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Original Article

Bowline, a novel protein localized to the presomitic mesoderm, interacts with Groucho/TLE in *Xenopus*

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ABSTRACT Cells in the prospective somite of *Xenopus laevis* embryos rotate in an orchestrated manner to form a segregated somite. The prospective somite boundaries are prepatterned by gene expressions in the unsegmented presomitic mesoderm (PSM). However, the roles of polarized gene expression in this boundary formation are not well elucidated. Here we identified a novel gene, *bowline*, which localizes to the anterior halves of S-II, III in the PSM of *X. laevis*. Bowline associated with corepressor XGrg-4, a *Xenopus* homolog of Groucho/TLE protein. A WRPW tetrapeptide motif in Bowline was prerequisite for coprecipitation with XGrg-4 and for downregulation of *X-Delta-2* by *bowline* RNA injection. This study indicates that Bowline is a novel protein interacting with Groucho/TLE and may play a role in somitogenesis in *X. laevis*.

KEY WORDS: *Xenopus laevis, somitogenesis, presomitic mesoderm, XGrg-4, Groucho/TLE, X-Delta-2*

Introduction

The somites of a vertebrate embryo have a metameric structure that develops into the axial skeleton, muscles and dermis of the trunk. This structure also provides a design for other segmental patterns, including vascular and peripheral nervous systems. The repeated structure of the somites is formed along the anteroposterior axis as groups of cells bud off one by one from the anterior end of the presomitic mesoderm (PSM) at a species-specific constant rate. In the case of *X. laevis,* the PSM of the embryo is subdivided in two major regions separated by a transition zone (TZ); a posterior tailbud domain (TBD) and a somitomeric domain containing four prospective somites. During segmentation, the cells of a somitomere rotate through 90 degrees in an orchestrated manner, so that the cells lie parallel to the anteroposterior axis. After the rotation, each elongated myotomal cell of a formed somite spans the full length of the somite (Hamilton, 1969, Jen *et al.*, 1999, Keller, 2000, Youn *et al.*, 1980).

In a vertebrate, the spatial and temporal control of somitic boundary formation is made through a molecular oscillator, the segmentation clock, which has been revealed by the cyclic expression of genes in the PSM (reviewed in Pourquié, 2003). The region of the cyclic gene expression narrows increasingly as it slides along the anterior-posterior axis toward the anterior end of the PSM before the anterior front of the region stops at the border of the prospective somite segment (Palmeirim *et al.,* 1997). Genes related to Notch and Wnt signallings showing such an oscillatory behaviour have been discovered in mouse, chick, *X. laevis* and zebrafish (reviewed in Aulehla and Herrmann, 2004, Dubrulle and Pourquié, 2004, Rida *et al.*, 2004). The anterior end of the cyclic gene expressions has been proposed to be regulated by a posterior-to-anterior gradient of the Wnt and Fgf signallings, which regresses with an elongation of the axis of an embryo (Aulehla *et al.*, 2003, Dubrulle *et al.*, 2001, Sawada *et al.*, 2001). A retinoic acid signal is also implicated in modulating the Fgf signalling by opposing an anterior-to-posterior gradient (Kawakami *et al.*, 2005, Moreno and Kintner, 2004, Vermot *et al.*, 2005, Vermot and Pourquié, 2005).

Prior to somitic segmentation, the prospective somitic cells of a vertebrate within the anterior end of its PSM have striped expressions of genes; these gene expressions prefigure the positions of the prospective segmentation and the anteroposterior polarities within a segment. In *X. laevis* embryos, anteriorly polarized segmental expression in the somitomere region and involvement in somite segmentation have been reported for the Notch ligands, *X-Delta-2*, bHLH transcription factors, *Thylacine1* (*Thy1)*, *ESR-4, 5, esr-9, 10* and for a member of the cadherin superfamily, *paraxialprotocadherin (PAPC)*. In contrast, *hairy2A* has been reported to be localized to the posterior half of the

Abbreviations used in this paper: IP, immunoprecipitation; PAPC, paraxial-protocadherin; PSM, presomitic mesoderm; TBD, tailbud domain; Thy, thylacine; TLE, transducine-like enhancer of split; TZ, transition zone; WB, Western blot.

