A twist in sea urchin gastrulation and mesoderm specification

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Abstract

The bHLH (basic helix-loop-helix) transcription factor, sea urchin myogenic factor-1 (SUM-1), plays an important role in myogenic determination during sea urchin embryogenesis. SUM-1-mediated transactivation is restricted to the mesenchyme lineages in transgenic sea urchin embryos, suggesting that other factors, either positive or negative, influence the activity of SUM-1 in different embryonic cell types. While post-translational regulation of vertebrate myogenic factors has been suggested from in vitro studies, it has never been demonstrated in vivo. The most compelling in vitro experiments have shown that the mesodermal bHLH, twist, negatively regulates myogenic bHLHs. However, in the vertebrate embryo, twist and myogenic bHLHs are not expressed coincidentally, and different concentrations of twist play a role in the differentiation of different muscle lineages (somatic versus visceral) in Drosophila embryos. The gene expression studies in vertebrates and the genetic experiments in Drosophila suggest disparate roles for twist in these organisms. To gain a better understanding of the role of twist in mesodermal and myogenic specification, we cloned a sea urchin twist homologue and characterized its role in gastrulation and myogenesis in this simple embryo. Our data suggest that twist from Lytechinus variegatus functions after gastrulation and initial specification of the embryonic mesoderm of the sea urchin.

Introduction

The experiments of Hörstadius in the first half of this century highlighted the necessity for cell–cell interactions in the development of different cell types of the sea urchin embryo [1]. His experiments established a role for cell–cell signalling in the specification of cell fates in the sea urchin. Hörstadius showed that the micromeres influenced the differentiation of other embryonic cells, setting the stage for present day experiments which have begun to unravel the molecular pathways involved in micromere signalling and the specification of other embryonic lineages. It is now thought that the derivatives of the micromeres (the skeletogenic or primary mesenchyme cells) are autonomously specified during the
early cleavage stages. However, it is still not clear when and how the different secondary mesenchymal lineages are specified during sea urchin embryonic development [2].

Cell-lineage analysis of the vegetal plate of the mesenchyme blastula-stage embryo suggested that lineages that arise from this region, the endoderm and secondary mesenchyme cells, are specified prior to the initiation of gastrulation [3]. Cell dissociation experiments have substantiated this for the endoderm [4], but when and how the different secondary mesenchyme lineages are specified is uncertain.

The only secondary mesenchyme lineage for which we have sufficient molecular information concerning its specification is the myogenic lineage, which is specified during early to mid-gastrulation through a programme involving a sea urchin homologue of the vertebrate MyoD family, SUM-1 [5,6]. This transcription factor was cloned from *Lytechinus variegatus* and shown to be expressed in the presumptive muscle cells before overt myogenic differentiation takes place towards the end of gastrulation [5,6]. When we examined the activity of SUM-1 in different embryonic lineages we found that the transactivational activity of SUM-1 was restricted to the mesenchymal lineages (results not shown). Further analysis of SUM-1 and its role in the specification of the myogenic lineage has led us to examine other factors which might play a role in mesoderm specification in the sea urchin embryo.

Studies of mesodermal development in *Drosophila* have identified a gene, *twist*, which is essential for the earliest formation of the mesoderm in the fly [7]. *Twist*, like SUM-1, is a member of the HLH family of transcriptional regulators which are important in a variety of determinative events during development. Twist homologues have now been identified in the human [8], mouse [9], frog [10], lancelet [11], *Drosophila virilis* [12], Tribolium [13] leech [14], and *Caenorhabditis elegans* [15].

*Drosophila twist* homozygous-null embryos fail to make mesoderm [16], suggesting a role in the earliest specification of this tissue. However, initial analysis of mouse embryo twist mutants suggests that murine twist functions after gastrulation [17]. Whereas twist from *Drosophila* was shown in vivo to act as a transcriptional activator for early mesoderm-specific genes, in vitro analysis of murine twist demonstrated that it can act as a repressor of myogenic genes [18]. Gene expression studies in vertebrates [19] and genetic experiments in *Drosophila* [20] suggest disparate roles for twist in these different organisms. We therefore wish to use our transactivation assay as a functional test to better understand the role of twist in mesodermal and myogenic specification. To begin, we have identified a sea urchin twist homologue and characterized its role in gastrulation and myogenesis. Surprisingly, higher levels of *Lv-twist* (twist from *L. variegatus*) transcripts are expressed following the initiation of gastrulation, suggesting that the primary function of sea urchin twist occurs after the early specification of the mesoderm.

### Materials and methods

#### Sea urchin embryos

*Lytechinus variegatus* (Sea Grant, University of Massachusetts, U.S.A.) or *B. variegatus* embryos ([6,7]).

#### Molecular cloning

Two degenerate primers, 

(G/A/T/C)ATG

(T/C)TT(G/A/T/C)ATG

were used to PCR-amplify *Lytechinus* stage endomesoderm cDNA. These PCR-amplified cDNA was subcloned into a plasmid vector, pBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.). Clones were isolated and sequenced. The sequence of each primer was determined with a Sequenase kit (U.S.A.) and were analysed using the GCG software package.

