LvDelta is a mesoderm-inducing signal in the sea urchin embryo and can endow blastomeres with organizer-like properties

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SUMMARY

Signals from micromere descendants play a critical role in patterning the early sea urchin embryo. Previous work demonstrated a link between the induction of mesoderm by micromere descendants and the Notch signaling pathway. In this study, we demonstrate that these micromere descendants express LvDelta, a ligand for the Notch receptor. LvDelta is expressed by micromere descendants during the blastula stage, a time when signaling has been shown to occur. By a combination of embryo microsurgery, mRNA injection and antisense morpholino experiments, we show that expression of LvDelta by micromere descendants is both necessary and sufficient for the development of two mesodermal cell types, pigment cells and blastocoelar cells. We also demonstrate that LvDelta is expressed by macromere descendants during mesenchyme blastula and early gastrula stages. Macromere-derived LvDelta is necessary for blastocoelar cell and muscle cell development. Finally, we find that expression of LvDelta is sufficient to endow blastomeres with the ability to function as a vegetal organizing center and to coordinate the development of a complete pluteus larva.

Key words: Delta, Notch, Sea urchin, Micromere, Induction, Mesoderm, Endoderm

INTRODUCTION

The establishment and patterning of the three germ layers in the sea urchin embryo are regulated by a signaling cascade that originates from the micromere descendants at the vegetal pole of the embryo (reviewed by Angerer and Angerer, 2000; Ettensohn and Sweet, 2000). The powerful signaling properties of micromeres were first reported by Hörstadius, who showed that these cells can induce the formation of an ectopic archenteron when recombined with animal blastomeres (reviewed by Hörstadius, 1973). Micromeres are necessary and sufficient for the development of the vegetal plate (Ransick and Davidson, 1993; Ransick and Davidson, 1995), and this gives rise to the endoderm and non-skeletogenic mesoderm and expresses distinct molecular markers. Removal of micromeres results in a delay in endoderm and mesoderm development (Ransick and Davidson, 1995; Sweet et al., 1999). Micromere removal also causes deficiencies in the development of certain types of mesoderm (Sweet et al., 1999), and micromeres transplanted to ectopic regions induce ectopic non-skeletogenic mesoderm (Khaner and Wilt, 1991; Amemiya, 1996; Sweet et al., 1999). This evidence indicates that a signal from the micromeres plays an important role in the development of non-skeletogenic mesoderm (Sweet et al., 1999).

The Notch signaling pathway also plays an important role in mesoderm specification during sea urchin development (Sherwood and McClay, 1997; Sherwood and McClay, 1999;

Sherwood and McClay, 2001). This conserved pathway controls many cell fate decisions in diverse animal embryos (reviewed by Artavanis-Tsakonis et al., 1999). In the sea urchin embryo, activation of the Notch signaling pathway causes excess non-skeletogenic mesoderm development, whereas blocking the pathway causes severe deficiencies in the development of all non-skeletogenic mesodermal cell types (Sherwood and McClay, 1999).

The Notch signaling pathway is normally activated during the blastula stages (Sherwood and McClay, 1999) and the presence of the micromeres is necessary for this activation, suggesting that a signal from the micromeres might directly activate the Notch receptor (Sweet et al., 1999). Providing further evidence for this idea, a signal from blastula stage micromere descendants (eight through tenth cleavage) is sufficient to activate the Notch protein (McClay et al., 2000). Eighth cleavage stage micromere descendants have signaling abilities, as micromere derivatives from this stage have a strong potential to induce animal cells to generate an archenteron (Minokawa and Amemiya, 1999). These studies support the hypothesis that descendants of the micromeres activate the Notch signaling pathway during the blastula stage by providing a signal in the form of a Notch ligand.

The purpose of this study is to examine further the molecular mechanisms of non-skeletogenic mesoderm specification. We cloned and characterized the sea urchin homologue of Delta and found it to be expressed by micromere derivatives during the blastula stage, and by macromere derivatives during later

stages. Experiments with chimeric embryos demonstrate that micromere-derived LvDelta is necessary and sufficient for induction of pigment and blastocoelar cells, whereas macromere-derived LvDelta is involved in the development of blastocoelar and muscle cells. In addition, we find that LvDelta expression is sufficient to endow blastomeres with the powerful inductive properties first recognized by Hörstadius – the ability to act as an organizing center to coordinate the development of animal cells into a pluteus larva.

MATERIALS AND METHODS

Animals
Adult sea urchins (*Lytechinus variegatus*) were obtained from Jennifer Keller (Duke University Marine Laboratory) and Susan Decker (Davie, FL, USA). Gametes were collected following intracoelomic injection of 0.5 M KCl. Eggs were fertilized and raised in artificial sea water at 23°C.

Cell isolation and transplantation
Micromere removal at the 16-cell stage, animal cap isolation at the 8-cell stage, and cell transplantations were performed by hand using a glass needle as described (Sweet et al., 1999).

Antibody staining
Antibody staining was performed as described (Sweet et al., 1999). Primary antibodies included 6a9 (Ettensohn and McClay, 1988) and 6e10 (Ingersoll, 1993) (both are markers for skeletogenic mesoderm), SMC2 and SMC1 (a blastocoelar cell marker and a marker specific for prospective secondary mesenchyme cells (SMCs) in the vegetal plate, respectively) (Hodor, 1998; Sweet et al., 1999), Endo1 (an endodermal marker) (Wessel and McClay, 1985), anti-myosin (a muscle marker) (Wessel et al., 1990), and CAD-1 (an antibody against LvG-cadherin) (Miller and McClay, 1997). Secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM for SMC1, SMC2 and 6a9, Texas Red-conjugated goat anti-mouse IgG for 6e10, and Cy3-conjugated goat anti-guinea pig IgG for CAD-1 (Jackson ImmunoResearch Laboratories, Inc.).

The SMC2 antibody is specific for blastocoelar cells in the pluteus larva; however, antibody staining with SMC2 is often prone to high background staining (Hodor, 1998; Sweet et al., 1999). To distinguish between SMC2-positive blastocoelar cells and background staining, confocal microscopy was used to image whole larvae stained with SMC2. In each confocal *z*-section, blastocoelar cells were identified by SMC2 staining, cell morphology and location in the larva, and were counted. The total number of blastocoelar cells for a single larva is the sum of the blastocoelar cells counted from each *z*-section. Images of SMC2 staining in Figs 5 and 8 correspond to one or two confocal *z*-sections.

Cloning of LvDelta
A cDNA clone from *Strongylocentrotus purpuratus* was originally identified as a putative Delta or Serrate homologue, based on a single sequencing reaction from the 5′ end of the clone (Zhu et al., 2001). We sequenced and characterized the remainder of the clone and verified that it was a homologue of Delta ($P=1\times10^{-135}$ by BLAST analysis). Because the SpDelta clone was incomplete, and because *L. variegatus* offered certain technical advantages for future experiments, we cloned Delta from *L. variegatus*. To clone LvDelta, total RNA was isolated from 8 hour (hatched blastula) *L. variegatus* embryos using Trizol (Life Technologies) and used as a template for RT-PCR with sequencing primers designed for SpDelta. A 291-bp fragment was amplified and cloned into Bluescript SK (Stratagene). Sequencing confirmed that this fragment was homologous to Delta. A digoxigenin (DIG)-labeled DNA probe was synthesized from the 291-bp LvDelta fragment (DIG-High Prime kit, Roche Molecular Biochemicals) and used to screen a mesenchyme blastula stage cDNA library (Guss and Ettensohn, 1997). Nylon membranes (Osmonics) were hybridized overnight with probe in DIG Easy Hyb (Roche) according to the manufacturer's instructions. Positive plaques were identified using chemiluminescence (Roche). One full-length cDNA clone was isolated and sequenced on both strands.

Northern blotting
Total RNA was isolated from embryos using Trizol. 10 μg of RNA from each developmental stage was loaded onto a 1% agarose formaldehyde gel, separated by electrophoresis and blotted onto Nylon membrane (Roche) using Turboblot (Schleicher and Schuell). A DIG-labeled antisense RNA probe was synthesized from the LvDelta clone (base pairs 1-1927) using a T7 Megascript Kit (Ambion) and was used to probe the RNA blot using DIG Easy Hyb (Roche). The RNA blot was developed using chemiluminescence (Roche).

