

Functional Interactions between *Drosophila* bHLH/PAS, Sox, and POU Transcription Factors Regulate CNS Midline Expression of the *slit* Gene

Yue Ma,¹ Kaan Certel,² Yanping Gao,¹ Emily Niemitz,¹ Jack Mosher,³ Ashim Mukherjee,¹ Mousumi Mutsuddi,¹ Neda Huseinovic,¹ Stephen T. Crews,³ Wayne A. Johnson,² and John R. Nambu¹

¹Department of Biology, University of Massachusetts, Amherst, Massachusetts 01003, ²Genetics Ph.D. Program and Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242, and ³Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

During *Drosophila* embryogenesis the CNS midline cells have organizing activities that are required for proper elaboration of the axon scaffold and differentiation of neighboring neuroectodermal and mesodermal cells. CNS midline development is dependent on Single-minded (Sim), a basic-helix-loop-helix (bHLH)-PAS transcription factor. We show here that Fish-hook (Fish), a Sox HMG domain protein, and Drifter (Dfr), a POU domain protein, act in concert with Single-minded to control midline gene expression. *single-minded*, *fish-hook*, and *drifter* are all expressed in developing midline cells, and both loss- and gain-of-function assays revealed genetic interactions between these genes. The corresponding proteins bind to DNA sites present in a 1 kb midline enhancer from the *slit* gene and

regulate the activity of this enhancer in cultured *Drosophila* Schneider line 2 cells. Fish-hook directly associates with the PAS domain of Single-minded and the POU domain of Drifter; the three proteins can together form a ternary complex in yeast. In addition, Fish can form homodimers and also associates with other bHLH-PAS and POU proteins. These results indicate that midline gene regulation involves the coordinate functions of three distinct types of transcription factors. Functional interactions between members of these protein families may be important for numerous developmental and physiological processes.

Key words: *Drosophila*; CNS midline; bHLH/PAS; Sox; POU; *slit*; gene expression

During *Drosophila* embryogenesis, a group of specialized CNS midline neurons and glia provide signals that are essential for the differentiation of neighboring ectodermal and mesodermal cells (for review, see Crews, 1998). The CNS midline also serves as an intermediate axon guidance target that expresses chemotactic factors, including Slit, Commissureless, and D-Netrin, which control axon crossing at the midline (Harris et al., 1996; Mitchell et al., 1996; Tear et al., 1996; Kidd et al., 1999). In particular, Slit is a conserved epidermal growth factor (EGF)-repeat protein that is strongly expressed by midline glia and interacts with the Roundabout receptor to prevent commissural axons from recrossing the midline (for review, see Guthrie, 1999; Van Vactor and Flanagan, 1999; Zinn and Sun, 1999). Development of the entire CNS midline lineage requires the gene regulatory functions of Single-minded (Sim), a basic-helix-loop-helix (bHLH)-PAS transcription factor (Nambu et al., 1991). Sim protein forms functional heterodimers with Tango (Tgo), a bHLH-PAS protein that ex-

hibits widespread embryonic expression (Sonnenfeld et al., 1997; Ward et al., 1998). Sim::Tgo heterodimers act through the CNS midline element (CME), an ACGTG sequence motif present in the regulatory regions of *slit* and other midline-expressed genes (Wharton et al., 1994; Sonnenfeld et al., 1997; Kasai et al., 1998). A CME present in a 380 bp *slit* regulatory DNA fragment is essential for midline expression of a linked reporter gene (Wharton et al., 1994), although sequences in addition to the CME are required for high levels of gene expression (Wharton and Crews, 1993). Interestingly, results from Sim ectopic expression experiments suggest that in addition to Tgo, Sim functionally interacts with other factors more specifically expressed in the midline and lateral and cephalic neuroectoderm (Nambu et al., 1991; Muralidhar et al., 1993).

One candidate for such a Sim cofactor is the Sox HMG domain protein Fish-hook (Fish) (Nambu and Nambu, 1996; Russell et al., 1996). Fish is strongly expressed in the early neuroectoderm and is required for proper differentiation of midline and lateral CNS cells (Nambu and Nambu, 1996; Ma et al., 1998; Sánchez Soriano and Russell, 1998). The Fish HMG domain binds DNA sequences related to AACAAAT and AACAAAG and induces strong DNA bending (Ma et al., 1998). This suggests that Fish may provide chromatin architectural functions to facilitate the assembly of higher-order protein/DNA complexes. In this regard, the closely related vertebrate Sox2 protein directly associates with the Oct3 POU domain protein to synergistically activate expression of the *fgf4* gene (Yuan et al., 1995; Ambrosetti et al., 1997). *fish* mutants exhibit genetic interactions with mutations in *Drosophila* POU genes (Ma et al., 1998; Sánchez Soriano and Russell 1998), and one of these POU genes, *drifter* (*dfr*), is expressed in

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Correspondence should be addressed to Dr. John R. Nambu, Biology Department, Morrill Science Center, University of Massachusetts, Amherst, MA 01003. E-mail: jnambu@bio.umass.edu.

E. Niemitz's present address: Predoctoral Training Program in Human Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

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the CNS midline and is essential for proper midline glial migrations (Anderson et al., 1995).

In this study we use genetic, biochemical, and cell culture assays to show that Sim, Fish, and Drifter (Dfr) act together to regulate CNS midline development and gene expression. In particular, these proteins synergistically influence *slit* transcription by acting through a 1 kb midline regulatory region that contains a single CME, as well as binding sites for Fish and Dfr. Regulation of *slit* expression appears to involve direct interactions between Fish, Sim, and Dfr. Fish directly associates with the Sim PAS domain and the Dfr POU domain, and the three proteins can form a ternary complex in yeast cells. These results identify novel regulatory interactions between bHLH-PAS, Sox, and POU proteins.

MATERIALS AND METHODS

Fly strains. The following strains were used in this study: the *fish*⁸⁷ null mutant (Nambu and Nambu, 1996), the *dfr*^{ES2} lethal mutant (Anderson et al., 1995), P[1.0*slit-lacZ*] (Nambu et al., 1991; Wharton et al., 1994), P[UAS-*fish*] (Mukherjee et al., 2000), P[UAS-*sim*] (Xiao et al., 1996), and P[GMR-Gal4] (obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN); generated by M. Freeman). A *dfr*^{ES2}-*fish*⁸⁷/TM3, P[*ftz-lacZ*] strain was generated via meiotic recombination.

Immunostaining of Drosophila embryos. For single-label immunostaining experiments, embryos were collected from wild-type and mutant strains, as well as from genetic crosses, and fixed in PEM buffer (0.1 M PIPES, pH 6.9, 2 mM EGTA, 1 mM MgSO₄) with 4% formaldehyde (Patel, 1994). The embryos were incubated overnight with primary antibodies in PTN (1× PBS, 0.2% Triton X-100, 5% normal horse serum). Vectastain biotinylated secondary antibodies and streptavidin/horseradish peroxidase reagents (Vector Laboratories, Burlingame, CA) were used with H₂O₂/diaminobenzidine histochemistry to detect primary antibody binding. The monoclonal antibody (mAb) BP102 (Developmental Studies Hybridoma Bank, Iowa City, IA) was used at a 1:4 dilution. A monoclonal antibody against β-galactosidase (β-Gal) (Promega, Madison, WI) was used at a 1:500 dilution. Stained embryos were dehydrated through an ethanol series, mounted in methyl salicylate and Permount (Fisher), and examined via Nomarski optics using a Nikon Optiphot compound microscope.

For double-label fluorescence immunostaining experiments, embryos were fixed in PEM buffer with 4% formaldehyde. They were labeled by following previously described protocols (Mitchison and Sedat, 1983; Johnson, 1992). β-Gal expression was detected using either a rabbit polyclonal antiserum (Cappel, West Chester, PA) at a 1:500 dilution or a mouse mAb 40-1a (Developmental Studies Hybridoma Bank) at a 1:3 dilution. Pre-absorbed rat anti-Dfr serum was used at a final dilution of 1:3000 and rabbit anti-Fish serum at a dilution of 1:1000. After repeated washes in PBT (1× PBS, 0.5% bovine serum albumin, 0.2% Triton X-100), embryos were incubated with FITC-conjugated anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine-conjugated anti-mouse (Jackson ImmunoResearch Laboratories) or anti-rabbit (Biosource, Camarillo, CA) secondary antibodies at 1:200 dilution. Stained embryos were mounted in Vectashield (Vector Laboratories) and observed using a Bio-Rad MRC-1024 confocal microscope.