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somitomere (Jen *et al.*, 1999, Jen *et al.*, 1997, Kim *et al.*, 2000, Li *et al.*, 2003, Sparrow *et al.*, 1998). It has been proposed that the polarity within a somitomere is determined at the TZ and established at the caudal-most somitomere, S-III and that during polarity determination, *Thy1*, a Mesp2-like bHLH gene, which is expressed at the anterior halves of somitomeres S-I~S-III, is involved (Jen *et al.*, 1999, Moreno and Kintner, 2004, Sparrow *et al.*, 1998). It remains unclear the role of the anteroposterior polarity within somitomeres in the formation of regularly shaped somites in *X. laevis* embryo.

Under these circumstances, we screened for novel genes based on an expression patterns and successfully isolated a gene which is expressed in the anterior halves of somitomeres S-II~III. The characterization of the novel protein showed that the protein named as 'Bowline' interacts with Groucho/transducine-like enhancer of split (TLE). As generally recognized, Groucho/TLE is an important protein and functions as a corepressor in various phenomena. As Bowline interacts with such an important protein, Bowline could be added to a group of proteins having a novel and unknown role in transcriptional regulation (Chen and Courey, 2000).

Results

Isolation of bowline *cDNA*

A clone coding a partial sequence of the *bowline* gene was isolated from a cDNA library made from *X. laevis* animal caps stimulated with Activin A. The cDNA library was constructed by subtraction-hybridization; for tester, *X. laevis* animal caps were cultured with Activin A (100 ng/ml) for 1 hour and successively cultured for 5 hours without Activin A and for driver, animal caps were cultured for 1 hour without Activin A. Forward-subtracted cDNAs were used to probe the subtracted cDNA library. The isolated clones were selected based on RNA localization at the blastula and neurula stages and one clone, designated as A21, showed a segmented expression pattern at the dorsal region. We screened a cDNA library derived from *X. laevis* neurula-stage lateral mesoderm (Satow *et al.,* 2002) by using the A21 clone as a probe and recovered one clone. Sequence determination of this clone and 5' RACE revealed the *bowline* gene consisting of 1842 base pairs (bp) and encoding a protein of 153 amino acids from the first possible initiation site at the 42nd nucleotide (nt) (Fig. 1). No in-frame stop codon was found in the upstream sequence of the predicted initiation site. The sequence in the vicinity of the first methionine codon partially matches the Kozak consensus sequence (A/G residue at position 3 and G residue at position +4) and hence is highly likely to be the translation initiation site (Kozak, 1987).

No significant motifs were identified in the amino acid sequence of Bowline according to motif analysis against PROSITE, although PSORTII predicted that the *bowline* gene product would localize in the nucleus with a probability of 43.5 % (Nakai and Kanehisa, 1992). A protein-protein BLAST search and a BLAST search against translated database of cDNA and EST clones revealed three *X. laevis* homologs, Ledgerline (Chen *et al.,* unpublished) and two cDNA clones (accession no. BJ036197 and DR718559), in addition to DSCR6, Amphisom and Ripply1-3 (Kawamura *et al.,* 2005, Li *et al.,* 2005, Shibuya *et al.,* 2000). Bowline-related proteins have been found in chordates including human, chimpanzee, dog, mouse, rat, chick, *X. laevis, X. tropicalis,* zebrafish, *Tetraodon nigroviridis* and *Branchiostoma belcheri*.

Two conserved regions were found by aligning the amino acid sequences of Bowline with its homologs: (1) a tetrapeptide motif, WRPW; and, (2) 36 amino-acid residues at the C-terminal region designated as the Bowline-DSCR6-Ledgerline conserved region or "BDLC-region" (Fig. 1). Further comparisons with the BDLC-region revealed that Bowline is closely related to the following proteins and DNA sequences: NP001009994 (human, identity of 75%; similar to DSCR6), XP518613 (chimpanzee, identity of 75%; similar to DSCR6), Ripply2 (mouse, identity of 75%; BAE53720), XP343445 (rat, identity of 75%; similar to DSCR6), XM849178 (dog; identity of 75%), XP419859 (chick, identity of 75%), Ledgerline (*X. laevis*, identity of 75%; BAB90857), DR718559 (*X. laevis*, identity of 89%; cDNA clone) and CR760245 (*X. tropicalis,* identity of 75%; cDNA clone). The remaining homologs have the amino acid-sequence identities of 44-67%. A cDNA clone (accession no. DR718559) encoded for a protein with overall identity of 80%. It is not clear whether this gene is an allele of *bowline*. With this exception, we found no protein with more than a 40% identity to the N-terminal 92 amino acids of Bowline.