In situ hybridization
Whole-mount in situ hybridizations were carried out as described previously (Zhu et al., 2001). The same probe used in the northern analysis of LvDelta was used for in situ hybridization.

mRNA injection
The full-length LvDelta clone was linearized with *Xho*1 and used as a template to generate 5′ capped mRNA using the T3 mMessage mMachine kit (Ambion). In addition, mRNA encoding a truncated form of LvDelta was used as a control. Using the Quik Change Site-Directed Mutagenesis kit (Stratagene) a G-to-T point mutation was introduced at nucleotide position 981, which generated an internal stop codon following amino acid 135. This mutation results in the translation of a short, N-terminal fragment of the protein lacking any signaling activity (Henderson et al., 1997). Truncated LvDelta mRNA was generated as described above. mRNA injection solution consisted of 20% glycerol, 0.5% RITC-dextran (Sigma) and 2 μg/μl mRNA. Fertilized eggs were placed in a row onto protamine sulfate-treated Petri dish covers and injected with 45-65 pg mRNA solution using Picospritzer II (General Valve Corporation). Embryos were used for further analysis only if they had four micromeres at the 16-cell stage.

Morpholino injection
Morpholino antisense oligonucleotides were designed against LvDelta sequence (5′-CAAGAAGGCAGTGCAGCCGATTCGT-3′; from –32 to –8 relative to the translational start codon) and produced by GeneTools, LLC. To account for possible nonspecific toxic effects of morpholino solution, a morpholino complementary to the 5′-UTR of the SpAristaless transcript (Zhu et al., 2001) was used as a control. It is presumed that the sequence of SpAristaless is sufficiently divergent from the sequence of LvAristaless mRNA because SpAristaless morpholino causes a phenotypic effect when injected into the eggs of *S. purpuratus* (C. A. E., unpublished observations) but not when injected into the eggs of *L. variegatus*. Morpholino injection solution consisted of 3.5 mM morpholino, 20% glycerol and 0.5% RITC-dextran. Fertilized eggs were injected with 22-34 pl of morpholino solution.

RESULTS

Cloning and sequence analysis of LvDelta
Because the Notch signaling pathway is involved in mesoderm specification (Sherwood and McClay, 1999) and because micromeres are required for activation of the Notch signaling pathway (Sweet et al., 1999; McClay et al., 2000), we
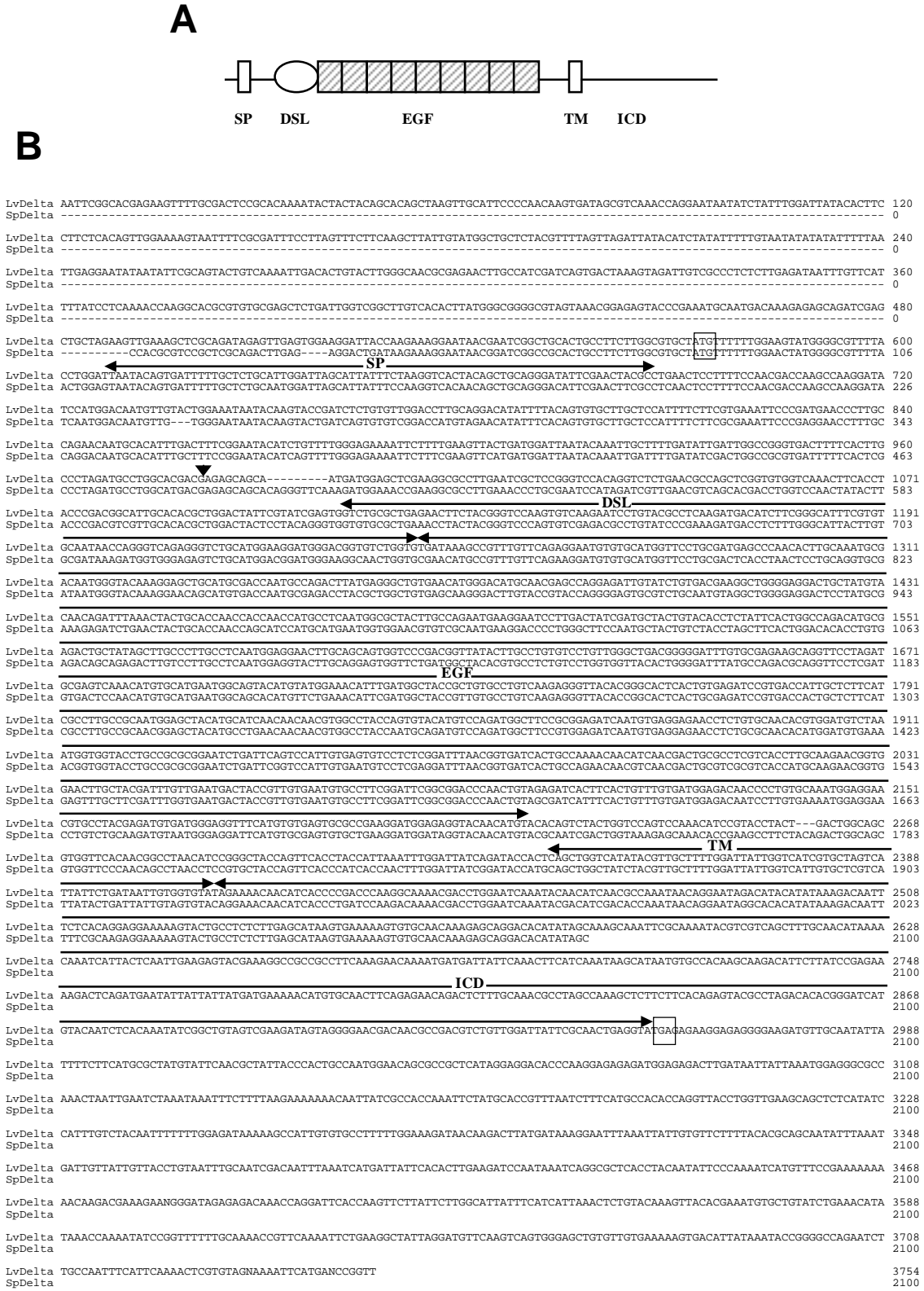
Fig. 1. Structure of sea urchin Delta homologues. (A) Schematic of LvDelta protein. SP, signal peptide; DSL, Delta/Serrate/LAG-2 domain; EGF, EGF repeats; TM, transmembrane domain; ICD, intracellular domain. (B) Nucleotide sequence of the LvDelta and SpDelta clones. Translational start and stop codons are boxed. Motifs common to Delta homologues are indicated. The site of the G-to-T mutation used to generate a premature stop codon and a truncated form of LvDelta is labeled with an arrowhead. The LvDelta clone continues to the poly(A) tail although the 3′ untranslated region was not completely sequenced. The SpDelta clone terminates in the region encoding the intracellular domain. GenBank accession numbers for LvDelta and SpDelta are AY074791 and AY074792, respectively.

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                   1                                                                                        100
                                    _____SP_____
                    <-------------------------------->
   S. purpuratus    MFFGTMGRFN WSNTVIFALQ WISIISKVTT AAGTFELRLN SFSNDQAKDI NGQCC.GNN. TSTDQCVGPC RTYFTVCLLH FLREIPEEPL PGQCTFAFRN
   L. variegatus    MFFGSMGRFT WINTVIFALH WISIISKVTT AAGIFELRLN SFSNDQAKDI HGQCCTGNN. TSTDLCVGPC RTYFTVCLLH FLREIPDEPL PEQCTFDFRN
       X. laevis    ~~~~~~~~~M GQQRMLTLLV LSAVLCQI.S CSGLFELRLQ EFVNKKGLLG NMNCCRPGSL ASLQRC..EC KTFFRICLKH YQSNVSPEP. ..PCTYGGAV
      M. musculus    ~~~~~~~~~M GRRSALALAV VSALLCQV.W SSGVFELKLQ EFVNKKGLLG NRNCCRGGSG PP...C..AC RTFFRVCLKH YQASVSPEP. ..PCTYGSAV
   D. melanogaster  ~~~~~~~~MH WIKCLLTAFI CFTVIVQV.H SSGSFELRLK YFSNDHGRDN EGRCCSGESD GATGKCLGSC KTRFRVCLKH YQATIDTTS. ..QCTYGDVI