DNA sequence analysis. DNA sequencing of the *slit* 1 kb midline regulatory region was performed by Retrogen, Inc. (San Diego, CA). The DNA fragment was sequenced by primer walking approaches, and the sequences from both strands were obtained in multiple runs. The analysis revealed this fragment to be 970 bp in length and flanked by two *HindIII* sites.

Gel mobility shift assays. For Fish gel mobility shift assays, two sets of complementary 26 mer oligonucleotides corresponding to Sox consensus DNA binding sites from the *slit* regulatory fragment were synthesized and annealed in 1× T4 polynucleotide kinase buffer (Promega). The sequence of the upper strand of each pair of oligonucleotides is shown below, and the underlined sequence corresponds to Sox consensus DNA binding sites (see Fig. 4A for location of probe sequences): TA-CAAT probe—5'-ACTATACTAATTGTATTATGCACAG-3'; TTC-AAT probe—5'-ACTGTATTCAATTTTCATTGAAACAAA-3'.

The annealed oligonucleotides were end-labeled using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD) and γ-³²P-ATP (New England Nuclear) and gel-purified. Gel mobility shift assays were

performed using a purified 6XHis-HMG Fish fusion protein as described previously (Ma et al., 1998).

For Dfr gel mobility shift assays, probes were generated by end-labeling double stranded oligonucleotides with γ-³²P-ATP (ICN Biochemicals, Costa Mesa, CA) and T4 polynucleotide kinase (New England Biolabs). The sequence of the upper strands of each pair of oligonucleotides is shown below, and the underlined sequence corresponds to consensus Dfr binding sites (see Fig. 4A for location of probe sequences): ATGCAAAT probe—5'-ATGCACGACATATTTGCATTTTAAAAT-AGAGAA-3'; CATAAAT probe—5'-ATATATGTCCCATTTATGTG-AGTGACATTCCA-3'.

Full-length Dfr protein was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein and purified over glutathione-Sepharose beads (Amersham Pharmacia, Arlington Heights, IL) as previously described (Certe et al., 1996). While on beads, the GST affinity tail was cleaved by using PreScission protease (Amersham Pharmacia) as per manufacturer's suggestions. End-labeled probes were incubated with full-length Dfr protein at room temperature for 15 min in binding buffer (25 mM HEPES, pH 7.6, 12.5 mM MgCl₂, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, 1 mM DTT). Immediately after incubation, the complexes were resolved on 5% native polyacrylamide gels at room temperature in 1× TBE buffer. Gels were dried and exposed to Kodak X-OMAT film.

Yeast two-hybrid assays. The yeast two-hybrid vectors pEG202 and pJG4-5 (Origene) were used to generate bait and prey constructs. Baits contained the LexA DNA binding domain fused to the protein of interest, and the prey contained fusions to the B42 transcriptional activation domain. A chromosomally integrated LexAop-*LEU2* reporter gene was used that contains six LexA operator sites fused to the *LEU2* gene. The bait and prey constructs were cotransformed into EGY48 host yeast cells (Mata *trp1 his3 ura3 leu2::6lexAop-LEU2*) according to the supplier's instructions (Origene). The transformed cells were plated on YNB/Leu/Ura (-His, -Trp) glucose medium and incubated at 30°C for 3–4 d. Resulting transformants were then plated on the YNB/Ura (-His, -Leu, -Trp) galactose/raffinose medium and incubated at 30°C for 3–7 d to assay for the presence of colonies that indicated interactions.

Bait constructs that express full-length Fish, or truncated versions consisting of the N-terminal 141 amino acids, the 79 amino acid HMG domain, the COOH-terminal 164 amino acids, the NH₂+HMG, the HMG+COOH, and the NH₂+COOH, are described in Ma et al. (1998). To generate a full-length Fish prey construct, the entire Fish 382 amino acid open reading frame was amplified via PCR from full-length *fish* cDNA clone 2-5 (Nambu and Nambu, 1996) using the following primers: 5'-AGAGAATTCATGGCCACCTTATCGACACACCC-3'; 5'-TGTGAATTCCTACTAATAGAGCACCAGGACCGGTGCGCT-3'.

The resulting DNA fragment was digested with *EcoRI*, purified via agarose gel electrophoresis, and cloned into the pJG4-5 vector.

Prey constructs that express full-length Dfr or the Dfr POU domain were generated via PCR using a full-length *dfr* p128 cDNA clone (Anderson et al., 1995) and the following primers: full-length Dfr—5'-CATGGAATTCACCGATGATGATCTGGAGGCC-3', 5'-CATGCTCGAGTTACTAGTGGCCGCCAAGTGTGCGCCGC-3'; Dfr POU domain (amino acids 210–362)—5'-CCCCGAATTCACGTCCGATGATCTGGAGGCC-3', 5'-GGGGCTCGAGCGTCATGCGCTTCTCCTTCTG-3'.

The PCR products were digested with *EcoRI* and *XhoI*, purified via agarose gel electrophoresis, and subcloned into the pJG4-5 vector.

A prey construct expressing the POU domain of Pdm-1 (amino acids 420–601) was generated via PCR from the full-length C616A cDNA clone (Billin et al., 1991), kindly provided by Steve Poole (University of California Santa Barbara), and the following primers: 5'-CATGGAAATTCGGAGGAAACCACCGATCTAGAA-3'; 5'-CATGCT CGA-GTTACTAGGGACTGTCCAGGG-AGGGATTGAT-3'.

A similar *pdm-2* POU domain (amino acids 283–457) prey construct was generated via PCR from the full-length C9A cDNA clone (Billin et al., 1991) and the following primers: 5'-CATGGAATTCGAACAATC-GCCGGAAGAGACCACC-3'; 5'-CATGCTCGAGTTACTAGTCCA-GATCCAGCGAGGGATTGAT-3'.

Both *pdm-1* and *pdm-2* PCR products were digested with *EcoRI* and *XhoI*, purified via agarose gel electrophoresis, and subcloned into the pJG4-5 vector.

A prey construct that expresses the full-length mouse Oct3 protein was generated via PCR from a full-length pGEX-2-Oct3 cDNA clone, kindly provided by Lisa Dailey (New York University) (Yuan et al., 1995), and the following primers: 5'-GGGGAATTCATGTTCCGAGAAGGTG-

GAACCAA-3'; 5'-GGGCTCGAGTCACCCTGTAGCCTCATACT-CTT-3'.

The PCR product was digested with *EcoRI* and *XhoI*, purified via agarose gel electrophoresis, and subcloned into the pJG4-5 vector.

The following Sim and Trachealless (Trh) bait or prey constructs (in the pEG202 and pJG4-5 vectors) were used: full-length Sim (amino acids 1–673), Sim bHLH-PAS (amino acids 1–461), Sim bHLH-PAS-A (amino acids 1–183), Sim bHLH (amino acids 1–92), Sim PAS (amino acids 58–370), full-length Trh (amino acids 1–929), and Trh bHLH-PAS (amino acids 1–689) (Sonnenfeld et al., 1997; M. Sonnenfeld and S. T. Crews, unpublished results). The Period (Per) bait (Per-C2) construct containing the PAS domain and a COOH-extended region (amino acids 233–685) was kindly provided by Michael Rosbash (Brandeis University) (Huang et al., 1995).

To express native full-length Fish protein in yeast, the entire Fish open reading frame was amplified via PCR from *fish* cDNA clone 2–5 using the following primers: 5'-GGGGGAAGCTTATGGCCACCTTATCGACA-3'; 5'-CCCCAAGCTTCTAATAGAGGACCGGAAC-3'.

The resulting DNA fragment was digested with *HindIII*, purified via agarose gel electrophoresis, and cloned into the pDB20 yeast expression vector (Becker et al., 1991). pDB20 permits high level of transgene expression driven by the ADC1 (ADH1) promoter and contains the selectable URA3 marker.

To analyze interactions between Fish, Sim, and Dfr, full-length Sim bait, full-length Dfr prey, and full-length native Fish constructs were transformed into EGY48 host cells. The cells were grown on YNB/Leu (-His, -Ura, -Trp) glucose plates and incubated at 30°C for 3–4 d. Transformed colonies were then streaked on YNB (-His, -Leu, -Trp, -Ura) galactose/raffinose plates and incubated at 30°C for 4 d. For controls, full-length Sim bait and native Fish or full-length Dfr prey and native Fish constructs were transformed into EGY48 cells that were grown on YNB/Trp/Leu (-His, -Ura) glucose plates at 30°C for 3–4 d. Transformed colonies were then streaked on the YNB/His (-Leu, -Trp, -Ura) or YNB/Trp (-His, -Leu, -Ura) galactose/raffinose plates and incubated at 30°C for 4 d.