Localization of bowline *transcripts in* X. laevis *embryos*

The distribution of *bowline* mRNA expression in *X. laevis* embryos was determined by *in situ* hybridization using probes directed against the coding region of the *bowline* gene (Fig. 2 A-C). At stage 13, the *bowline* transcripts were localized as bilateral, regularly spaced stripes (Fig. 2A). The expression of bilateral stripes was observed at neurula stage and tailbud stage (Fig. 2B, C). The number of stripes was found to vary between two and three as shown in Fig 2 B (arrowheads). The same striped

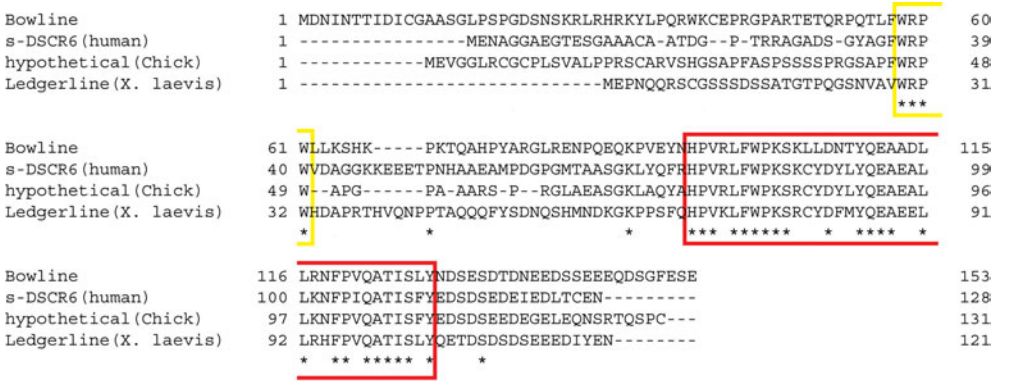

Fig. 1. Structural features of Bowline. *The complete peptide sequence of Bowline deduced from its nucleotide sequence is aligned with human "similar to DSCR6" (s-DSCR6; NP001009994), chick hypothetical protein (XP419859) and* X. laevis *Ledgerline (BAB90857). Asterisks under the sequences indicate the position of conserved amino acids. A yellow box indicates the position of the conserved WRPW sequence and a red box indicates the BDLC-region. Sequences were aligned using GENETYX software. The Genbank accession number for the bowline nucleotide sequence is AB105905.*

distribution of expression was also detected using a probe against the 3' untranslated region (3'-UTR) of *bowline*. At stage 30, signal was sometimes detected in the head region using the 3'-UTR probe, but not when the open reading frame (ORF) probe was used, implying that *bowline* is localized only as bilateral stripes at stage 30.

The expression of *bowline* was next compared with that of other known genes expressed in the PSM, so as to precisely define the location of the mRNA expression. Sequential sections of stage-20 embryos were hybridized to detect *bowline* (probe against 3'- UTR) and *X-Delta-2* or *Thy1* mRNA expression (Fig. 2 D-I). *X-Delta-2* was expressed in the anterior half of somitomeres, S0, S-I, S-II, S-III and in the TBD in the PSM of *X. laevis* embryos (Jen *et al.,* 1997, Sparrow *et al.,* 1998). *In situ* hybridization showed that (1) the anterior and posterior ends of the *bowline*-expressing region in somitomeres, S-II and S-III, correspond to those of a continuous section stained for *X-Delta-2* and (2) *X-Delta-2* was expressed in a salt-and-pepper pattern while *bowline* is expressed uniformly (n= 2, Fig.2 D, E); *Thy1* is localized to the anterior half of S-I to S-III (Moreno and Kintner, 2004, Sparrow *et al.,* 1998). Two posterior stripes of *Thy1* expression were found to correspond to two major stripes of bowline expression and a very weak stripe of bowline transcripts was detected anterior to the two major stripes. A band at S-I is consistent with the results from the whole-mount *in situ* hybridization in that the number of stripes varied. As shown in Fig. 2 H and I, the *Thy1* expression region is slightly narrower than the corresponding region for *bowline* expression in the somitomere domain (n= 3).

