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GSK3β/shaggy mediates patterning along the animal-vegetal axis of the sea urchin embryo

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SUMMARY

In the sea urchin embryo, the animal-vegetal axis is defined before fertilization and different embryonic territories are established along this axis by mechanisms which are largely unknown. Significantly, the boundaries of these territories can be shifted by treatment with various reagents including zinc and lithium. We have isolated and characterized a sea urchin homolog of GSK3β/shaggy, a lithium-sensitive kinase which is a component of the Wnt pathway and known to be involved in axial patterning in other embryos including *Xenopus*. The effects of overexpressing the normal and mutant forms of GSK3β derived either from sea urchin or *Xenopus* were analyzed by observation of the morphology of 48 hour embryos (pluteus stage) and by monitoring spatial expression of the hatching enzyme (*HE*) gene, a very early gene whose expression is restricted to an animal domain with a sharp border roughly coinciding with the future ectoderm / endoderm boundary. Inactive forms of GSK3β predicted to have a dominant-negative activity, vegetalized the embryo and decreased the size of the *HE* expression domain, apparently by shifting the boundary towards the animal pole. These effects are similar to, but even stronger than, those of lithium. Conversely, overexpression of wild-type GSK3β animalized the embryo and caused the *HE* domain to enlarge towards the vegetal pole. Unlike zinc treatment, GSK3β overexpression thus appeared to provoke a true animalization, through extension of the presumptive ectoderm territory.

These results indicate that in sea urchin embryos the level of GSKβ activity controls the position of the boundary between the presumptive ectoderm and endoderm territories and thus, the relative extent of these tissue layers in late embryos. GSK3β and probably other downstream components of the Wnt pathway thus mediate patterning both along the primary AV axis of the sea urchin embryo and along the dorsal-ventral axis in *Xenopus*, suggesting a conserved basis for axial patterning between invertebrate and vertebrate in deuterostomes.

Key words: Embryonic axis, Wnt, GSK3β, Sea urchin

INTRODUCTION

In the sea urchin embryo, the animal-vegetal (AV) axis is established before fertilization. Early blastomeres are formed through a cleavage pattern which is invariable and oriented with respect to the AV axis. At the 60-cell stage the embryo consists of a stack of horizontal cell tiers which have different capacities to differentiate, and at the blastula stage, the fate map defines several presumptive territories whose limits are perpendicular to the AV axis. Just before gastrulation, the mesoderm forms by migration of the progeny of micromeres from the vegetal pole into the blastocoel and at gastrulation, presumptive endoderm cells invaginate from the vegetal pole. Meridional halves obtained by bisection of eggs or early embryos along the AV axis give rise to half-sized but nearly normal embryos. In contrast, animal and vegetal halves obtained by cutting unfertilized eggs or early embryos along the equatorial plane develop with widely different phenotypes: animal halves develop into permanent blastulae with extended apical tufts and no endodermal structures, while vegetal halves can form almost complete embryos. Developmental potential and cell fate are thus dependent on position along the AV axis.

Cell specification along the AV axis involves both autonomous processes and cell interaction (Hörstadius, 1973; Davidson, 1986, 1989). While isolated micromeres can differentiate fully under appropriate conditions (Okasaki, 1975) suggesting that they are specified autonomously by inheritance of maternal factors localized at the vegetal pole, conditional cell specification of other blastomeres have been demonstrated by blastomere recombination, grafting and deletion experiments (Hörstadius, 1973; Ransick and Davidson, 1993, 1995). Micromeres grafted at ectopic positions can induce formation of secondary archenterons whereas their deletion from the vegetal pole impairs or delays differentiation of the overlying cells to form the vegetal plate. The relative contribution of autonomous processes and cell interactions to the specification and patterning of the AV axis remains to be established, and neither the determinants underlying the maternal primordial pattern, nor the signaling pathways which mediate inductive events have been identified.