GST pull-down assays. Full-length ³⁵S-labeled Sim protein was generated from *sim* cDNA clone F1 (Nambu et al., 1991) using an *in vitro* transcription/translation reaction (Promega) and ³⁵S-methionine (Amersham Pharmacia). For the GST–Fish fusion protein, a DNA fragment encoding the full-length Fish protein, was generated by PCR using *fish* cDNA clone 2–5 as a template and the following two oligonucleotide primers: 5'-GGCCGAATTCATGCCACCTTATCGACACACCC-AT-3'; 5'-CCGGGAATTCCTACTAATAGAGCACCAGGACCGG-3'.

The resulting PCR product was digested with *EcoRI*, purified via agarose gel electrophoresis, and subcloned into the pGEX-2T vector (Amersham Pharmacia) to generate an in-frame fusion with GST. The resulting construct was transformed into *Escherichia coli* BL-21 (Amersham Pharmacia), and expression of GST–Fish or GST alone was induced in 500 ml cultures via isopropyl-1-thio-β-D-galactopyranoside. Induced cells were pelleted and lysed in 4 ml of B-Per solution (Pierce, Rockford, IL). The debris was pelleted by centrifugation, and 200 μl of supernatant (containing ~2 μg of GST–Fish or GST protein) was incubated with 10 μl of glutathione-Sepharose-4B beads (Amersham Pharmacia) and 5 μl of ³⁵S-labeled Sim *in vitro* translation mix for 2 hr at 4°C. The beads were washed five times with BC100N buffer (Ambrosetti et al., 1997) and then boiled with 2 μl of SDS gel loading buffer and 8 μl of H₂O. The supernatants were electrophoresed on a 10% SDS-polyacrylamide gel along with 1 μl of the *in vitro* translation mixture (20% of the input used in the binding assays). The gel was dried and analyzed via autoradiography.

To test Fish dimerization, 1–4 μg of purified GST–Fish was incubated with glutathione-Sepharose-4B beads in NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP40) at 4°C for 2 hr. This was followed by several washes with NETN. ³⁵S-labeled Fish protein was generated via an *in vitro* transcription/translation reaction (Promega) using *fish* cDNA clone 2–5 as template. Five microliters of ³⁵S-labeled Fish were separately incubated with glutathione-Sepharose-4B beads in NETN at 4°C for 2 hr. The supernatant containing ³⁵S-labeled Fish was then incubated for 2 hr at 4°C with the GST–Sepharose-4B beads, which had been preincubated with GST–Fish. The beads were washed several times with NETN, and bound protein was eluted with buffer containing reduced glutathione (Amersham Pharmacia). These samples, along with 2 μl of the *in vitro* translation mixture (40% of the input used in the binding assays), were mixed with SDS sample buffer, boiled, and elec-

trophoresed on an 8% SDS-polyacrylamide gel. The gel was fixed in methanol/acetic acid (1:1), dried, and analyzed via autoradiography.

Schneider line 2 cell transient expression assays. *Drosophila* Schneider line 2 (S2) cells were cotransfected with the P[1.0*slit*-lacZ] reporter plasmid or the lacZ control vector C4PLZ along with combinations of plasmids that provide a source of Sim, Tgo, Fish, and Dfr proteins. The reporter plasmid contained the *slit* 1 kb midline glial enhancer fragment cloned into the C4PLZ enhancer/tester vector (Wharton and Crews, 1993). Expression plasmids pAct-*sim*, pAct-*tgo*, pAct-*fish*, and pAct-*dfr* were made by subcloning cDNA fragments containing the complete coding regions into pAct5C (*sim*, *tgo*, *dfr*) or pAct5CSRS (*fish*), each containing the *Drosophila* actin5C promoter and poly(A) site (Han et al., 1989; Sonnenfeld et al., 1997; K. Burtis, personal communication). S2 cells were transiently transfected using Ca₂PO₄ (Fehon et al., 1990). Transfections were performed in triplicate or more using 5 μg/plasmid and were normalized using 2.5 μg of copia-luc, which has a luciferase (luc) reporter (pGL3-Basic; Promega) under the control of the copia long terminal repeat (LTR) promoter. The cells were lysed 48 hr after transfection, and β-Gal and luciferase assays were performed using a LacZ/β-Gal Quantitation Kit (Molecular Probes, Eugene, OR) and Luciferase Assay System (Promega), respectively.

RESULTS

sim, *fish*, and *dfr* are expressed together in developing CNS midline cells

To address whether the *sim*, *fish*, and *dfr* genes might functionally interact to regulate development of the embryonic CNS midline, we first analyzed whether they exhibit overlapping expression in developing midline cells. This was accomplished using anti-Fish and anti-Dfr sera, as well as a P[3.7*sim*-lacZ] marker that mimics *sim* midline expression (Nambu et al., 1991; Kasai et al., 1998). P[3.7*sim*-lacZ] embryos were immunostained using anti-β-gal and either anti-Fish or anti-Dfr sera. Prominent overlapping expression was detected between Sim and Fish in developing CNS midline cells from stage 8 throughout the remainder of germ band extension. Overlap was also detected in a subset of prospective foregut cells. Similar overlapping expression was also detected between Sim and Dfr. Midline coexpression of Fish and Dfr was detected by immunostaining wild-type embryos with anti-Fish and anti-Dfr sera. Both genes are expressed together in the CNS midline throughout germ band extension. Examples of this overlapping expression in germ band-extended embryos are presented in Figure 1. In germ band-retracted embryos, Fish exhibited overlapping expression with Sim and Dfr in the midline glia (data not shown). Fish and Dfr were also detected together in lateral cells of the thoracic ganglia and a subset of ventral epidermal cells (data not shown). Consistent with previous studies (Ma et al., 1998), Fish protein was not detected in the trachea, a prominent site of Dfr expression (Anderson et al., 1995; Certel et al., 1996). These analyses indicated that *sim*, *fish*, and *dfr* are coexpressed in developing CNS midline cells. The midline expression of these three genes also overlaps that of the *slit* gene, which is a downstream target of Sim (Wharton and Crews, 1993; Wharton et al., 1994).

Genetic interactions reveal cooperative functions between *sim*, *fish*, and *dfr*

Both loss-of-function and gain-of-function assays were used to detect genetic interactions between *sim*, *fish*, and *dfr*. Significantly, a previous study had revealed genetic interactions between *fish* (also called *Dichaete*) and *dfr* (also called *ventral veins lacking*) mutants in CNS midline differentiation and Slit protein expression (Sánchez Soriano and Russell, 1998). We have extended these studies to analyze potential cooperative interactions between *sim*, *fish*, and *dfr* in regulating *slit* gene transcription through use of a P[1.0*slit*-lacZ] marker. This reporter contains a

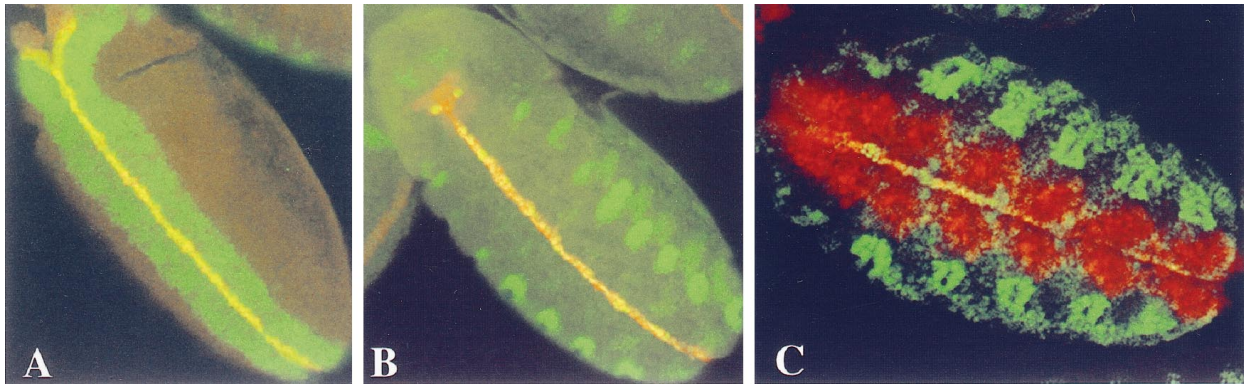


Figure 1. Coexpression of Sim, Fish, and Dfr in embryonic CNS midline cells detected via double-label immunostaining of P[3.7*sim-lacZ*] (*A, B*) and wild-type (*C*) embryos and confocal microscopy. *A*, A stage 9 P[3.7*sim-lacZ*] embryo immunostained with anti- β -gal (red) and anti-Fish (green) sera. Note strong overlapping expression (yellow) in the CNS midline cells. Fish is also strongly expressed in the lateral and cephalic neuroectoderm. *B*, A stage 10 P[3.7*sim-lacZ*] embryo immunostained with anti- β -gal (red) and anti-Dfr (green) sera. Note strong overlapping expression (yellow) in the CNS midline cells. Dfr is also strongly expressed in developing tracheal cells. *C*, A wild-type stage 11 embryo immunostained with anti-Fish (red) and anti-Dfr (green) sera. Note overlapping expression (yellow) in the CNS midline cells. Fish expression is not detected in tracheal cells. All views are ventral with anterior to left.