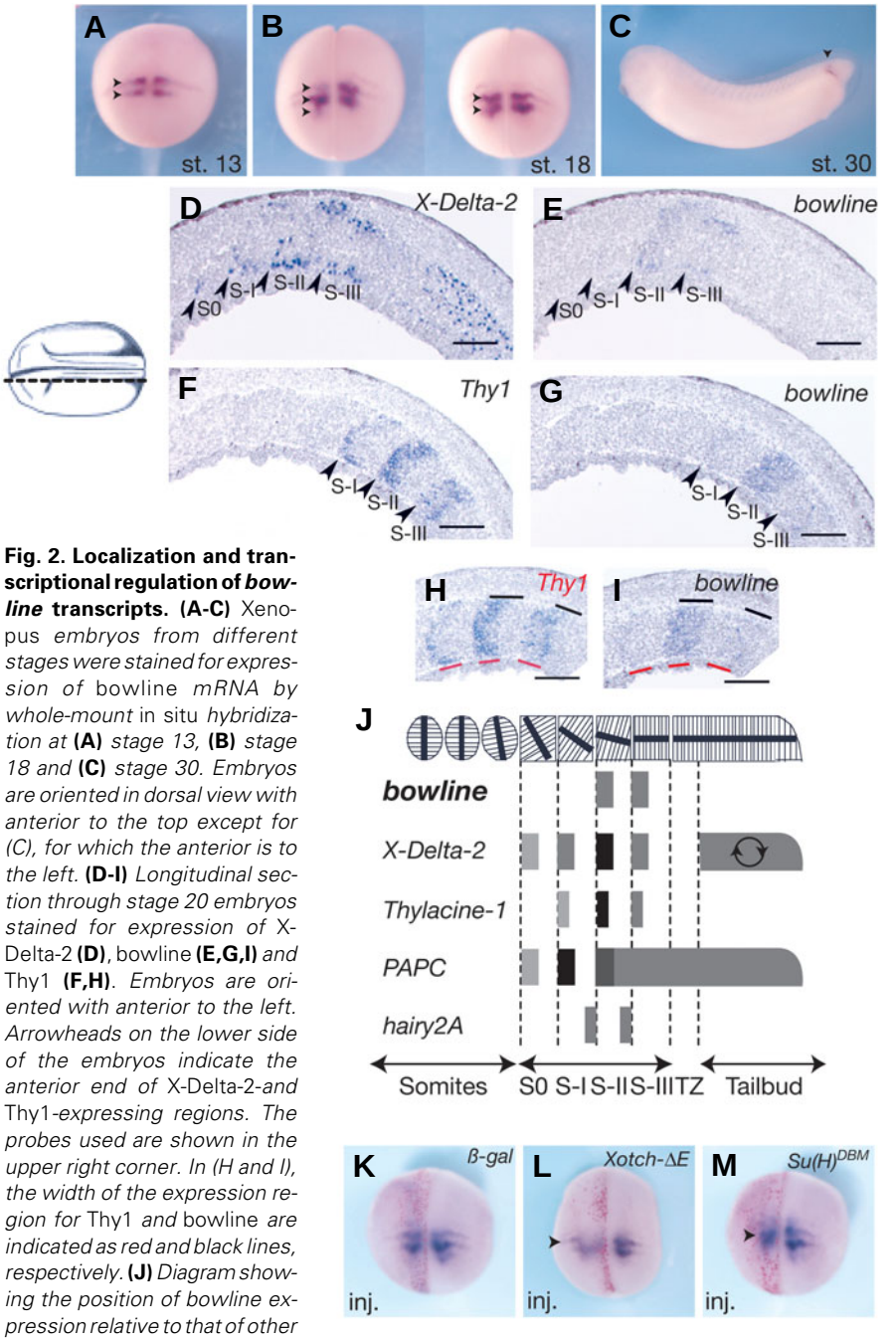

Fig. 2. Localization and transcriptional regulation of *bowline* transcripts. (A-C) Xenopus *embryos from different stages were stained for expression of* bowline *mRNA by whole-mount* in situ *hybridization at* **(A)** *stage 13,* **(B)** *stage 18 and* **(C)** *stage 30. Embryos are oriented in dorsal view with anterior to the top except for (C), for which the anterior is to the left.* **(D-I)** *Longitudinal section through stage 20 embryos stained for expression of* X-Delta-2 **(D)**, bowline **(E,G,I)** *and* Thy1 **(F,H)**. *Embryos are oriented with anterior to the left. Arrowheads on the lower side of the embryos indicate the anterior end of* X-Delta-2-*and* Thy1-*expressing regions. The probes used are shown in the upper right corner. In (H and I), the width of the expression region for* Thy1 *and* bowline *are indicated as red and black lines, respectively.* **(J)** *Diagram showing the position of bowline expression relative to that of other genes known to be expressed segmentally in* X. laevis *embryos (Jen* et al., *1997, Kim* et al., *2000, Sparrow* et al., *1998).* **(K-M)** *Embryos were unilaterally injected with* β-gal *RNA* **(K)**, XotchΔE *RNA* **(L)**, or XSu(H)^{DBM} *RNA* **(M)** *and analyzed for the expression of* bowline *(K,L,M) by whole-mount* in situ *hybridization at stage 20. RNA encoding the lineage tracer* β-gal *was co-injected to identify the injected side (red staining). Dorsal views with anterior towards the top are shown. Injected sides are indicated as "inj." Somitomeres were demarcated as S0, S-I, S-II, S-III based on the annotation of Pourquié and Tam (Pourquié and Tam, 2001). Bars, 100 μm.*