#### Reverse transcription and PCR

RNA was isolated from *Lytechinus* embryos using Tri-reagent (Molecular Research. Inc., U.S.A.). Clones 1 and 2 were amplification with 30 cycles at 94°C (25 s), 59°C (25 s), and 72°C (30 s) with 3 min of DNA polymerase (Klenow fragment, 25:24:1, by vol. Promega, Madison, WI). PCR-amplified RNA was annealed with 10× amplification using (Gibco, BRL). A reverse transcription carried out using oligo(dT)~18 (T/AT)~18 and (25:24:1, by vol. Promega, Madison, WI). Reverse transcription was PCR-amplified with 30 cycles at 94°C (25 s), 59°C (25 s), and 72°C (30 s) with 3 min of DNA polymerase (Klenow fragment, 25:24:1, by vol. Promega, Madison, WI). PCR product was cloned into a plasmid vector, PBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.) and were sequenced.

#### Immunocytochemistry

Embryos were stained with monoclonal antibodies to myoglobin minimum of 20 round embryos. Embryos were fixed in 3% paraformaldehyde, 0.1% Triton X-100, 0.1 M sodium phosphate buffer (PBST); subsequent steps were run in PBST.
Materials and methods

Sea urchin embryo culture

*Lytechinus variegatus* adults were obtained from Susan Decker (Davie, FL, U.S.A.) or Beaufort Biologicals (Duke Marine Lab, Beaufort, NC, U.S.A.). *L. variegatus* embryos were cultured by stirring at 17°C as described previously [6,7].

Molecular cloning of the bHLH domain of the *Lv-twist*

Two degenerate primers (primer 1: 5’-CCCTCGAG(C/A)G(G/A/T/C)GT (G/A/T/C)ATGGC(G/A/T/C)AA(T/C)GT-3’ and primer 2: 5’-CCGTCGAC(T/C)TT(G/A/T/C)A(G/A)(G/A/T/C)GT(T/C)TG(G/A/T)AT(T/C)TT-3’) were used to PCR-amplify a twist-specific bHLH domain from an *L. variegatus* prism stage endomesoderm enriched cDNA library (provided by Gary Wessel, Brown University, Providence, RI, U.S.A.). A 1 µl portion of cDNA library and 0.5 µg of each primer were mixed in 20 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 25 mM KCl, 100 µg/ml gelatin, 50 mM dNTP and 5 units of Taq DNA Polymerase (Fisher Scientific, Pittsburgh, PA, U.S.A.) in a 50 µl reaction volume. Forty cycles (30 s at 94°C and 3 min at 50°C) of PCR reaction was followed by a single 8 min extension at 72°C. PCR reaction products were extracted with phenol, precipitated with ethanol and resuspended in deionized H₂O. The DNA was digested with SalI, gel purified and ligated into pBluescriptII KS+ (Stratagene, La Jolla, CA, U.S.A.). Clones were sequenced using the dideoxy chain-termination method [21] and were analysed with the BLAST search engine (NCBI).

Reverse transcriptase-PCR (RT-PCR) analyses of embryonic RNA

*L. variegatus* embryos were cultured to appropriate developmental stages in Millipore-filtered artificial sea water (MFASW) at 17°C. Total RNA was prepared using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH), followed by 30 min of DNase I treatment at 37°C, phenol/chloroform/iso-amyl alcohol (25:24:1, by vol.) extraction and ethanol precipitation. A 10 µg portion of total RNA was annealed with 5 pg of a sequence-specific oligonucleotide (5’-ATCTT-TTGATCTTCTTGCACT-3’) at 65°C for 2 min. Reverse transcription was carried out using SuperScript II RTase, following the manufacturer’s instructions (Gibco, BRL). About 2 µl of the 20 µl total RT reaction was used for PCR amplification with the reverse primer mentioned above and a forward primer (5’-CCACAATATTTATCGAGAAGGACT-3’). PCR amplification was performed with 30 cycles at 94°C for 2 min, 55°C at 1 min and 72°C at 1.5 min.

Immunocytochemistry

Embryos were stained as whole mounts after fixation in −20°C methanol for a minimum of 20 min, or in 3.7% paraformaldehyde in MFASW for 30 min at room temperature with similar results. Methanol-fixed embryos were washed in phosphate buffered saline (PBS) and incubated with antibodies diluted in PBS. Embryos fixed in paraformaldehyde were washed in PBS with 0.1% Tween 20 (PBST); subsequent incubations and washes were performed in PBST. Primary
antibodies were incubated for 1 h to overnight, followed by 3 × 5 min washes. Secondary antibodies were incubated for a minimum of 30 min, followed by 3 × 5 min washes. Embryos were mounted in PBS/glycerol (50:50, v/v) and viewed with a Zeiss Axiovert 100 Epifluorescence microscope. Embryos were photographed with an Olympus OM-1 35 mm camera using Kodak 400 ASA Gold print film, or a Dage video camera attached to a Power Computing computer equipped with Scion imaging software.