portion of a *slit* intron that drives *lacZ* expression mimicking that of the native *slit* gene in developing midline glia; P[1.0*slit-lacZ*] expression is first detected in germ band-extended stage 11 embryos and is maintained throughout the remainder of embryogenesis (Fig. 2*A*) (Nambu et al., 1991; Wharton and Crews, 1993; Wharton et al., 1994). *fish* null mutant embryos exhibited a misplacement and loss of midline glia, as detected via anti- β -gal immunostaining (Fig. 2*B*). P[1.0*slit-lacZ*] was expressed normally in stage 11 *fish* mutant embryos, but during germ band retraction the number of midline glia became reduced from wild type, and many cells were located at aberrant ventral positions within the nerve cord. Similar, although less severe, defects were observed in *dfr* mutant embryos, where some midline glia were displaced from their normal positions (Fig. 2*C*). Notably, β -gal-expressing midline glia were still detected in both *fish* and *dfr* mutants, indicating that unlike Sim, Fish and Dfr are not absolutely required for P[1.0*slit-lacZ*] expression or midline glial development.

We then used a *dfr-fish* double mutant strain to examine whether *fish* and *dfr* might act together to regulate midline gene expression. Embryos mutant for both *fish* and *dfr* were shown to exhibit much more severe defects in P[1.0*slit-lacZ*] expression than either *fish* or *dfr* single mutants. Although P[1.0*slit-lacZ*] was activated normally in stage 11 *dfr-fish* double mutant embryos, there was a striking loss of midline P[1.0*slit-lacZ*] expression during germ band retraction (Fig. 2*D*). This synergistic effect strongly suggests that Fish and Dfr function together to regulate *slit* transcription. These functions may be mediated directly through Fish and Dfr binding sites present in the *slit* 1 kb regulatory region (see below). Another, nonexclusive possibility is that Fish and Dfr might indirectly control *slit* transcription by regulating the expression of *sim*. To address this possibility we examined P[3.7*sim-lacZ*] expression in wild-type and *dfr-fish* embryos. Compared with wild-type embryos, *dfr-fish* double mutants exhibited a severe decrease in P[3.7*sim-lacZ*] expression, a phenotype that first became apparent during germ band retraction (Fig. 2*E, F*). Thus, *fish* and *dfr* also influence *sim* expression and hence may indirectly influence the expression of a wide array of midline genes.

Because homozygous *sim* mutants exhibit severe CNS midline defects, it was not informative to analyze the phenotypes of *fish-sim* or *dfr-sim* double mutants. Instead, we examined poten-

tial interactions between *fish* and *sim* via a gain-of-function approach using the Gal4/UAS targeted gene expression system (Brand and Perrimon, 1993). A P[GMR-Gal4] strain that drives Gal4 expression in and behind the morphogenetic furrow in the developing eye imaginal disk was crossed to P[UAS-*fish*] and P[UAS-*sim*] strains. P[GMR-Gal4]/+;P[UAS-*fish*]/+ animals exhibited a moderate eye roughening with disruption of ommatidia organization and loss of mechanosensory bristles (Mukherjee et al., 2000) (Fig. 3*A, D*). In contrast, ectopic *sim* expression resulted in essentially normal eye morphology (Fig. 3*B, E*). The effects of *fish* and *sim* coexpression revealed a nonadditive phenotype; there was a stronger disorganization of ommatidia and mechanosensory bristles than seen in flies expressing *fish* or *sim* alone, and there was also a dramatic loss of eye pigmentation (Fig. 3*C, F*). These results indicated that ectopic expression of *fish* and *sim* synergistically alters normal eye development, and along with other data described below supports the hypothesis that these genes can interact functionally.

Sim, Fish, and Dfr directly regulate *slit* transcription

Previous DNA sequence analysis of a 380 bp *slit* midline regulatory fragment indicated the presence of a single CME, through which Sim::Tgo heterodimers act (Wharton et al., 1994; Sonnenfeld et al., 1997). The CME is located within 300 bp from the distal end (farther from the promoter in the native *slit* gene) of this fragment (Wharton et al., 1994). We additionally noted the presence of an inverted TTCAAT repeat (TTCAATTTTCATTGAA) located 20 bp proximal to the CME. This sequence resembles a (A/T)(A/T)CAAT consensus binding site for Sox proteins, although to our knowledge, binding of Sox proteins to a TTCAAT sequence has not been reported. Because sequences present in an extended 1 kb *slit* DNA fragment are required for normal levels of *slit* expression *in vivo* (Wharton and Crews, 1993), we obtained additional DNA sequences. This analysis indicated that no other CMEs are present in the 1 kb *slit* DNA fragment. However, we did identify two perfect Dfr consensus binding sites (Certel et al., 1996), ATGCAAT and CATAAAT, located within 500 bp of DNA proximal to the CME (Fig. 4*A*). These two Dfr binding sites are separated by ~150 bp and flank a consensus Fish binding site, TACAAT (Fig. 4*A*). These data suggest that Fish, Sim, and Dfr may all bind to sites present in the 1 kb *slit*