Effect of Notch signalling on the polarized expression of bowline

The possible involvement of Notch signalling in the regulation of *bowline* expression was examined by injecting the following RNAs unilaterally into embryos: (1) *Xotch-ΔE*, which encodes an activated form of the Notch receptor (extracellular deletion construct of Notch) (Coffman *et al.,* 1993); and, (2) *Su(H)^{DBM}*, a dominant-negative Su(H) (Wettstein *et al.,* 1997). *Xotch-ΔE* injection diminished the expression level and abrogated the on-off pattern of bowline expression in the PSM (500 pg; 16/17, Fig. 2L), while *Su(H)^{DBM}* RNA injection resulted in the segments of expression being into one narrow, blurred spot (500 pg; 29/31, Fig. 2M). In embryos injected with *β-gal* RNA, the segmental pattern of *bowline* expression remained intact (500 pg; 26/28, Fig. 2K). These findings show that the on-off pattern of *bowline* expression depends on Notch signalling.

Interaction of Bowline with XGrg-4

Alignment of the amino acid sequence of Bowline with its homologs showed that the WRPW tetrapeptide motif is conserved

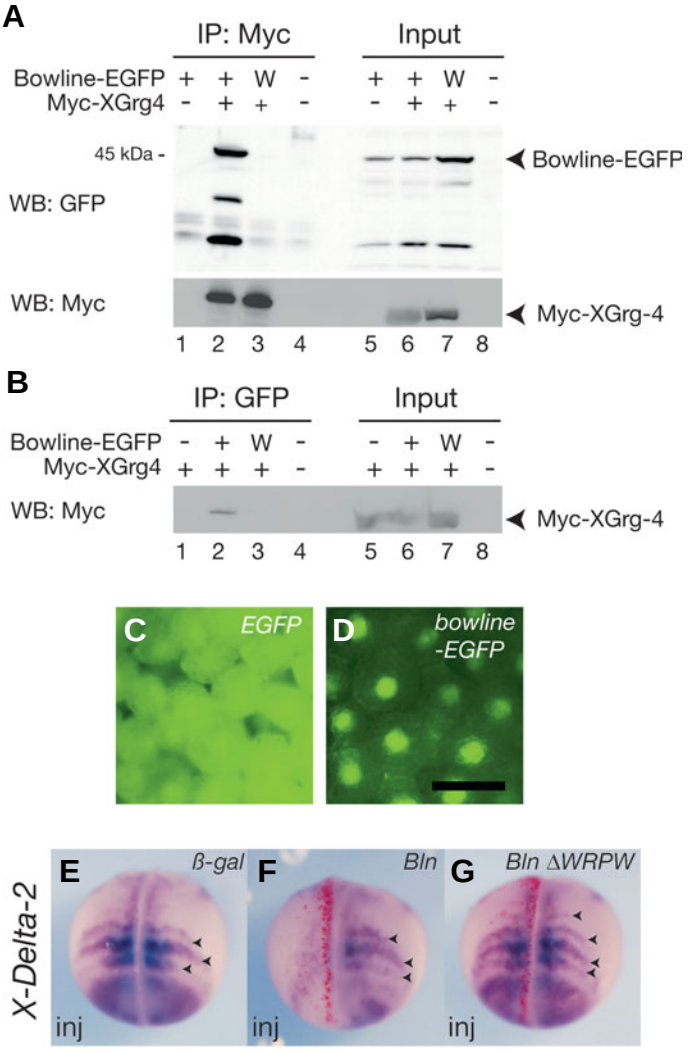

Fig. 3. The WRPW motif is required for Bowline interaction with XGrg-4. (A) *Extracts prepared from embryos injected with* bowline-EGFP *alone (lane 1),* Myc-XGrg4 *and* bowline-EGFP *(lane 2), or* Myc-XGrg4 *and* bowlineΔWRPW-EGFP *(lane 3) were subjected to immunoprecipitation (IP) with an anti-myc antibody followed by Western blotting (WB) with either anti-GFP or anti-myc antibody.* **(B)** *Extract prepared from embryos injected with* Myc-XGrg4 *alone (lane 1),* Myc-XGrg-4 *and* bowline-EGFP *(lane 2), or* Myc-XGrg-4 *and* bowlineΔWRPW-EGFP *(lane 3) were subjected to IP with an anti-GFP antibody, followed by WB with the anti-myc antibody. Lane 4 was an uninjected negative control.* **(A,B)** *5% of embryo extracts were loaded as input to indicate the expression (A,B; lanes 5-8).* **(C,D)** *Subcellular localization of Bowline-EGFP protein.* EGFP *RNA (1 ng)* **(C)** *or* bowline-EGFP *RNA (1 ng)* **(D)** *was injected into two animal blastomeres of two-cell stage embryos. Animal caps were excised at stage 9-10 and examined for GFP fluorescence. Bars, 50 μm.* **(E-G)** *Embryos injected unilaterally at the 4-cell stage with* β-gal *RNA* **(E)**, bowline RNA **(F)** *or* bowlineΔWRPW **(G)** *and analyzed for the expression of* X-Delta-2 *(E,F,G) by whole-mount* in situ *hybridization at stage 20. RNA encoding the lineage tracer* β-gal *was co-injected to identify the injected side (red staining). Dorsal views with anterior towards the top are shown. Injected sides are indicated as "inj."*