**Antibodies**

Anti-(Drosophila twist) polyclonal antibodies were obtained from Bruce Patterson (NCI, NIH, Bethesda, MD, U.S.A.) and Han Nyugen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) and used at 1:200 dilution. Goat anti-mouse FITC secondary antibody was diluted 1:100 in PBS (Cappel, ICN Pharmaceuticals, Inc., Costa Mesa, CA, U.S.A.).

**Results and discussion**

**Isolation of a twist homologue in *L. variegatus***

To isolate a *twist* homologue from *L. variegatus*, degenerate primers were designed to two conserved regions of the *Drosophila twist* bHLH domain (Figure 1A).

![Figure 1 A](image-url)

**PCR cloning of the **Lv-twist** bHLH domain**

(A) Degenerate oligonucleotide primers to conserved regions of the *Drosophila twist* bHLH were used to amplify *Lv-twist* from a prism-stage sea urchin mesendoderm-enriched cDNA library. These oligonucleotide primers recognize residues in the bHLH that are specific to twist. (B) Comparison of the *Lv-twist* bHLH sequence with those of the murine (M-Twist), human (H-Twist), *Xenopus laevis* (X-twist), *lancelet* (Bb-Twist), *D. virilis* (Dv-twist), *D. melanogaster* (Dm-twist), and *C. elegans* (Ce-twist) twist sequences shows the twist bHLH is highly conserved amongst these different species.
5 min washes. Followed by 3 × 5 washes and viewed with photographic print film, or equipped with photographic equipment from Bruce Albert Einstein lab. Goat anti-bodies were designed (Cappel, ICN

**Figure 2**

**RT-PCR analysis of Lv-twist expression**

RNA isolated from different-stage L. variegatus embryos was analysed by RT-PCR using a different set of primers from those used to clone the bHLH. The highest levels of twist transcripts are found after gastrulation has ceased. This suggests that Lv-twist functions after the initial specification of the mesoderm. Abbreviations: HB, hatching blastula; E- and L-MB, early and late mesenchyme blastula; E- and M-Gast, early and mid-stage gastrula; L-Gast and L'-Gast, two separate late gastrula; Pr, prism; Cont, control.

and used to amplify a ~123 bp DNA fragment from a late gastrula-stage mesoderm-enriched cDNA library. The deduced amino acid sequence of the 123 bp fragment was compared with the bHLH regions of twists from other species (Figure 1B). An average of 87% identity and over 90% similarity was observed for the bHLH regions of twists from other species. Lv-twist was found to be most similar to the bHLH regions of *Xenopus*, mouse and ascidian twists. In addition, high similarities were shared with other vertebrate mesodermal-specific bHLH proteins, including Dermo-1 [22], Paraxis/Meso1 [23], Scleraxis [24] and eHAND [25].

**Temporal expression pattern of Lv-twist**

RNA from different embryonic stages was analysed by RT-PCR to determine when *Lv-twist* transcripts are expressed during development. Transcripts of *Lv-twist* are present at low levels at the blastula stage but then disappear and reappear during gastrulation, with highest levels at the pluteus stage (Figure 2). The zygotic expression pattern of *Lv-twist* transcripts reveals highest levels after the completion of gastrulation. These data suggest that *Lv-twist* functions subsequent to the establishment of the different germ-layers and has an important role in later developmental events.

**Spatial expression of Lv-twist**

Immunocytochemical analysis of *Lv-twist* expression employed polyclonal antibodies generated to the *Drosophila* twist protein. The immunocytochemical localization of twist in developing sea urchin embryos suggests that most *Lv-twist* is spatially restricted to the mesendoderm of the gastrula stage embryo, indicating a potential role in the specification of lineages that arise from this region (Figure 3C).

**Summary**

The sea urchin *twist* homologue, *Lv-twist*, has high sequence similarity to *twists* of other species, suggesting conserved function through its DNA binding and dimerization motifs. Expression of *Lv-twist* suggests that its primary function
Indirect immunofluorescent localization

Polyclonal antibodies to the full length Drosophila twist protein was used to examine the spatial pattern of Lv-twist expression at different embryonic stages. No expression is observed in the blastula stage (A) and only weak expression is observed in the vegetal plate at the early gastrula (B). Highest levels of protein were detected in the mesendoderm at the gastrula stage (C).

occurs after the initial specification of the embryonic mesoderm of the sea urchin. While our data do not preclude the possibility that low levels of maternal or zygotic Lv-twist protein may function to specify the embryonic mesoderm, high levels of expression after the initial events of gastrulation suggest that Lv-twist also serves a later function. It remains to be determined if Lv-twist influences the activity of the sea urchin myogenic bHLH SUM-I.

References


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