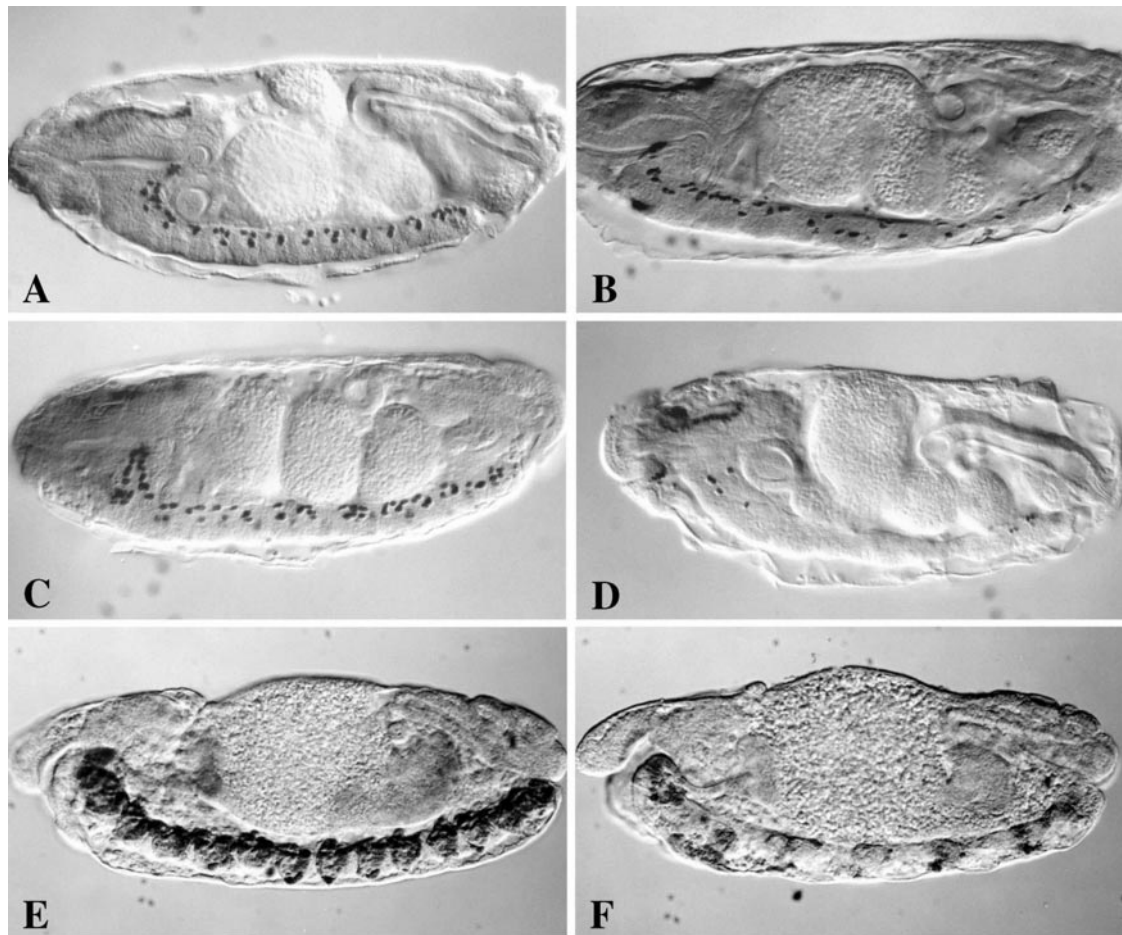


Figure 2. Genetic interactions between *fish* and *dfr* in CNS midline gene expression and development. Anti- β -gal immunostaining of stage 15 wild-type (*A*), *fish*⁸⁷ (*B*), *dfr*^{E82} (*C*), and *dfr*^{E82}-*fish*⁸⁷ double mutant (*D*) embryos carrying the P[1.0*slit*-lacZ] marker. In addition, anti- β -gal immunostaining was also performed on stage 15 wild-type (*E*) and *dfr*^{E82}-*fish*⁸⁷ double mutant (*F*) embryos carrying the P[3.7*sim*-lacZ] marker. *A*, Note the predominantly dorsal positions of P[1.0*slit*-lacZ]-expressing midline glia in each segment of the ventral nerve cord in a wild-type embryo. *B*, In *fish*⁸⁷ mutant embryos there is a loss and disorganization of P[1.0*slit*-lacZ]-expressing midline cells. *C*, In *dfr*^{E82} mutant embryos there is only modest misplacement of P[1.0*slit*-lacZ]-expressing midline cells. *D*, In *dfr*^{E82}-*fish*⁸⁷ double mutant embryos there is a dramatic loss of P[1.0*slit*-lacZ]-expressing cells. This phenotype is much more severe than that seen in either *fish*⁸⁷ (*B*) or *dfr*^{E82} (*C*) single mutant embryos. *E*, In wild-type embryos the CNS midline cells express *sim* and are organized into segmentally reiterated clusters of cells along the ventral nerve cord. *F*, In *dfr*^{E82}-*fish*⁸⁷ double mutant embryos there is a severe decrease in *sim* expression and disorganization of midline cells. All views are sagittal with anterior to left.

regulatory DNA fragment. To test this possibility, DNA gel mobility shift assays were performed using the Fish HMG domain and full-length Dfr protein on double-stranded oligonucleotide probes corresponding to sequences from the *slit* 1 kb fragment. The Fish HMG domain bound strongly to a 26 mer probe containing the TACAAT site (Fig. 4*B*). In contrast, Fish did not bind consistently to a 26 mer probe containing both TTCAAT sites, suggesting that Fish can distinguish between closely related DNA sequences. Dfr protein bound very strongly to a 33 mer probe that contained the ATGCAAAT site, and less strongly to a 32 mer probe containing the CATAAAT site (Fig. 4*B*). Dfr bound the ATGCAAAT site both as an apparent monomer and a dimer, because two distinct bands with reduced mobilities were detected. The 1 kb *slit* fragment thus may integrate the actions of at least three different types of regulatory proteins, represented by Sim, Fish, and Dfr.

We next examined the ability of Fish, Dfr, Sim, and Tgo to directly control *slit* transcription using transient transcription assays in cultured *Drosophila* S2 cells (Fig. 5). The P[1.0*slit*-lacZ] construct was used as a reporter with various combinations of

plasmids that express Fish, Dfr, Sim, or Tgo. Fish modestly activated P[1.0*slit*-lacZ] transcription (4 units of β -gal activity), indicating that in both yeast (Ma et al., 1998) and fly cells, Fish can function as a direct transcriptional activator. Dfr resulted in little if any activation of P[1.0*slit*-lacZ] (<1 unit), and Dfr and Fish together did not exhibit any increased activation over the levels observed for Fish alone. Neither Sim nor Tgo alone was able to activate the P[1.0*slit*-lacZ] reporter, because only background levels of expression were detected (<0.4 units of activity). Furthermore, Sim and Tgo together yielded only minimal P[1.0*slit*-lacZ] activation (1 unit). These results imply that although Sim::Tgo heterodimers strongly activate expression of a P[6XCME-lacZ] reporter (>150 units) that contains six multimerized CMEs (Sonnenfeld et al., 1997), additional factors are required to achieve high levels of P[1.0*slit*-lacZ] expression. Significantly, the combination of either Fish and Sim::Tgo or Dfr and Sim::Tgo both resulted in relatively high levels of P[1.0*slit*-lacZ] activation (23 units for Fish and Sim::Tgo and 12 units for Dfr and Sim::Tgo). Thus, both Fish and Dfr strongly enhanced the ability of Sim::Tgo heterodimers to activate *slit* transcription. Compara-

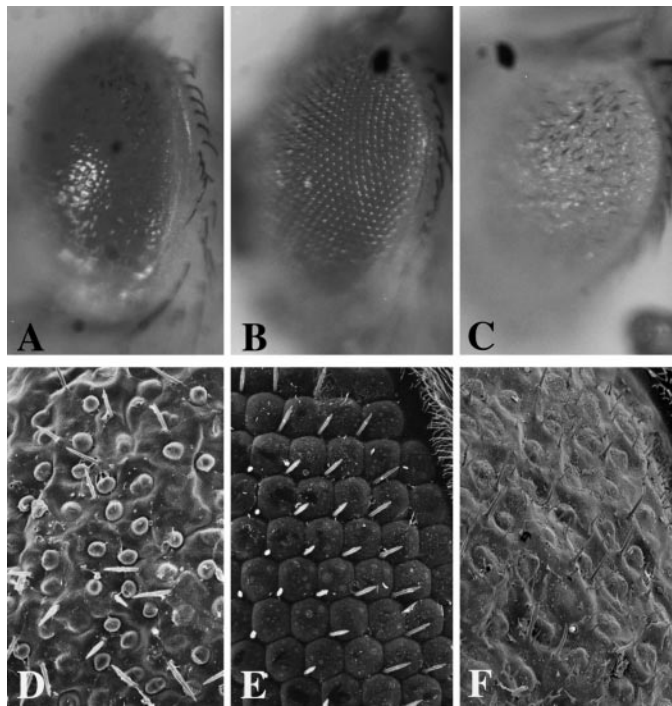


Figure 3. Interactions between Fish and Sim detected via gain-of-function assay. Light microscope (*A–C*) or scanning electron microscope (*D–F*) analysis of eyes from adults in which ectopic Fish and/or Sim expression was driven in developing eye imaginal disks via P[GMR-Gal4]. *A, D*, A P[GMR-Gal4]/+; P[UAS-*fish*]/+ animal. Note that the eye is roughened, and there is a disorganization of ommatidia and loss of most mechanosensory bristles. There is a slight loss of eye pigmentation. *B, E*, A P[GMR-Gal4]/+; P[UAS-*sim*]/+ animal. Note essentially normal organization of ommatidia and mechanosensory bristles and uniform eye pigmentation. *C, F*, A P[GMR-Gal4]/+; P[UAS-*sim*]/P[UAS-*fish*] animal. Note more severe disorganization of ommatidia and mechanosensory bristles as well as a strong loss of eye pigmentation. *A–C*, Light microscope images at 20 \times magnification. *D–F*, Scanning electron microscope images at 1000 \times magnification.

ble levels of P[1.0*slit*-lacZ] activation (14 units) were observed when all four proteins were expressed together. Taken together, the DNA binding and transcriptional activation assays provide additional evidence that regulation of *slit* expression in the midline glia requires functional interactions between Fish, Dfr, Sim, and Tgo.