(Fig 1). It has been previously reported that interaction of Groucho/TLE transcription corepressor proteins and Hairy-family basic helix-loop-helix domain-containing transcription factors are mediated by the WRPW tetrapeptide motifs at C-terminal ends of the transcription factors (reviewed in Chen and Courey, 2000). Therefore, we assume that Bowline functions by interacting with the Groucho/TLE proteins. To test this assumption, we investigated (1) the *in vitro* interaction of Bowline with a *Xenopus* homolog of the Groucho/TLE proteins, XGrg-4, (2) the subcellular localization of Bowline and (3) the requirement of Bowline-XGrg-4 interaction in Bowline function *in vivo* (Roose *et al.,* 1998).

Interaction of Bowline with XGrg-4 was examined by immunoprecipitation. Embryos were injected with RNAs encoding myc-tagged XGrg-4 *(Myc-XGrg4),* a fusion protein of Bowline and EGFP *(bowline-EGFP), bowlineΔWRPW-EGFP* RNA in which the WRPW motif was replaced with a GGGG tetrapeptides and combinations thereof. The injected embryos were homogenized and their extracts were immunoprecipitated with an anti-myc antibody or with an anti-GFP antibody. Bowline-EGFP was co-immunoprecipitated with Myc-XGrg-4 (Fig. 3 A, lane 2) and vice versa (Fig. 3 B, lane 2). In contrast, BowlineΔWRPW-EGFP was not co- immunoprecipitated with Myc-XGrg-4 (Fig. 3 A, lane 3) and Myc-XGrg-4 was not co-immunoprecipitated with BowlineΔWRPW-EGFP (Fig. 3 B, lane 3).

The possibility of co-localization of Bowline with XGrg-4 was examined from subcellular distribution of Bowline. *bowline-EGFP* was injected into the animal pole of two-cell stage embryos. At stage 9-10, ectodermal cells were excised from the embryos and observed under a fluorescence microscope. Bowline-EGFP was accumulated in the nucleus of each cell following injection of *bowline-EGFP* RNA, while EGFP protein was distributed uniformly in each cell as shown in Fig. 3 C, D.

Finally, the necessity of the WRPW motif in Bowline function was investigated *in vivo*. Embryos injected with 50 pg of *bowline* or *bowlineΔWRPW* RNA were examined for the expression *X-Delta-2* by whole-mount *in situ* hybridization. In embryos injected with *bowline* RNA, expression of *X-Delta-2* was suppressed or vanished both in the somitomere domain and in the TBD, while it remained intact in the embryos injected with *bowlineΔWRPW* RNA (number of downregulated embryos: *bowline;* 22/ 23, *bowlineΔEGFP;* 0 /22, Fig. 3 E-G).