                   101                                        ▼                                            <-----200
   S. purpuratus    TSVLGENSFE VHDGL..... .....IQIDF DIDWPRDFSL ALDAWHDESS TGFKDGNRRR L.ETLRIHRS LNVSTTWSNY TYPTSLHTLD YSYRVVCAET
   L. variegatus    TSVLGENSFE VTDGL..... .....IQIAF DIDWPGDFSL ALDAWHDESS ...NDGARRR L.ESLRVHRS LNASSVWSNF TYPTALHTLD YSYRVVCAEN
       X. laevis    TPVLGTNSFV VPESSNA.DP TFSNPIRFPP GFTWPGTFSL IIEAIHADSA DDLNTENPER LISRLATQRH LTVGEQWSQD LHSSDRTELK YSYRFVCDEY
      M. musculus    TPVLGVDSFS LPDGAGI.DP AFSNPIRFPP GFTWPGTFSL IIEALHTDSD DDLATENPER LISRLTTQRH LTVGEEWSQD LHSSGRTDLR YSYRFVCDEH
   D. melanogaster  TPILGENSVN LTDAQRFQNK GFTNPIQFPF SFSWPGTFSL IVEAWH.DTN NSGNARTNKL LIQRLLVQQV LEVSSEWKTN KSESQYTSLE YDFRVTCDLN

                   201                _____DSL                            _____EGF 1                  _____EGF 2         300
                                                 <----------                          <----------                    <----------
   S. purpuratus    YYGSQCRDAC IPKDDLFGHY LCDKDGGRVC MDGWEGNWCE HAVCSEGC.. VHGSCDSPNS CRCDNGYKGT ACDQCETYAG CEQGTCTVPG ECVCNVGWGG
   L. variegatus    FYGSKCQESC TPQDDIFGHF VCNNQGQRVC MEGWDGVWCD KAVCSEEC.. VHGSCDEPNT CKCDNGYKGA ACDQCQTYEG CEHGTCNEPG DCICDEGWGG
       X. laevis    YYGEGCSDYC RPRDDAFGHF SCGEKGEKLC NPGWKGLYCT EPICLPGCDB HHGYCDKPGE CKCRVGWQGR YCDECIRYPG CLHGTCQQPW QCNCQEGWGG
      M. musculus    YYGEGCSVFC RPRDDAFGHF TCGDRGEKMC DPGWKGQYCT DPICLPGCDD QHGYCDKPGE CKCRVGWQGR YCDECIRYPG CLHGTCQQPW QCNCQEGWGG
   D. melanogaster  YYGSSGCAKFC RPRDDSFGHS TCSETGEIIC LTGWQGDYCH IPKCAKGCE. .HGHCDKPNQ CVCQLGWKGA LCNECVLEPN CIHGTCNKPW TCICNEGWGG

                   301       <--------- _____EGF 3                    <--------  _____EGF 4                   <----------     400
   S. purpuratus    LLCERDLNYC TNQHPCMNGG TCRNEGPLGF QCYCLPSFTG HTCETAE.TC .....PCLNG GTCRSGS... DGYTCLCPGG YTGDLCQTQV PRCDSNMCMN
   L. variegatus    LLCTTDLNYC TNHQPCLNGA TCQNEGILDY RCYCTPLFTG QTCETAI.AC .....PCLNG GTCSSGP... DGYTCLCPVG LTGDLCEKQV PRCESNMCMN
       X. laevis    LFCNQDLNYC THHKPCENGA TCTNTGQGSY TCSCRPGYTG SNCEIEVNEC DA..NPCKNG GSCSD...LE NSYTCSCPPG FYGKNCELSA MTCADGPCFN
      M. musculus    LFCNQDLNYC THHKPCRNGA TCTNTGQGSY TCSCRPGYTG ANCELEVDEC AP..SPCKNG ASCTD...LE DSFSCTCPPG FYGKVCELSA MTCADGPCFN
   D. melanogaster  LYCNQDLNYC TNHRPCKNGG TCFNTGEGLY TCKCAPGYSG DDCENEIYSC DADVNPCQNG GTCIDEPHTK TGYKCHCANG WSGKMCEEKV LTCSDKPC.H

                   401    _____EGF 5                       <--------- _____EGF 6                   _____EGF 7       500
                                                                                                          <----------
   S. purpuratus    GSTCSETFD. ......GYRC ACQEGYTGTH CEIR.DHCSS SPCRNGATCL NNNVAYQCRC PDGFRGDQCE ENL..CATHG CENGGTCRAE SDSVHCECPR
   L. variegatus    GSTCMETFD. ......GYRC ACQEGYTGTH CEIR.DHCSS SPCRNGATCI NNNVAYQCTC PDGFRGDQCE ENL..CATRG CLNGGTCRAE SDSVHCECPL
       X. laevis    GGRCADNPDG ......GYIC FCPVGYSGFN CEKKIDYCSS NP........ .......... .......... CANGARCEDL GNSYICQCQE
      M. musculus    GGRCSDNPDG ......GYTC HCPLGFSGFN CEKKMDLCGS NP........ .......... .......... CSNGAKCVDL GNSYLCRCQA
   D. melanogaster  QGICRNVRPG LGSKGQGYQC ECPIGYSGPN CDLQLDNCSP NPCINGGSCQ PSG...KCIC PAGFSGTRCE TNIDDCLGHQ CENGGTCIDM VNQYRCQCVP

                   501          _____EGF 8                        <--------- _____EGF 9                  600
   S. purpuratus    GFNGDHCQNN VNDCVASPCK NGGVCFDLVN DYRCECAFGF GGPNCSDHFT VCDGDNPCEN GGTCLQDVMG GFMCECAEGW IGTTCTQSTG KEQTPKPSTD
   L. variegatus    GFNGDHCQNN INDCASSPCK NGGTCTDLLN DYRCECAGF VCDGDNPCAN GGTCLRDVMG GFMCECAEGW RGTTCTQSTG PVQTSVP.TD
       X. laevis    GFSGRNCDDN LDDCTSFPCQ NGGTCQDGIN DYSCTCPPGY IGKNCNSMPIT KCE.HNPCHN GATCHERN.N RYVCQCARGY GGNNC.QFLL PEEK..PVVV
      M. musculus    GFSGRYCEDN VDDCASSPCA NGGTCRDSVN DFSCTCPPGY TGKNCSAPVS RCE.HAPCHN GATCHQRG.Q RYMCECAQGY GGPNC.QFLL PEPPPGPMVV
   D. melanogaster  GFHGTHCSSK VDLCLIRPCA NGGTCLNLNN DYQCTCRAGF TGKDCSVDID ECS.SGPCHN GGTCMNRV.N SFECVCANGF RGKQC.D... .EESYDSVTF

                   601                           _____TM_____                                            700
                                                <-------------->
   S. purpuratus    WQRGSQQPNL RATSSPITNF GLSDTMQLAI YVAFGLLVIV LVIILIIVVY RKQHHPDPRQ NDLESNTTST PNNRNRHTYK DNFSQEEKVL PLLSISEKVC
   L. variegatus    WQRGSQRPNI RATSSPTIKF GLSDTTQLVI YVAFGLLVIV LVIILIIVVY RKQHHPDPRQ NDLESNTTST PNNRNRHTYK DNFSQEEKVL PLLSISEKVC
       X. laevis    DLTEKYTEGQ S...GQFPWI AVCAGIVLVL MLLLG..CAA VVVCVRVRVQ KRRHQPEACR G..ESKTMNN LANCQRE... ....KDISVS FIGTTQIKNT
      M. musculus    DLSERHMESQ G...GPFPWV AVCAGVVLVL LLLLG..CAA VVVCVRLKLQ KHQPPPEPCG G..ETETMNN LANCQRE... ....KDVSVS IIGATQIKNT
   D. melanogaster  DAHQYGATTQ ARADGLTNAQ VVLIAVFSVA MPLVAVIAAC VVFCMKRKRK RAQEKDDAEA R..KQNEQNA VATMHHN... ...GSGVGVA LASASLGGKT

                   701                            _____ICD                                     800
   S. purpuratus    NKEQDTYS~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   L. variegatus    NKEQDTYSKA NSQNTSSALQ HKTNHYSIEE YERPPPSKNK MMIIQTSSNK HNVPQARHSY PRKDSDEYYY YDEKHVQLQR TDSLQTPSQS SSSQSTPRHT
       X. laevis    NKKIDFLSES NNEKNGYKPR YPSVDVNLVR E..LKNEDSP .KEE...RSK CEAKCSSNDS DSED.VNSVH SKRDSSERRR PDSAYSTSKD TKYQSVYVIS
      M. musculus    NKKADFHGDH GAKKSSPKVR YPTVDYNLVR D..LKGDEAT VRDT...HSK RDTKCQSQSS AGEEKIAPTL RGGEIPDRKR PESVYSTSKD TKYQSVYVLS
   D. melanogaster  GSNSGLTFDG GN.PNIIKNT WDKSVNNICA SAAAAAAAAA AADECLMYGG YVASVADNNN ANSDFCVAPL QRAKSQKQLN TDPTLMHRGS PAGSSAKGAS