Interactions between Sim, Fish, and Dfr proteins

The ability of Sim, Fish, and Dfr to regulate P[1.0*slit*-lacZ] expression and the location of their respective DNA binding sites in the 1 kb *slit* DNA fragment suggests that their regulatory functions may involve direct protein–protein interactions. We initially addressed this possibility using yeast 2-hybrid assays, in which combinations of bait (fusions to the LexA-DNA binding domain) and prey (fusions to the B42 activation domain) constructs that express full-length or truncated Sim, Fish, and Dfr proteins were tested for their ability to activate expression of a *LEU2* reporter gene. Full-length Fish yielded strong interaction with both full-length Sim and full-length Dfr (Fig. 6*A*). In addition, Fish exhibited the ability to self-associate, suggesting that it may form homodimers. We then analyzed which regions of Sim and Dfr associate with Fish. Interactions were detected between full-length Fish and Sim bHLH-PAS and PAS constructs, but not a Sim bHLH-only construct (Fig. 6*A*). Thus, Fish specifically in-

A

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AAGCTTTCGATCAGCAGCGGCGAGTGGCAGCATTAACTGTTATAGCATTATA 50
GCCAGTTATAAGCAGTCGGTCGGGCCAACTCGGTGCAAGTTGCCTGGC
TGACTGGTTGGTTGGCTGTGCGACTGTGCTCAGGGACACATTTGGCTGCT
GTTATTTGCGCTCTTTTTTTTATTTGTGTTTTTTATTTTTTTTATTTGTATT
TATTGTTTTTTAATGCTGCATACAGAGAGAGTAATATAAAACACAGAAAC
CAGCCAGCAGTGAGCCAAACAGAAACAGAAACATATCAACATGCACGACA
TATTTGCATTTTAAATAGAGAACCGAACCGATGCGAACCCAACTGAACT
Dfr binding site
GGGCACGGCATATATGTGCGCTACTATATCTATACTATATTGTATTATGCA
Fish binding site

CAGGGTGCCTCAACGACCACGGATCAAATTTATATATGTCCTCCATTATGCT
Dfr binding site
GAGTGACATTCCATGCGGAGCGATGAGGTTCCGGGTGTCAAAATGTGTGT
GTGTGTGTGTGTCAGGAGAATGTGTCAAGAGTCTTAAGCTGAGCTTAAG
TGGGGCAGCGAAAGAACCAAAATAAGCGACTGGCAACCCAGGTGAGGATGT
GATGAAAGGCTCTTGGGGAAATTATGATTTCAACAGATTATATAGTAGA
AGCGGGGAATTAATTCCAAAGCCATCGAGGGGAGAGGGGAACTCTA
AAGGTGAAAAGTACTTTTGTTCATGAAATGAAATACAGTCTAAGTCGC
consensus Sox sites

ATTGCTGCGGCacGTGTTGAGTTTAAAGTACTTACCTAGCTCAAATTTCA
CME
TTATCTTATACTCCAGCATGGTAAGCAACTTAAATGCTTCGTTTTGGAAA
ACGGAAATCCAGCTACCTAAAAATGCTAAATACTTTTGGGCTTAGTTTA
TGCTAAAACGAGCAGTTATAGTTTCAGTCTTTTGAATTTGATTTTTAATAG
CCAAAGTAATTAAGGCTT
970

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B

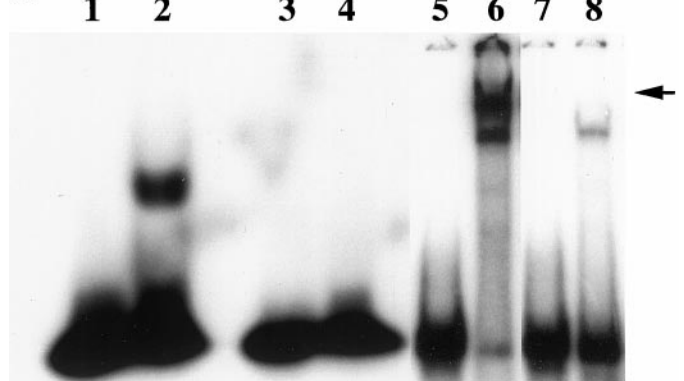


Figure 4. The *slit* 1 kb midline regulatory region contains binding sites for Sim::Tgo, Fish, and Dfr. *A*, Nucleotide sequence of a *HinDIII/HinDIII* restriction enzyme fragment from a *slit* gene intron (Wharton and Crews, 1993) that contains CNS midline regulatory elements. Indicated in *bold* are the single CME site through which Sim::Tgo heterodimers act, two closely linked TTCAAT consensus Sox sites, a TACAAT Fish binding site, and ATGCAAAT and CATAAAT Dfr binding sites. Sequences corresponding to the double-stranded oligonucleotide probes used in gel mobility shift assays with the Fish HMG domain or Dfr protein are underlined. *B*, Gel mobility shift assays performed with purified Fish HMG domain or Dfr protein and sequences from the *slit* 1 kb regulatory region. *Lane 1*, Free TACAAT probe. *Lane 2*, TACAAT probe and Fish HMG domain protein. Note strong binding of Fish protein. *Lane 3*, Free TTCAAT probe. *Lane 4*, TTCAAT probe and Fish HMG domain protein. Note lack of detectable binding by Fish protein. *Lane 5*, Free ATGCAAAT probe. *Lane 6*, ATGCAAAT probe and Dfr protein. Note that Dfr binds strongly to this sequence as both an apparent monomer and a dimer (arrow). *Lane 7*, Free CATAAAT probe. *Lane 8*, CATAAAT probe and Dfr protein. Note that Dfr also binds to this sequence, although less strongly than to the ATGCAAAT sequence.

teracts with the PAS domain of Sim. Only a single PAS region was required for this interaction, because Fish also exhibited interactions with a Sim bHLH-PAS-A construct. Full-length Fish was also found to interact with the POU domain of Dfr (Fig. 6*A*). This is consistent with the ability of the vertebrate Sox2 protein to associate with the POU domain of Oct3 (Ambrosetti et al., 1997). In an attempt to map which region of Fish is responsible for

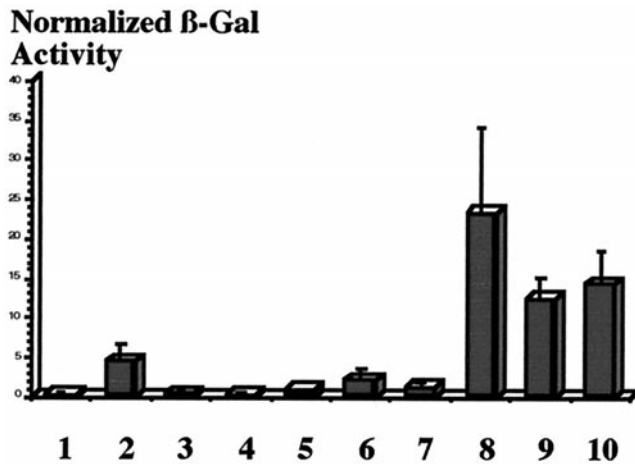


Figure 5. Fish and Dfr enhance Sim::Tgo transcription of the P[1.0*slit-lacZ*] transgene in cultured *Drosophila* S2 cells. S2 cells were cotransfected with P[1.0*slit-lacZ*] reporter alone (1) or P[1.0*slit-lacZ*] with various combinations of the expression plasmids pAct-*sim*, pAct-*tgo*, pAct-*fish*, and pAct-*dfr* (2–10). Transfection efficiencies were normalized using a copia-luc plasmid. Cells were lysed 48 hr after transfection, and β -gal and luciferase activity were assayed. Normalized β -gal activity is expressed in arbitrary fluorescence units as the mean (SEM of 3–5 independent transfections). (1) 1.0*slit-lacZ*; (2) 1.0*slit-lacZ* + Fish; (3) 1.0*slit-lacZ* + Tgo; (4) 1.0*slit-lacZ* + Sim; (5) 1.0*slit-lacZ* + Dfr; (6) 1.0*slit-lacZ* + Fish + Dfr; (7) 1.0*slit-lacZ* + Sim + Tgo; (8) 1.0*slit-lacZ* + Sim + Tgo + Fish; (9) 1.0*slit-lacZ* + Sim + Tgo + Dfr; (10) 1.0*slit-lacZ* + Sim + Tgo + Fish + Dfr.

interactions with Sim and Dfr, we used several bait constructs that express truncated versions of Fish, including the HMG domain, NH₂-terminal region, COOH-terminal region, NH₂+HMG, HMG+COOH, or NH₂+COOH regions. None of these constructs exhibited consistent interaction with any Sim or Dfr prey construct. Although it is possible that structural determinants present only on full-length Fish are required for interactions with PAS or POU domains in the yeast 2-hybrid assay, on the basis of previous studies on Sox2 (Ambrosetti et al., 1997) it is likely that the Fish HMG domain is a key contributor to these interactions.

To verify the interactions between Fish and Sim and the self-association of Fish, we additionally performed GST pull-down assays. Fish and Sim interaction was tested using bacterially expressed GST or GST–Fish fusion protein, and ³⁵S-labeled Sim generated via *in vitro* translation. The ³⁵S-labeled Sim migrated on an SDS polyacrylamide gel as a doublet, with one band near the predicted molecular weight of Sim protein (73 kDa) and the second at a somewhat lower apparent molecular weight (Fig. 6B). Equal amounts of labeled Sim protein were incubated with either GST or GST–Fish, and the mixtures were subjected to glutathione-Sepharose chromatography followed by SDS-PAGE and autoradiography. As expected, Sim did not bind to the GST because no labeled bands were detected (Fig. 6B). However, Sim did associate with GST–Fish, because both of the labeled Sim bands were observed (Fig. 6B). These data confirm the results of the yeast 2-hybrid assays and indicate that Fish and Sim are capable of direct physical association. Similar GST pull-down assays were performed to verify Fish self-association using GST–Fish and ³⁵S-labeled *in vitro*-translated Fish protein. The major band present in the ³⁵S-labeled Fish reaction migrated at ~45 kDa (Fig. 6C), close to the predicted molecular weight of Fish protein (40 kDa). This 45 kDa band was specifically bound by GST–Fish but not GST alone (Fig. 6C). This result also con-

firmed the yeast 2-hybrid data indicating that Fish is able to self-associate.