Discussion

In *X. laevis*, the prospective boundary of a somite is initially marked by anteroposteriorly polarized gene expression at S-II and –III of the corresponding somitomere. The genes that exhibit polarized expressions in S-II and -III are transcription factors, *Thy1, ESR-4, 5, esr-9, 10* and *hairy2A,* as well as Notch ligand, *X-Delta-2* (Jen *et al.,* 1999, Jen *et al.,* 1997, Li *et al.,* 2003, Sparrow *et al.,* 1998). In this connection, it has been proposed that the polarity within a somitomere is determined at the TZ and established first at the caudal-most somitomere, S-III (Jen *et al.,* 1999, Moreno and Kintner, 2004). However, the role of polarized gene expression in boundary formation is not well understood.

In this study, we reported a novel gene *bowline*, which is localized at the anterior halves of somitomeres,S-II and -III. Bowline is a novel type of protein, which functions together with X-Grg-4. The present findings with the aid of previous studies

implicate Bowline in transcription regulation mediated by Groucho/TLE-type corepressors, as discussed below.

Bowline interaction with corepressor Groucho/TLE

The present study provides the following information about the molecular functions of Bowline: (1) Bowline-EGFP was co-immunoprecipitated with Myc-XGrg-4; (2) Bowline-EGFP was accumulated at the nuclei; (3) the WRPW motif was required for co-immunoprecipitation of Bowline-EGFP with Myc-XGrg-4; and, (4) the WRPW motif was also necessary for the downregulation of *X-Delta-2* observed in embryos injected with *bowline*. Together, these findings (1-4) indicate that Bowline downregulates *X-Delta-2* expression in cooperation with XGrg-4, which is localized in the PSM at tailbud stage (Molenaar *et al.,* 2000). In this downregulation, it is considered that Bowline regulates the activity of XGrg-4 by (i) modulating interaction of Groucho/TLE with a DNA binding transcription factor which interacts with Groucho/TLE via the WRPW or WRPY motif (Hairy/HES for example), or by (ii) mediating interaction of Groucho/TLE with a DNA binding protein which alone does not interact with Groucho/TLE, since no DNA binding motifs are identified in the Bowline sequence (Chen and Courey, 2000, Fisher *et al.,* 1996, Grbavec and Stifani, 1996). Note that Groucho/TLE proteins are transcriptional corepressors that lack DNA binding motif, but interact with DNA-bound transcription factors (Chen and Courey, 2000). If case (i) described above is operative, for instance, it is plausible that Bowline regulates the activity of the Hairy family bHLH transcription factors, such as ESR-4, -5, -9 and -10 at the somitomere region, because they have the WRPW motif at their C-terminus which is known to interact with Groucho/TLE proteins (Jen *et al.,* 1999, Li *et al.,* 2003). Localization of Bowline at nuclei is consistent with the involvement of Bowline in transcription.

The involvement of Groucho/TLE proteins in somitogenesis has been reported previously for zebrafish *groucho2* (Takke and Campos-Ortega, 1999). Miss-expression of *groucho2* led to the strong downregulation of *MyoD* expression. Recently, involvement of a Groucho-interacting protein in somitogenesis has just been reported in zebrafish. Ripply1 represses mesp-b expression in the PSM through a Groucho-interacting WRPW motif. As for Bowline and its homologs in chordates, Ripply1 in zebrafish also contains a motif corresponding to the BDLC-regions of Bowline. Amino acid sequence comparison of the BDLC-regions of Ripply1-3 with that in Bowline, together with their respective expression patterns, suggests that Ripply2 might be the counterpart of Bowline in zebrafish (Kawamura *et al.,* 2005).

In the present study, we discovered Bowline classified into a novel group of proteins implicated in somitogenesis. Bowline is a Groucho/TLE-interacting protein without having any known DNA binding motif as far as we know and could have novel functions in modulating activities of Groucho/TLE. Bowline could be fallen into a group of proteins having a novel and unknown role in transcriptional regulation (Chen and Courey, 2000).

Materials and Methods

***Isolation of* bowline**

A subtracted cDNA library was constructed by using RNA extracted from animal caps dissected from stage 9 *X. laevis* embryos treated as follows (Ariizumi and Asashima, 1994): for the tester, animal caps were cultured for 1 hour in medium containing 100 ng/ ml Activin A, followed by 5 hour-culture without Activin A; for the driver, animal caps were cultured for 1 hour in medium without Activin A. Hybridization screening was performed against the constructed cDNA library. Forward- and reverse-subtracted cDNAs were used as probes and differentially expressed clones were isolated as positive clones. Screening by RNA localization was done against gastrula and neurula embryos, using the selected clones as probes for *in situ* hybridization. For isolation of the full cDNA clone of bowline, a cDNA library constructed from the lateral region of stage 14-20 embryos was screened (Satow *et al.,* 2002). The RACE method was done using RLM-RACE kit (Ambion). The DNA sequence of *bowline* has been submitted to Genbank (accession no. AB105905).