                   801                                                           868
   S. purpuratus    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
   L. variegatus    GSCTISQISA VVEDSRGTTT PTSVGLFATE V~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
       X. laevis    DEKDECIIAT EV~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
      M. musculus    AEKDECVIAT EV~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
   D. melanogaster  GGGPGAAEGK RISVLGEGSY CSQRWPSLAA AGVAGACSSQ LMAAASVAGS GAGTAQQQRS VVCGTPHM
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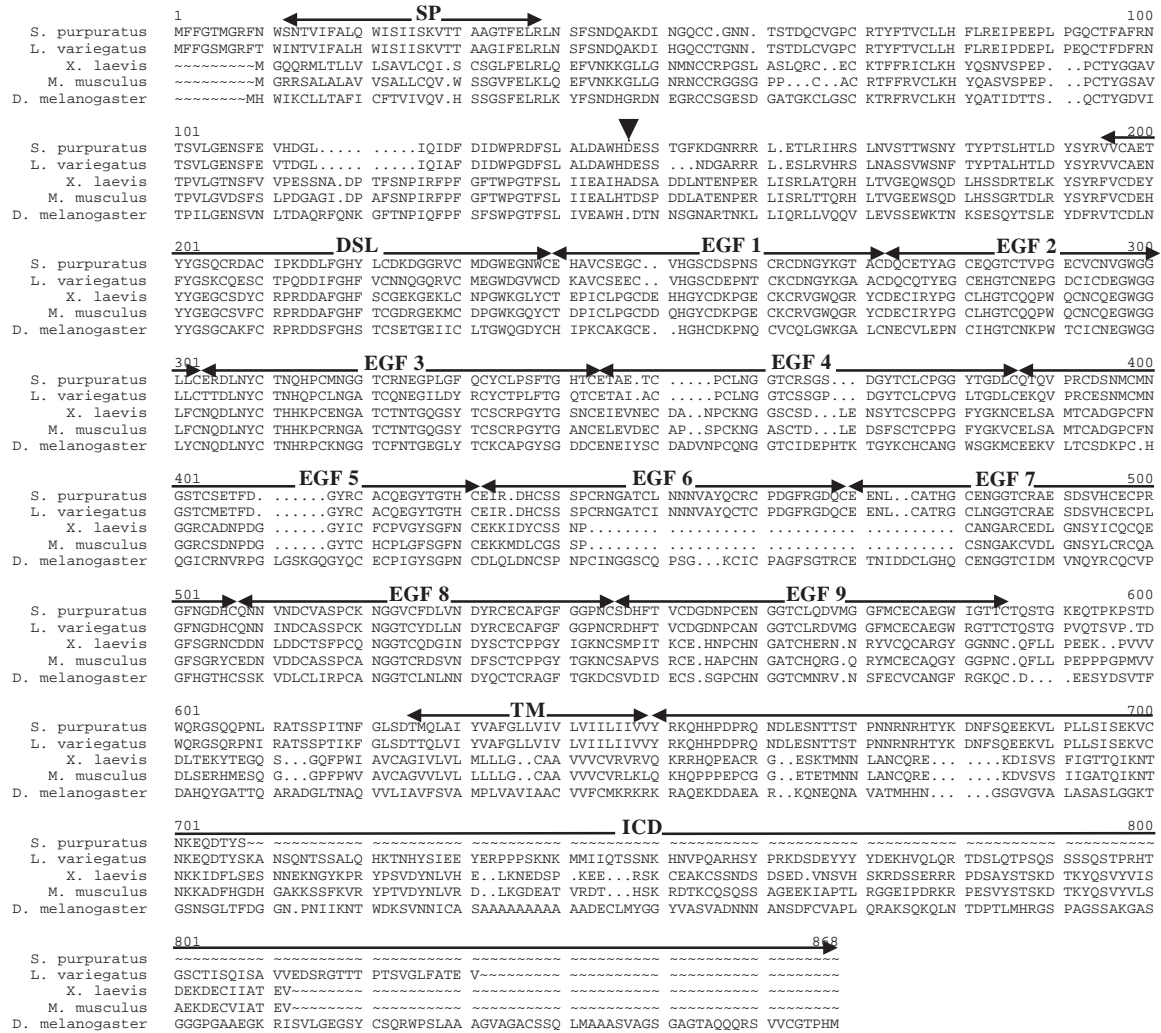
Fig. 2. Alignment of the predicted amino acid sequences of several Delta proteins. Amino acid sequences of Delta proteins from *S. purpuratus*, *L. variegatus*, *X. laevis,* (Chitnis et al., 1995), *M. musculus* (Bettenhausen et al., 1995) and *D. melanogaster* (Kopczynski et al., 1988) were aligned using Pileup and Pretty in the GCG Wisconsin Package. Conserved domains are labeled. The mutation site used to generate truncated LvDelta is indicated by an arrowhead.

characterized the sea urchin homologue of Delta, a ligand for the Notch receptor. A putative *S. purpuratus* homologue of Delta or Serrate was originally identified by us (Zhu et al., 2001). We sequenced both strands of this clone and verified that it encodes a homologue of Delta (Fig. 1). The SpDelta clone was not complete, however, as it terminated in the intracellular domain. We used information from the SpDelta sequence to clone a full-length cDNA encoding the *L. variegatus* homologue, LvDelta (Fig. 1). This cDNA clone is approximately 4.4 kb; however, the 3′ untranslated region was not completely sequenced.

LvDelta has a predicted open reading frame of 794 amino acids. Alignment of the predicted amino acid sequence of LvDelta and SpDelta with other Delta homologues showed that both sea urchin proteins have the characteristic domain structure of Delta family members, including a signal peptide, a Delta/Serrate/LAG-2 (DSL) domain, multiple EGF repeats, a single transmembrane domain, and a C-terminal cytoplasmic domain (Fig. 2).

LvDelta is expressed during blastula stages by micromere and macromere derivatives

Developmental northern analysis showed that LvDelta transcripts first accumulated between 4-6 hours after fertilization; i.e. between the sixth and seventh cleavage stages (Fig. 3A). Initially, only one transcript of approximately 5.3 kb was present; however, a smaller transcript (about 4.8 kb) began to accumulate by 8 hours, which was the time of greatest accumulation. After this time, transcripts decreased in abundance and they were barely detectable by 24 hours (prism stage). A comparison of the sizes of the LvDelta transcripts and the LvDelta cDNA (about 4.4 kb) suggests that the cDNA corresponds to the smaller transcript. The larger transcript could be a result of alternative splicing of LvDelta or a product of a closely related gene.

In situ hybridization was used to determine the spatial localization of LvDelta transcripts. Transcripts were first detected in 5-hour embryos (7th cleavage stage) in a ring of approximately eight cells surrounding about four unlabeled

cells (not shown). The signal was stronger at later stages and transcripts were consistently found in a ring of cells at the vegetal pole (Fig. 3B). This spatial pattern suggests that the cells expressing LvDelta at this stage are the descendants of the large micromeres that are found in a ring surrounding the descendants of the small micromeres (at the 32-cell stage,
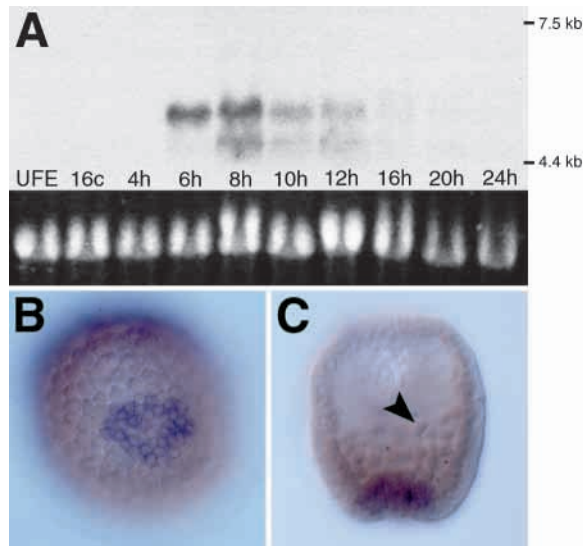

Fig. 3. *LvDelta* is activated zygotically and is expressed transiently during the blastula and early gastrula stages. (A) Developmental northern blot. Total RNA (10 μg) from each of the stages indicated was hybridized with DIG-labelled LvDelta RNA probe. Ethidium bromide staining of the 18S rRNA bands served as a loading control. Molecular mass markers (kb) are indicated. A large transcript (5.3 kb) begins to accumulate by 6 hours after fertilization (seventh cleavage). A second, smaller transcript (4.8 kb) accumulates by 8 hours after fertilization. Both transcripts decrease in abundance after 8 hours until they are barely detectable by 16 hours (gastrula stage). UFE, unfertilized embryo. (B,C) In situ hybridization. (B) At the late blastula stage (8 hours), LvDelta is expressed in a ring of cells at the vegetal pole. (C) At the late mesenchyme blastula/early gastrula stage (12 hours), LvDelta is expressed by macromere derivatives in the vegetal plate, but not by PMCs (large micromere derivatives; arrowhead).

the large and small micromeres are the daughters of the micromeres from the 16-cell stage). At the mesenchyme blastula stage to early gastrula stage (10-12 hours after fertilization), LvDelta transcripts were no longer detectable with this assay in the micromere derivatives, which had ingressed to form primary mesenchyme cells. Transcripts were detectable, however, in the central region of the vegetal plate (Fig. 3C), which is composed mostly of macromere descendants and gives rise to the non-skeletogenic mesoderm (Ruffins and Ettensohn, 1996). Consistent with the northern blot analysis, LvDelta transcripts were not detectable in 16 hour (midgastrula stage) embryos or at later stages (not shown).