No interactions were detected between Sim bait and Dfr prey constructs in the yeast 2-hybrid assays (Fig. 6A), indicating that at least in yeast, they do not associate directly. Because both proteins did associate with Fish, we tested whether Fish might facilitate interactions between Sim and Dfr. This was pursued using Sim bait, Dfr prey, and a pDB20-*fish* construct to express full-length native Fish in yeast (see Materials and Methods). The combinations of pDB20-*fish* and either Sim bait or Dfr prey did not result in any detectable interaction, as indicated by the inability of transformed yeast cells to activate *LEU2* reporter gene expression and grow on leu⁻ medium (Fig. 7). However, the simultaneous presence of pDB20-*fish*, Sim bait, and Dfr prey constructs did permit growth on leu⁻ medium (Fig. 7), indicating that Sim, Fish, and Dfr are able to form a ternary complex in yeast cells.

Additional yeast 2-hybrid assays were performed to examine potential interactions between Fish and other PAS and POU proteins. Full-length Fish interacted with the POU domains of both *Drosophila* Pdm-1 and Pdm-2, which are involved in embryonic segmentation and nervous system development (Bhat et al., 1995; Yeo et al., 1995; Ma et al., 1998), as well as full-length mouse Oct3 (Fig. 6A). Fish also interacted with the bHLH-PAS region of *Drosophila* Tracheless (Trh), a protein required for formation of several types of tubular tissues (Wilk et al., 1996). As well, Fish interacted with a PAS domain-containing fragment of *Drosophila* Per (Per-C2) (Huang et al., 1995), a PAS-only protein that associates with the Timeless protein and plays a key role in regulating biological rhythms (for review, see Hardin, 1998; Young, 1998). Because Fish also interacted with PerL, a mutant version of Per that exhibits reduced ability to bind Timeless (Gekakis et al., 1995), it appears that distinct residues are important for Per/Fish versus Per/Timeless interactions. Together, these data indicate that Fish can associate with multiple POU and PAS domain proteins and suggest that functional interactions between Sox, PAS, and POU proteins may be important in diverse developmental and physiological processes.

DISCUSSION

Functions of Sim, Fish, and Dfr in the regulation of CNS midline gene expression

The *Drosophila slit* gene encodes a large extracellular protein that is required for normal midline glial migration and axon projection patterns (Rothberg et al., 1988, 1990). *slit* expression in the CNS midline glia was mimicked by a P[1.0*slit-lacZ*] transgene that is first expressed in fully germ band-extended embryos (Wharton and Crews, 1993). This expression is completely dependent on the functions of the bHLH-PAS protein Sim, because *sim* mutant embryos exhibit a complete absence of P[1.0*slit-lacZ*] expression (Nambu et al., 1991). In this study, we show that the combined functions of the Sox protein Fish and the POU domain protein Dfr are also essential for P[1.0*slit-lacZ*] expression, because *dfr-fish* double mutant embryos exhibit a dramatic loss of P[1.0*slit-lacZ*] expression in germ band-retracted embryos. Additionally, Fish and Dfr significantly enhanced the ability of Sim and Tgo to activate P[1.0*slit-lacZ*] expression in cultured *Drosophila* S2 cells.

The 1 kb *slit* midline regulatory region was found to contain binding sites for Fish and Dfr, as well as a single CME through which Sim::Tgo heterodimers function. Fish bound strongly to a TACAAT site and Dfr bound to two sites, ATGCAAAT and CATAAAT, that flank the Fish site within a 150 bp interval.

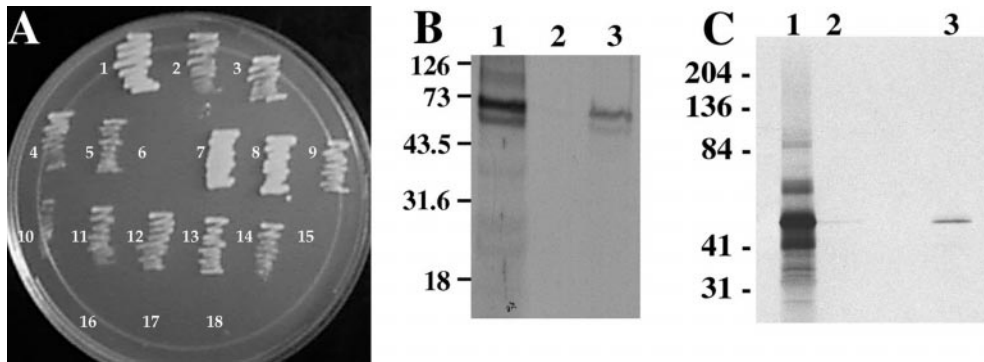


Figure 6. Fish directly associates with POU and PAS domain proteins. *A*, Interactions between Fish, Sim, and Drifter revealed via yeast 2-hybrid assays. The following combinations of bait and prey constructs were transformed into yeast and assayed for growth on Leu⁻ medium: (1) Fish/TrhbHLH-PAS; (2) Fish/PerL; (3) Fish/Per; (4) Fish/Sim; (5) Fish/SimPAS; (6) Fish/SimbHLH; (7) Fish/SimbHLH-PAS; (8) Fish/SimbHLH-PASA; (9) Fish/Fish; (10) Fish/Drifter; (11) Fish/DrifterPOU; (12) Fish/Pdm-1; (13) Fish/Pdm-2; (14) Fish/Oct-3; (15) Drifter/Sim; (16) Drifter/SimbHLH-PAS;

(17) DrifterPOU/Sim; (18) DrifterPOU/SimbHLH-PAS. Note that interactions were detected between Fish and the POU domain of Drifter, as well as the PAS domain but not the bHLH region of Sim. No interactions were detected between any Sim and Drifter constructs. *B*, Binding of GST–Fish fusion protein to ³⁵S-labeled Sim generated via *in vitro* translation. *Lane 1*, *In vitro*-translated Sim analyzed via SDS-PAGE and autoradiography. Note a radiolabeled band at 73 kDa corresponding to full-length Sim protein, as well as a second band of lower molecular weight. *Lane 2*, *In vitro*-translated Sim does not bind GST. Sim protein was incubated with GST and glutathione-Sepharose resin. The mixture was washed, and the bound material was eluted and analyzed via SDS-PAGE and autoradiography. Note the absence of labeled Sim protein. *Lane 3*, *In vitro*-translated Sim binds to GST–Fish. Sim protein was incubated with GST–Fish and glutathione-Sepharose resin. The mixture was washed, and the bound material was eluted and analyzed via SDS-PAGE and autoradiography. Note the presence of labeled Sim proteins. *C*, Binding of GST–Fish fusion protein to ³⁵S-labeled Fish generated via *in vitro* translation. *Lane 1*, *In vitro*-translated Fish analyzed via SDS-PAGE and autoradiography. Note a major radiolabeled band at ~45 kDa corresponding to full-length Fish. *Lane 2*, *In vitro*-translated Fish does not bind efficiently to GST. Fish protein was incubated with GST and glutathione-Sepharose resin. The mixture was washed, and the bound material was eluted and analyzed via SDS-PAGE and autoradiography. Note the absence of labeled Fish protein. *Lane 3*, *In vitro*-translated Fish does bind GST–Fish. Fish protein was incubated with GST–Fish and glutathione-Sepharose resin. The mixture was washed, and the bound material was eluted and analyzed via SDS-PAGE and autoradiography. Note the presence of labeled Fish protein.