Embryo manipulation, RNA synthesis and injections

X. laevis embryos were collected according to standard procedures as previously described (Abe *et al.,* 2004). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop, 1956). Capped mRNAs were synthesized with the mMESSEGE mMachine kit (Ambion). Embryos were injected with mRNA in a volume of 10 nl in 5% Ficoll at two- or four-cell stage. *β-galactosidase* RNA was co-injected as a lineage tracer when unilaterally injected. When pigmented embryos were used, embryos were bleached with a solution containing 1% H_2O_2, 5% formamide and 0.5 x SSC after the Red-GAL staining (Mayor *et al.,* 1995). For generating synthetic RNA *in vitro*, the coding region of the *bowline* cDNA was inserted into the pCS2+ vector (Turner and Weintraub, 1994) to produce a construct in which the 3' UTR of *bowline* cDNA was deleted (pCS2+bowline). For *bowlineΔWRPW*, the tetrapeptide WRPW at aa 58-61 was replaced with GGGG by introducing mutation into pCS2+bowline by PCR using primers (5'- CTCAAACATTGTTCgGGgGAggTgGGCTTTT GAAATC –3' and 5'- GATTTCAAAAGCCcAccTCcCCcGAACAATGTTT GAG –3') (pCS2+bowlineΔWRPW). To generate the EGFP fusion protein of Bowline or BowlineΔWRPW, fragments containing the open reading frame of pCS2+bowline and pCS2+bowlineΔWRPW were subcloned together with EGFP (accession no. AAF62891) at its 5' into the pCS2+. For myc-epitope-tagged XGrg-4, the coding region of *XGrg-4* was subcloned 5' to the myc-epitope fusion site in the pCS2+MT vector (Roose *et al.,* 1998, Turner and Weintraub, 1994). The templates for the synthesis of *Xotch ΔE* (Coffman *et al.,* 1993) and *Su(H)^{DBM}* (referred to as *X-Su(H)1^{DBM}*) (Wettstein *et al.,* 1997) have been previously described.

***In situ* hybridization**

Whole-mount *in situ* hybridization was performed on staged embryos as described by Harland (Harland, 1991) with modifications (Abe *et al.,* 2004). *In situ* hybridization of sequential sections was carried out using the Discovery system (Ventana Medical Systems, Inc), according to the manufacturer's protocol. The reagents used were RiboMap and BlueMap kits except for SA-HRP, protease 2 and anti-DIG alkaline phosphatase (Roche) instead of anti-DIG biotin antibody.

The probe for *bowline* 3'-UTR encompassed the 3'-UTR (658 nt-1080 nt). The majority of vitellogenin elements in the 3'-UTR were excluded from the probe sequence, so as to avoid cross-hybridization between transcripts having these elements (Schubiger *et al.,* 1985). The pCS2+bowline was used to generate a probe for the *bowline* coding region. Templates for the probes for *X-Delta-2* (Jen *et al.,* 1997) have been described. The probe for *Thy1* was isolated using RT-PCR with primers (Chen *et al.,* in preparation) based on published sequence (Sparrow *et al.,* 1998).

Subcellular localization of Bowline

Subcellular localization of Bowline protein was determined as previously described (Sakurai *et al.,* 2004) except for embryos injected at the two-cell stage.

Immunoprecipitation and Western blot analysis

Two blastomeres of two-cell stage embryos were injected at the animal pole with 1 ng of *XGrg-4-Myc, bowline-EGFP* and *bowlineΔWRPW-*

EGFP RNAs. Embryos were homogenized at stage 11-12 and lysate was prepared in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% NP-40 and 10% glycerol) containing protease inhibitors (protease inhibitor cocktail tablets; Roche Diagnostics). Twelve embryos were used for each immunoprecipitation and 5% of each lysate was used as input. The extract was incubated for 2 hours at 4˚C with 5 μg anti-myc antibody (9E10; Sigma) or anti-GFP antibody (Santa Cruz) coupled to 20 μl of ProteinG beads (Amersham). The beads were washed five times in NP-40 lysis buffer. The eluted samples were loaded for electrophoresis. Western blot analysis was performed using anti-myc antibody (9E10; Sigma) or anti-GFP antibody (FL; Santa Cruz).

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