The finding that micromere derivatives express LvDelta at the blastula stage is consistent with the hypothesis that LvDelta is the micromere-derived signal that activates the Notch signaling pathway and induces mesoderm. In addition, the expression of LvDelta by macromere derivatives at later stages raises the possibility that the protein has other developmental roles.

Perturbation of LvDelta function affects mesoderm development

To determine whether LvDelta is necessary for mesoderm development, an antisense morpholino oligonucleotide designed to interfere with endogenous LvDelta translation was injected into fertilized eggs. Effects on mesoderm development were assessed by morphology and by staining embryos with a collection of mesoderm-specific antibodies. Mesenchyme blastula-stage embryos were stained with SMC1, a marker for prospective SMCs (Sweet et al., 1999) and pluteus larvae were examined for the differentiation of pigment cells, blastocoelar cells (SMC2 staining) and muscle fibers (anti-myosin staining). To control for the possibility of non-specific effects of the LvDelta morpholino, embryos were injected with the same concentration of an unrelated morpholino designed to block SpAristaless function.

During gastrulation, control embryos produce SMCs at the tip of the archenteron (Fig. 4A). Following the injection of LvDelta morpholino, however, archenteron formation was delayed and gastrula-stage embryos exhibited a very smooth archenteron with few SMCs apparent at the tip (Fig. 4B). In normal late blastula-stage embryos, SMC1 stains prospective mesoderm cells in the vegetal plate (Fig. 4D) (Sweet et al.,
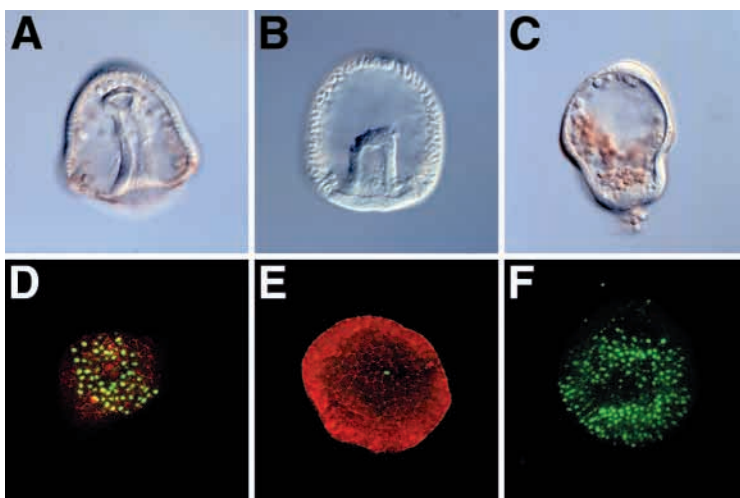
Fig. 4. Mesoderm development following manipulation of LvDelta levels. (A-C) Gastrulation morphology in living embryos; (D-F) Immunostaining of blastula stage embryos with SMC1 (green), a marker for non-skeletogenic mesoderm, and CAD-1 (red), which labels cell boundaries. (A,D) In control embryos, SMCs ingress from the archenteron during gastrulation. SMC1-positive cells are evident in the central region of the vegetal plate at the mesenchyme blastula stage. (B,E) Following injection of LvDelta morpholino, gastrulation is delayed and few SMCs ingress from the archenteron. SMC1 immunostaining shows that there are few prospective mesodermal cells in the vegetal plate (one SMC1-positive cell is visible in this embryo). (C,F) Following injection of LvDelta mRNA, the archenteron produces excessive numbers of SMCs and the vegetal plate contains greatly increased numbers of SMC1-positive cells.
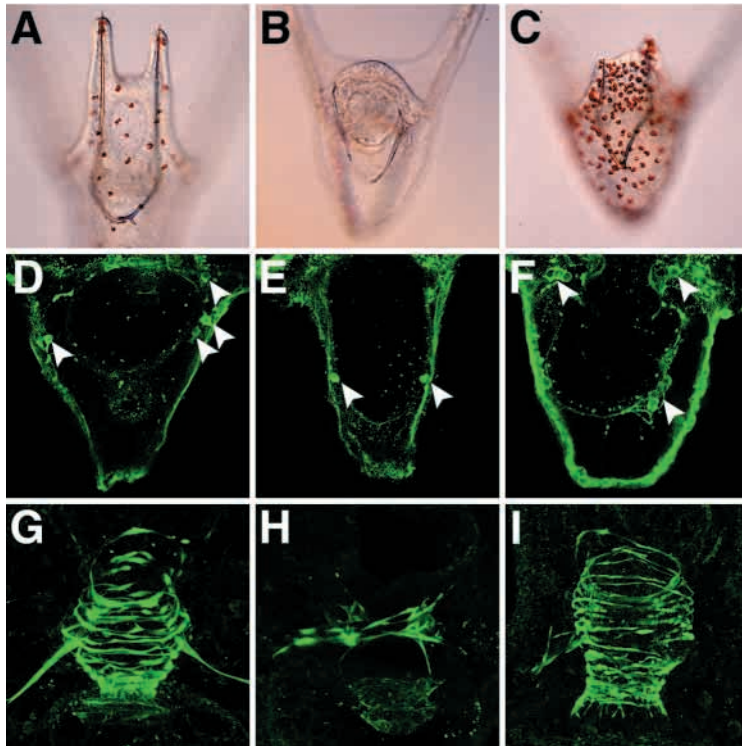
Fig. 5. Later differentiation of mesodermal cells following manipulation of LvDelta levels. (A-C) Pigment cells; (D-F) SMC2 staining (blastocoelar cells are indicated by arrowheads; there is background staining in the ectoderm and endoderm, showing the outline of the larva and the midgut, respectively); (G-I) anti-myosin staining showing muscle fibers. (A,D,G) Control pluteus larvae showing normal numbers of pigment cells, blastocoelar cells and muscle fibers. (B,E,H) Following injection of LvDelta morpholino, the resulting larvae have few pigment cells, blastocoelar cells and muscle fibers. (C,F,I) Following injection of LvDelta mRNA, increased numbers of pigment cells, clusters of blastocoelar cells and increased muscle fibers are apparent.

overexpressing a dominant negative form of the LvNotch receptor, based both on morphology and the expression of mesodermal markers (Sherwood and McClay, 1999). This suggests that, like LvNotch, LvDelta plays a critical role in the development of non-skeletogenic mesoderm.

To provide additional evidence that LvDelta plays a role in mesoderm development, mRNA encoding full-length LvDelta was injected into fertilized eggs. As a control, an equivalent amount of mRNA encoding a truncated form of LvDelta was injected. Overexpression of full-length LvDelta caused excess numbers of SMCs to form from the archenteron during gastrulation (Fig. 4C). These cells were often expelled into the surrounding sea water. Embryos injected with full-length LvDelta mRNA also exhibited a greatly expanded region of SMC1 staining at the mesenchyme blastula stage (Fig. 4F). Overexpression of full-length LvDelta led to the development of large numbers of pigment cells, blastocoelar cells and muscle fibers at later developmental stages, while the control truncated LvDelta mRNA had no significant effect (Fig. 5C,F,I; Table 1). These studies show that overexpression of full-length LvDelta and injection of LvDelta morpholino lead to essentially opposite phenotypes. Moreover, the Delta overexpression phenotype is very similar to that observed following hyperactivation of the Notch signaling pathway through the expression of a constitutive active form of the LvNotch receptor (Sherwood and McClay, 1999).

