognition helix of several homeodomain proteins, including BICOID, FTZ and ORTHODENTICLE, appears to be responsible for determining DNA-binding specificity (Driever et al., 1989; Schier and Gehring, 1992; Pankratz and Jäckle, 1993). Thus FISH could recognize target sequences distinct from those bound by other SOX proteins. Identification of these binding sites should prove useful in determining whether conformational changes in DNA that may be brought about by FISH binding are important for regulating pair-rule and segment polarity gene expression, perhaps by facilitating interactions between distantly located complexes of gap and pair-rule proteins bound to DNA.

The *fish* gene has been independently isolated by Michael Ashburner's group and they report similar findings on protein sequence, transcription patterns and mutant segmentation phenotypes (see Russell et al., 1996, this issue of *Development*). In addition, they have generated data indicating that *fish* mutants are allelic to *Dichaete*. We have found that all of the *fish* mutant alleles that we have generated are lethal over *Dichaete*. As *Dichaete* appears to be a dominant regulatory mutant defined by inversion breakpoints, we propose referring to *Dichaete* mutants as $fish^{D}$ alleles. The *fish* cDNA clone sequence has been deposited in GenBank, accession number U68056.

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