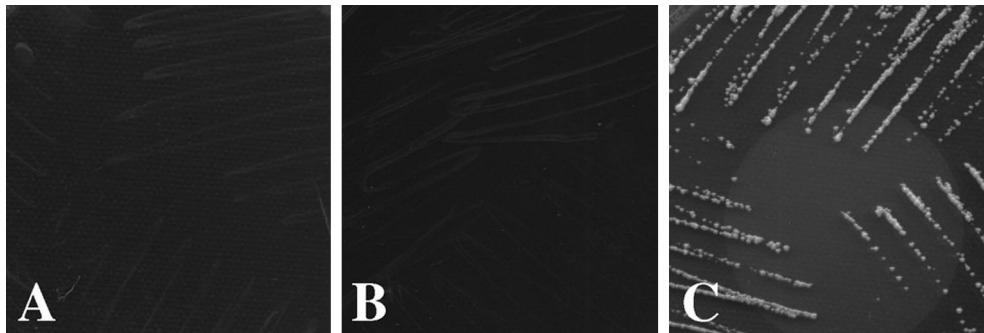


Figure 7. Fish, Sim, and Dfr can form a functional ternary complex in yeast cells. Combinations of full-length Sim bait, full-length Dfr prey, and full-length Fish expression constructs were tested for their abilities to interact in yeast cells. *A*, A combination of Sim bait and native Fish did not activate LEU2 expression, as indicated by the absence of cell growth on medium lacking leucine. *B*, A combination of Dfr prey and native Fish did not activate LEU2 expression, as indicated by the absence of cell growth on medium lacking leucine. *C*, A combination Sim

bait, Dfr prey, and Fish did result in activation of LEU2 expression, as indicated by significant cell growth on medium lacking leucine. Fish can facilitate interactions between Sim bait and Dfr prey in yeast cells.

Removal of the Fish and Dfr binding sites reduces *slit* expression, because a P[380*slit*-lacZ] strain that contains the CME but not the Fish or Dfr binding sites exhibits decreased levels of embryonic midline expression compared with P[1.0*slit*-lacZ] (Wharton and Crews, 1993). Fish did not consistently bind to two TTCAAT sequences located close to the CME; however, given the proximity of these sites, it will be of interest to determine whether Fish and Sim::Tgo might bind these sequences cooperatively. We also determined that Fish protein directly associates with the PAS domain of Sim and the POU domain of Dfr. In addition, all three proteins were able to form a ternary transcriptional regulatory complex in yeast. Together, these results provide strong evidence that these proteins act together to directly regulate midline gene expression. One model for the functions of these proteins is that although Sim::Tgo heterodimers are sufficient to activate embryonic expression of P[1.0*slit*-lacZ], Fish and Dfr are required to maintain high levels of LacZ expression (Fig. 8).

Functional interactions between Sim, Fish, and Dfr may also regulate the midline expression of other genes, including *sim* and *breathless* (*btl*). Thus, *sim* has autoregulatory functions (Nambu et al., 1991), and we have shown that the combined functions of

dfr and *fish* are also required for sustained midline *sim* expression. In addition, a 2.8 kb interval in the P[3.7*sim*-lacZ] transgene used in this study contains six evolutionarily conserved CMEs (Kasai et al., 1998) as well as several consensus Fish and Dfr binding sites. *btl* encodes an FGF receptor homolog whose expression in the CNS midline and tracheal cells has been shown to depend, respectively, on Dfr as well as Sim and Tgo, or Trh and Tgo (Anderson et al., 1996; Ohshiro and Saigo, 1997). A 200 bp *btl* midline/tracheal regulatory region contains three evolutionarily conserved CMEs (Ohshiro and Saigo, 1997). Inspection of this region also revealed the presence of a conserved consensus ATCAAT Fish binding site located in a 40 bp interval between CME2 and CME3, as well as a conserved consensus GATAAAT Dfr binding site (Anderson et al., 1996) located 40 bp downstream of CME3. Thus, functional interactions between Sim, Fish, and Dfr could be a general mechanism to regulate gene transcription during CNS midline development.

Like other Sox proteins, Fish can influence transcription through DNA bending, direct transcriptional activation, and protein–protein interactions. It will be of interest to determine the relative contributions of these activities in regulating *slit* expres-

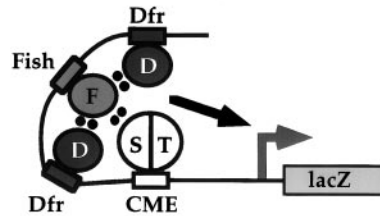
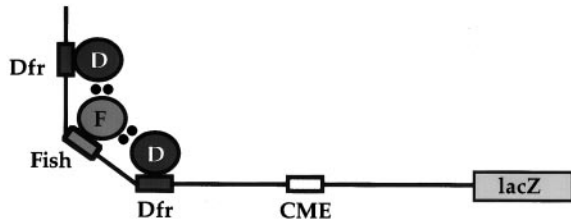
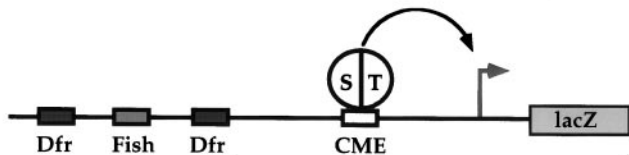
A. wild type embryo - maintained P[1.0*slit-lacZ*] expression**B. *sim* mutant embryo - P[1.0*slit-lacZ*] not expressed****C. *dfr-fish* mutant embryos - transient P[1.0*slit-lacZ*] expression**

Figure 8. A model for regulation of P[1.0*slit-lacZ*] expression in the CNS midline via interactions between Sim, Tgo, Fish, and Dfr. In wild-type embryos (*A*) the binding of a Sim: Tgo heterodimer to the CME and Fish and Dfr to nearby sites results in the formation of a ternary regulatory complex that permits sustained midline P[1.0*slit-lacZ*] expression. In the absence of Sim (*B*), Fish and Dfr are not sufficient to activate expression of P[1.0*slit-lacZ*]. In the absence of both Fish and Dfr (*C*), the initial presence of Sim is sufficient to activate midline P[1.0*slit-lacZ*] expression; however, this expression is not maintained in germ band-retracted *dfr-fish* mutant embryos. *D*, Drifter; *F*, Fish; *S*, Sim; *T*, Tgo. Dots indicate direct protein–protein interactions.

sion. In this regard, Sox proteins may act in distinct capacities depending on the presence of other regulatory proteins and the organization of control elements in a specific target gene. For example, close range interactions between Sox2 and Oct3 are required to synergistically activate a distal enhancer element from the vertebrate *fgf4* gene (Yuan et al., 1995; Ambrosetti et al., 1997). Increasing the distance between the Sox2 and Oct3 binding sites from 3 to 6 bp abolishes the ability of these proteins to activate transcription (Ambrosetti et al., 1997). In contrast, Sox2 represses Oct4-induced activation of a preimplantation enhancer from the mouse *osteopontin* gene by acting at a site 39 bp away from the Oct4 binding site (Botquin et al., 1998). This is similar to the interactions between Fish and Dfr through nonadjacent binding sites in the *slit* 1 kb midline enhancer. These findings indicate that there are different mechanisms through which Sox and POU domain proteins can interact to regulate transcriptional processes. Significantly, in the native *fgf4*, *osteopontin*, and *slit* genes, the relevant regulatory regions are all located several kilobases downstream of the promoter. This suggests that in addition to local DNA bending activities, the *in vivo* functions of Sox proteins may also involve DNA looping events (Lamb and

Rizzino, 1998). Because Sry and presumably other Sox proteins bind DNA as a monomer (Werner et al., 1995), DNA looping could be mediated via Sox protein dimerization, as detected for Fish and LSox5 and Sox6 proteins (Lefebvre and de Crombrughe, 1998), as well as through association between Sox and PAS or POU domain proteins.

Conserved PAS, Sox, and POU regulatory interactions in nervous system development?

There are several examples of functional association between vertebrate HMG domain and POU domain proteins (Zwilling et al., 1995; Ambrosetti et al., 1997; Botquin et al., 1998; Kuhlbrodt et al., 1998), and our studies have revealed similar association between *Drosophila* Sox and POU proteins. In addition, we have identified novel interactions between Sox and PAS domain proteins. Given the considerable sequence divergence between the PAS domains of Sim or Trh and Per, the ability of Fish to directly associate with each of these proteins suggests that interactions between Sox and PAS domain proteins may also be widespread. In this regard we have found that the mouse Sox2 protein, which contains an HMG domain highly related to that of Fish, also associates with Sim and Per (Y. Gao and J. R. Nambu, unpublished results). In addition, midline-targeted expression of Sox2 has been shown to partially rescue the axon scaffold defects in *fish* mutant embryos (Sánchez Soriano and Russell, 1998). Although the precise mechanism of PAS/Sox association is not clear, our results indicate that a single PAS region is sufficient for binding to a Sox protein, and they suggest that the HMG domain is crucial for this interaction. Further analyses of the regulatory interactions between Sim, Fish, and Dfr may ultimately provide a useful paradigm for a better understanding of the widespread functions of PAS, Sox, and POU genes in vertebrate neural development, and their involvement in specific human congenital disorders that result in deafness, obesity, and mental retardation (Pevny and Lovell-Badge, 1997; Crews, 1998; Crews and Fan, 1999; Latchman, 1999; McEvelly and Rosenfeld, 1999; Wegner, 1999).

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