LvDelta functions differently in micromere and macromere descendants

As LvDelta transcripts are expressed by both micromere and macromere descendants at different times during development, we examined the function of LvDelta in these different cells. Chimeric embryos were generated such that LvDelta function was blocked only in micromere descendants, or only in mesomere and macromere descendants. To block LvDelta function specifically in micromeres, the quartet of micromeres was removed from a normal embryo and replaced with micromeres containing LvDelta morpholino and a lineage tracer (Fig. 6A). The resulting embryos developed few pigment cells and blastocoelar cells compared to sham controls (Fig. 6B; Table 2). The same phenotype is observed following micromere removal (Sweet et al., 1999), supporting the hypothesis that LvDelta is the micromere-derived signal involved in the development of these cell types. Normal levels of muscle fibers developed in these chimeric embryos (Fig. 6C), however,

1999). SMC1-positive cells were rarely seen in embryos containing LvDelta morpholino (Fig. 4E), however, indicating that mesodermal specification had been affected prior to the start of gastrulation. Fig. 5 (A,D,G) shows examples of the numbers of pigment cells, blastocoelar cells and muscle fibers typically observed in control embryos (quantitative data are presented in Table 1). In embryos injected with LvDelta morpholino, few pigment cells, blastocoelar cells or muscle cells developed (Fig. 5B,E,H) (Table 1). Embryos injected with a control morpholino exhibited nearly normal levels of these mesodermal cell types (Table 1). Overall, embryos injected with LvDelta morpholino closely resembled those

Table 1. Development of mesodermal cells following manipulation of LvDelta levels

	Controls	LvDelta morpholino injection	SpAristaless morpholino injection	Full-length LvDelta mRNA injection	Truncated LvDelta mRNA injection
Pigment	80.3±28.3 (90)	6.7±14.3 (107)	93.4±17.9 (28)	133.7±65.3 (75)	83.7±31.4 (31)
Blastocoelar	72.1±17.0 (9)	33.1±24.7 (17)	93.6±16.5 (6)	100.0±31.8 (8)	85.6±9.3 (5)
Muscle fibers	16.3±3.0 (56)	1.7±2.4 (34)	16.7±2.5 (22)	20.9±3.8 (24)	16.0±2.9 (6)

Values are means ± s.d., with total number of embryos examined in parentheses.
Values underlined are significantly different from controls based on a two-sample *t*-test.
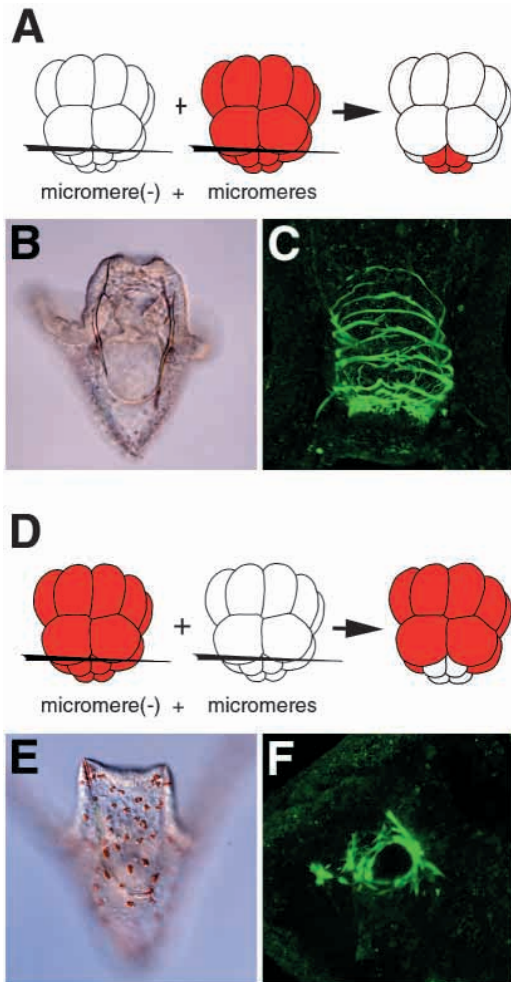
Fig. 6. LvDelta function is required in both micromere and macromere derivatives. Cell transplantation was used to test the effects of blocking LvDelta function in either the micromere descendants or the macromere and mesomere descendants. (A-C) Micromeres containing LvDelta morpholino and a lineage tracer were combined with normal macromeres and mesomeres. (A) Diagram of the experimental design. The resulting embryos have few pigment cells (B), but anti-myosin staining of muscle fibers appears normal (C). (D-F) Macromeres and mesomeres containing LvDelta morpholino and a lineage tracer were combined with normal micromeres. (D) Diagram of the experimental design. The resulting larvae produce many pigment cells (E) but few muscle fibers (F).

Table 2. Mesoderm development following elimination of LvDelta function in micromeres or macromeres and mesomeres

Micro macro+meso	LvDelta ML normal	Normal LvDelta ML	Combined Shams
Pigment	1.3±3.5 (15)	53.3±47.2 (15)	66.3±37.6 (21)
Blastocoelar	38.0±16.4 (4)	46.3±42.3 (6)	83.4±19.2 (10)
Muscle fiber	7.5±4.9 (2)	2.2±1.5 (5)	9.9±2.5 (9)

Values are means ± s.d., with total number of embryos examined in parentheses.

Values underlined are significantly different from the combined sham controls, based on a two-sample *t*-test.

consistent with other evidence that micromere signaling is not required for muscle development (Sweet et al., 1999).

To test whether LvDelta functions in other cells of the embryo, LvDelta function was blocked specifically in mesomere and macromere derivatives. LvDelta morpholino was injected into fertilized eggs, and at the 16-cell stage the micromeres of these embryos were removed and replaced with normal micromeres (Fig. 6D). Such chimeric embryos developed pigment cells (Fig. 6E), demonstrating that micromere-derived LvDelta is sufficient to induce this cell type and that LvDelta function is not required in the macromere or mesomere territories to respond to the signal that regulates pigment cell specification. These embryos developed reduced numbers of blastocoelar cells and muscle fibers (Fig. 6F; Table 2), however, indicating that LvDelta is required in macromere derivatives to generate these cell types. As controls for the chimera experiments, parallel cell transplantations were carried out using embryos that had been injected with SpAristaless morpholino instead of LvDelta morpholino. Results from the two types of control transplants were combined and used for comparison with the experimental embryos (Table 2).

LvDelta expression is sufficient for induction of mesoderm and endoderm

The experiments described above demonstrate that micromere-derived LvDelta is necessary for the induction of certain types of mesoderm. To test whether LvDelta is sufficient to induce mesoderm, fertilized eggs were injected with full-length LvDelta mRNA and a lineage tracer, and at the 16-cell stage, single mesomeres expressing LvDelta were transplanted to uninjected hosts from which the micromeres had been removed (Fig. 7A). To serve as a control, parallel transplants were performed using mesomeres expressing the truncated form of LvDelta mRNA. We scored the formation of pigment cells in these embryos because this mesodermal cell type is the most dramatically affected by micromere signaling (Sweet et al., 1999).

After transplantation of mesomeres expressing the truncated form of LvDelta, the resulting embryos developed few pigment cells (Fig. 7B; average 15.3, *n*=8). The transplanted mesomere contributed to the midgut, foregut (8/8; Fig. 7C) and coelomic pouches (5/8) of the pluteus larva. This finding demonstrated that the transplanted mesomere, which would normally give rise to ectoderm and possibly hindgut (Logan and McClay, 1997), is induced by neighboring macromeres to generate more vegetal cell types (Hörstadius, 1973).

In the presence of a mesomere expressing the full-length form of LvDelta, embryos developed many pigment cells (Fig. 7D; average 80.0, *n*=13). The transplanted mesomere gave rise to endoderm (9/13) and coelomic pouches, as well as other mesoderm (11/13; Fig. 7E). As the descendants of the transplanted cell generated mesoderm, it is likely that the Notch signaling pathway was activated within the clone of the original transplanted cell. It is clear, however, that the LvDelta-expressing cells also induced host cells to generate mesoderm, including pigment cells, because many mesodermal cells were not labeled with the RITC-dextran lineage tracer (Fig. 7E). These results indicate that LvDelta is sufficient to induce neighboring macromere derivatives to develop mesoderm.

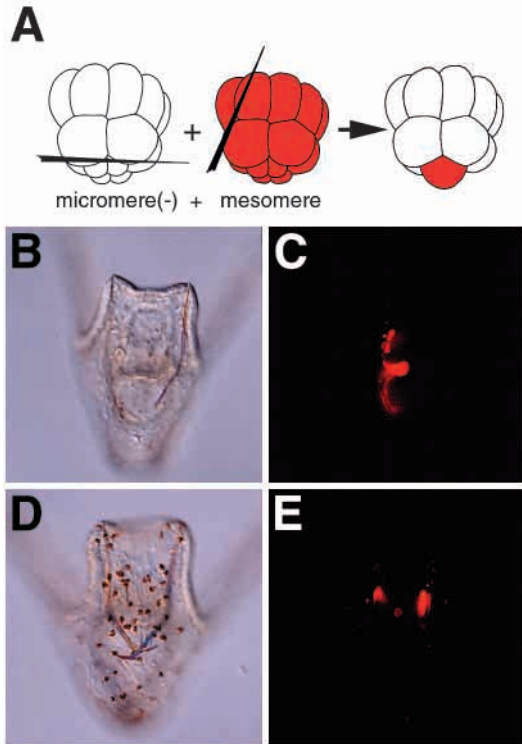To explore further whether LvDelta-mediated signaling

Fig. 7. LvDelta expressed by mesomeres is sufficient to induce pigment cell formation by macromeres. (A) Experimental design. Micromeres were removed from an unlabeled embryo to generate the host. Fertilized eggs were injected with the truncated or full-length form of LvDelta mRNA and one mesomere was isolated at the 16-cell stage and transplanted to the vegetal pole of the host embryo. (B) A micromere(–) embryo recombined with a mesomere expressing truncated LvDelta develops few pigment cells. (C) Epifluorescence image of the larva in B demonstrates that the transplanted mesomere contributes to the foregut and midgut. (D) A micromere(–) embryo recombined with a mesomere expressing full-length LvDelta develops many pigment cells. (E) Epifluorescence of the larva in D shows that the transplanted mesomere contributes to the foregut and mesoderm. Many mesodermal cells do not contain the lineage tracer and were derived from host tissue.

is sufficient for mesoderm induction, single mesomeres containing full-length or truncated LvDelta mRNA were transplanted to animal caps isolated at the 8-cell stage (Fig. 8A). Animal caps combined with a mesomere expressing truncated LvDelta rarely gastrulated (1/12) and usually formed hollow balls of ectoderm (Fig. 8B) in which the transplanted mesomere contributed to the epithelium (Fig. 8C). In contrast, animal caps recombined with a mesomere expressing full-length LvDelta, usually gastrulated (10/15) and frequently developed into miniature plutei (6/15; Fig. 8D). These plutei contained blastocoelar cells (Fig. 8F), skeletogenic cells (Fig. 8G) and pigment cells (average 4.2 cells; $n=15$; not shown). In embryos that gastrulated, the descendants of the transplanted mesomere formed endoderm (9/10) and mesoderm (8/10; Fig. 8E). The finding that some derivatives of the LvDelta-expressing mesomere generate mesoderm suggests that Notch signaling is activated within the clone of the transplanted cell. Endoderm and mesoderm also developed from unlabeled host cells (Fig. 8E), indicating that the descendants of the
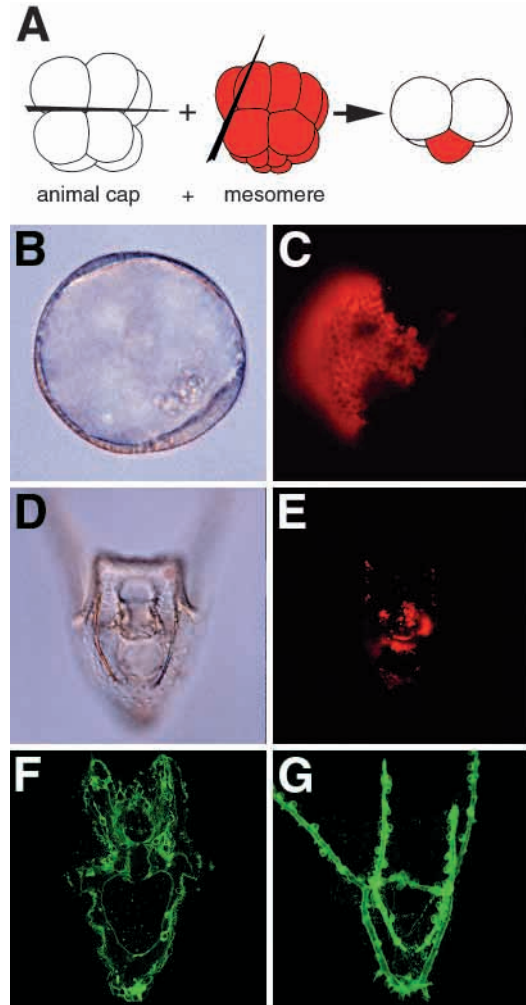
Fig. 8. LvDelta expressed by mesomeres is sufficient to induce animal caps to develop mesoderm and endoderm. (A) Experimental design. To generate the host, an animal cap was isolated at the 8-cell stage. Fertilized eggs were injected with the truncated or full-length form of LvDelta mRNA and one mesomere was isolated at the 16-cell stage and transplanted to the vegetal pole of the animal cap. (B) An animal cap recombined with a mesomere expressing truncated LvDelta develops into a hollow ball of ectoderm. (C) Epifluorescence image of the larva in B demonstrates that the transplanted mesomere contributes to the epithelium. (D) An animal cap recombined with a mesomere expressing full-length LvDelta develops into a pluteus larva. (E) Epifluorescence of the larva in D shows that the transplanted mesomere contributes to the foregut, midgut and mesoderm, though some endoderm and mesoderm is not labeled with the lineage tracer. These larvae develop blastocoelar cells (F) and skeletogenic cells (G).

transplanted LvDelta-expressing mesomere induced host cells to generate these cell types.

These blastomere transplantation experiments clearly show that LvDelta-mediated signaling is sufficient to induce macromeres and mesomeres to develop mesoderm and endoderm. Expression of LvDelta is sufficient to endow blastomeres with properties of an organizer; i.e. the ability to function as a signaling center that can coordinate the development of an animal cap into a pluteus larva.
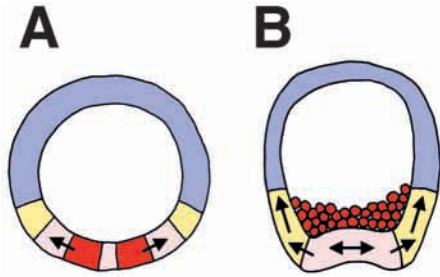
Fig. 9. Model for Delta/Notch signaling in the sea urchin embryo. There appear to be at least three roles for Delta/Notch signaling during early development. (A) Blastula stage (eighth-tenth cleavage stage). Micromere derivatives (red) express LvDelta (arrows), and activate the Notch protein in neighboring cells to promote the specification of pigment cells and blastocoelar cells in the region that will become the non-skeletogenic mesoderm territory (pink). (B) Mesenchyme blastula stage to early gastrula stage. Prospective nonskeletogenic mesoderm (pink) in the vegetal plate expresses LvDelta (double-ended arrow) to promote the development of prospective muscle cells and blastocoelar cells. LvDelta expression by these cells may also activate the Notch protein in neighboring cells (single arrows from pink to yellow), promoting the development of prospective endoderm (yellow). Delta/Notch signaling also results in the expression of a secondary signal (arrows entirely within the yellow region) that promotes endoderm development in more animal cells (Sherwood and McClay, 2001).

DISCUSSION

The LvDelta protein

Delta and Serrate (Jagged) proteins represent the two major known classes of Notch ligands in *Drosophila* and vertebrates. The SpDelta and LvDelta proteins described in this work are clearly members of the Delta family. Serrate family members contain a larger number of EGF repeats (14-16) and include a cysteine-rich region near the transmembrane domain, which is lacking from SpDelta and LvDelta proteins (Lissemore and Starmer, 1999). Vertebrate homologues of Delta typically contain eight EGF repeats, whereas *Drosophila* Delta has nine EGF repeats. LvDelta and SpDelta each contain nine EGF repeats, suggesting that one repeat may have been lost at some point along the chordate evolutionary lineage. This hypothesis could be explored further by determining the number of EGF repeats in the Delta proteins of non-vertebrate chordates (amphioxus and tunicates) and hemichordates. Because a putative Delta/Serrate duplication probably preceded the divergence of protostomes and deuterstomes, it is likely that echinoderms also possess at least one Serrate gene.

The two sea urchin homologues of Delta described here show 100% amino acid identity in the intracellular domain (ICD). Broader phylogenetic comparisons suggest, however, that the ICD is generally one of the least conserved domains of Delta (Lissemore and Starmer, 1999). The functions of the ICD are unclear and may vary among Delta/Serrate/Lag-2 (DSL) proteins (see Henderson et al., 1997). There is considerable evidence that soluble, monomeric Delta protein cannot signal and that clustering of the proteins is required for signaling (Varnum-Finney et al., 2000). In *Xenopus* and *Drosophila*, the ICD is essential for Delta function (Chitnis et al., 1995; Sun and Artavanis-Tsakonas et al., 1996) and it has been proposed that this domain might function to cluster Delta in the cell membrane. It is also possible that the ICD in *Drosophila* Delta is important for the endocytosis of Delta, which appears to be a critical part of the signaling process in this organism (Parks et al., 2000). In *C. elegans*, although membrane association is required for signaling, the ICD is dispensable (Henderson et al., 1997). The unusual degree of conservation seen between the SpDelta and LvDelta ICDs suggests that this domain has an important functional role in the sea urchin.

LvDelta is the micromere-derived signal that activates Notch and induces non-skeletogenic mesoderm

Previous studies have shown that a signal from the micromere descendants activates Notch in the central region of the overlying macromere territory and induces mesoderm (Sweet et al., 1999; McClay et al., 2000). Several studies suggest that the key signaling interaction occurs at the blastula stage, between the eighth and tenth cleavages (Minokawa and Amemiya, 1999; McClay et al., 2000) (H. C. Sweet and C. A. Ettensohn, unpublished observations). We find that micromere derivatives express LvDelta during the blastula stage, at the time when these cells are known to provide a mesoderm-inducing signal. Moreover, our morpholino and mRNA overexpression studies show that LvDelta function is both necessary and sufficient for mesoderm induction. The phenotypes of embryos described in this study are strikingly similar to those observed following expression of dominant-negative and activated LvNotch constructs (Sherwood and McClay, 1999). The effect of blocking LvDelta function in the micromeres closely resembles the effect of removing the micromeres, in that there is a reduction in the number of pigment cells and blastocoelar cells but little or no effect on the development of muscle cells. Taken together, our findings strongly support the hypothesis that LvDelta is the micromere-derived signal that activates the Notch signaling pathway and results in the development of non-skeletogenic mesoderm.

LvDelta and LvNotch play an additional role in micromere-independent mesoderm development

Following the removal of the micromeres, some mesoderm (mostly blastocoelar cells and muscle cells) develops. This suggests that there is a micromere-independent pathway leading to mesoderm development. The micromere-independent mesoderm pathway probably involves Notch signaling because the elimination of Notch function essentially eliminates all mesoderm development (Sherwood and McClay, 1999). We propose that the micromere-independent pathway also involves LvDelta. LvDelta transcripts are found in macromere derivatives within the vegetal plate domain of the mesenchyme blastula-stage embryo. More significantly, elimination of LvDelta function in macromeres results in a severe effect on blastocoelar cell and muscle cell development, a result similar to that observed following the elimination of Notch function throughout the embryo (Sherwood and McClay, 1999). This evidence supports the idea that Delta/Notch signaling occurs among macromere descendants in the vegetal plate, mediating the specification of blastocoelar and muscle cells.

Delta/Notch signaling and endoderm development

Although Delta/Notch signaling clearly plays a critical role in mesoderm induction, the role of this pathway in endoderm development is less clear. Activation of the Notch pathway in animal blastomeres is sufficient to induce ectopic endoderm and Notch signaling normally plays a role in positioning the ectoderm-endoderm boundary (Sherwood and McClay, 2001) (see below). Notch signaling does not appear to be necessary for endoderm specification, however (Sherwood and McClay, 1999). We report similar findings; expression of LvDelta by mesomeres is sufficient to induce animal cells to form endoderm, but suppression of LvDelta function throughout the embryo does not block endoderm development. It is not known whether endoderm specification in such embryos occurs by a normal mechanism or by an alternative pathway. Further analysis of the timing and pattern of expression of various endodermal gene markers should elucidate that point. The present results suggest, however, that the normal role of Delta/Notch signaling in the vegetal blastomeres may be limited to establishing the mesodermal domain within the vegetal plate and, possibly as a consequence, shifting the position of the prospective endoderm toward the animal pole.

It remains possible that the experimental methods used to block Delta/Notch function are not completely effective and that low levels of Delta/Notch function are sufficient to mediate endoderm specification. The normal expression patterns of LvDelta and LvNotch are consistent with the possibility that Delta/Notch signaling might be involved in normal endoderm development. At the mesenchyme blastula stage, vegetal plate cells express LvDelta and cells surrounding the vegetal plate express apical LvNotch (Sherwood and McClay, 1997). It is unclear whether the Delta morpholino and Notch dominant negative construct are effective at this relatively late stage, although our chimeric embryo experiments argue that Delta function in the macromeres at later stages is suppressed by the morpholino, as assayed by effects on muscle cell development.

Delta/Notch signaling in animal blastomeres

In a series of classic experiments in experimental embryology, Hörstadius demonstrated the organizing ability of the micromeres by transplanting the micromeres to the animal pole of the embryo and demonstrating the induction of a secondary archenteron and skeletal organizing centers (reviewed by Hörstadius, 1973). Hörstadius also transplanted micromeres to animal caps and found that micromeres can induce these cells, which would normally make only ectoderm, to form a complete pluteus larva. These results have been repeated and extended by many others (e.g. Khaner and Wilt, 1991; Ransick and Davidson, 1993; Amemiya, 1996; Sweet et al., 1999). Here we report for the first time that the expression of one signaling molecule can endow sea urchin blastomeres with powerful, organizer-like properties. We cannot exclude the possibility that there are multiple signaling molecules produced by micromeres, but signaling by LvDelta alone is clearly sufficient to entrain the repatterning of animal blastomeres to give rise to an organized pluteus.

The mechanism by which LvDelta-expressing cells mediate their organizing influence remains to be determined. One possibility is that the LvDelta-expressing cells act like micromere derivatives, inducing neighboring host cells (as well as some cells derived from the transplanted mesomere) to form pigment and blastocoelar cells, and entraining a normal cascade of signaling interactions that patterns the animal tissue. There is evidence that activation of Notch signaling shifts the endoderm/ectoderm boundary toward the animal pole through the production of a secondary signal (Sherwood and McClay, 2001). Sherwood and McClay found no evidence, however, of the activation of a secondary, non-autonomous signal when Notch signaling was activated specifically in animal cells. These two observations suggest that Notch signaling might act differently in animal and vegetal cells. It will be important to compare the effects of manipulating of Delta/Notch signaling on the expression of downstream regulatory molecules (Brachyury, Wnt-8, etc.) in vegetal cells and animal cells.

Patterning via Delta/Notch signaling: an overview

Delta/Notch signaling appears to be involved in at least three signaling events in the sea urchin embryo (Fig. 9). The first signaling event takes place during the blastula stage (eighth through tenth cleavage divisions), as LvDelta is expressed in the large micromere territory and activates the LvNotch receptor in neighboring macromere descendants (Fig. 9A). This results in the activation of a mesodermal developmental pathway specifically promoting the development of pigment cells and blastocoelar cells. In the second signaling event, LvDelta is expressed by macromere derivatives in the vegetal plate at the mesenchyme blastula and early gastrula stages (Fig. 9B; double arrow). We speculate that this second phase of Delta expression is independent of micromere signaling, although we have not yet tested this directly. Expression of LvDelta again activates the Notch signaling pathway within the macromere territory but now, perhaps as a result of changes in cell competence, stimulates a developmental pathway that specifies muscle cells as well as blastocoelar cells. As LvNotch is downregulated in the vegetal plate at this stage (Sherwood and McClay, 1997), it is possible that the second inductive event occurs only in cells at the periphery of the vegetal plate that still express LvNotch protein. During the third event, which may occur at the same time as the second, cells in the vegetal plate express LvDelta and activate LvNotch in adjacent cells (Fig. 9B; single arrows). This recruits cells at the periphery of the vegetal plate into the endoderm and shifts the position of the endoderm/ectoderm boundary toward the animal pole, possibly via the production of a secondary signaling molecule such as Wnt-8 (see Sherwood and McClay, 2001). To gain additional evidence either for or against this framework of a model it will be necessary to develop means of detecting and manipulating Delta/Notch signaling within the vegetal plate with even greater spatial and temporal resolution.

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