Patterning along the AV axis can be modified by chemical agents. Animalizing agents, such as zinc, cause embryos to develop into hyperciliated blastulae which resemble those derived from isolated animal hemispheres. Vegetalizing agents, such as lithium, produce exogastrulated larvae in which endoderm and derivatives, which normally arise from vegetal lineages, are greatly expanded and the ectodermal territory is reduced (Hörstadius, 1973; Nocente-McGrath et al., 1991). Since the original description of its dramatic effect on sea urchin development (Herbst, 1892) lithium has been shown to interfere with the development of many diverse organisms, including *Xenopus*, *Dictyostelium*, *Hydra* and zebrafish (Kao et al., 1986; Peters et al., 1989; Hassel et al., 1993; Stachel et al., 1993). Lithium is an inhibitor of inositol phosphate monophosphatases (Hallcher and Sherman, 1980) and so blocks the IP cycle, which may affect IP signaling by depleting the pool of inositol (Berridge et al., 1989). Lithium has been also shown to inhibit the GSK3β/shaggy intracellular kinase, an inhibitory component of the Wnt pathway, both in vitro (Klein and Melton, 1996), and in vivo (Stambolic et al., 1996; Hedgepeth et al., 1997) accounting for the remarkable similarities between the action of lithium and the effects of ectopic activation of the Wnt signaling pathway in *Xenopus* embryos. Lithium is now generally presumed to activate the Wnt pathway by counteracting the inhibitory effect of the GSK3β kinase on downstream components.

The Wnt pathway has been implicated in multiple developmental processes (Perrimon, 1994; Moon et al., 1997). In *Xenopus*, the Wnt pathway plays a crucial role in the definition of embryonic axes: misexpression of Wnt molecules or elements of their transduction pathway can provoke duplication of dorsal-axial structures or prevent formation of the endogeneous D-V axis (for review see Moon et al., 1997). Alterations induced by ectopic expression of normal and mutant GSK3β (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995, 1996) are consistent with GSK3β being the target for lithium.

In the sea urchin embryo, alteration of animal-vegetal patterning by lithium treatment, along with reports that β-catenin, an effector of the Wnt pathway, has a nuclear localization in the vegetal-most blastomeres of early cleavage stages (cited in Miller and Moon, 1996 and Logan and McClay, 1997) suggest that the Wnt pathway may be involved in the establishment of early embryonic territories. To understand patterning along the AV axis and the effect of lithium in sea urchin we focused our attention on GSK3β expression and function in early embryos.

MATERIALS AND METHODS

Embryos

Paracentrotus lividus adults were collected in the bay of Villefranche. Spawning, fertilization, and embryo cultures have been described previously (Lepage and Gache, 1990).

Cloning SU-GSK3 cDNAs

A cDNA fragment derived from the sea urchin GSK3 was cloned by RT-PCR. Total RNA was prepared from unfertilized eggs and 64-cell stage embryos as described below. cDNAs were synthesized using the mouse Moloney leukemia virus polymerase and oligo dT(15) as primer. Degenerate oligonucleotides corresponding to conserved regions of GSK3β were designed using alignment of published sequences from vertebrates and *Drosophila*. The 5′ and 3′ oligonucleotide primers were GTIGCIAT(ACT)AA(AG)AA-(AG)GTI(CT)TICA(AG)GA(CT) and (CT)TT(AG)(AT)(AG) (TC)-TCI(AG)T(AG)TA(AG)TTIGG(GA)TTCAT. Conditions for amplification with the Taq polymerase were: 95°C for 3 minutes; 40 cycles at 95°C for 1 minute, 45°C for 1 minute and 63°C for 3 minutes; 63°C for 10 minutes. A single fragment of the expected length (about 600 bp) was amplified with both egg and embryo RNA, and cloned into Bluescript (plasmids pG600). The 2 fragments were sequenced and were found to be identical and highly similar to the expected GSK3 partial sequences. The PCR fragment was excised, labeled by random priming and used as probe to screen about 5×10^5 recombinants from an amplified 64-cell-stage cDNA library (gift from M. Dibernardo, Palermo). Four different clones designated SU-G3, SU-G6, SU-G8 and SU-G9 were isolated and mapped. SU-G3 was entirely sequenced on both strands. SU-G8 was entirely sequenced on one strand. SU-G9 was partially sequenced on one strand, from the 5′ end to about 1 kb downstream of the stop codon, and over the end of the 3′ UTR (about 1 kb).

Southern and northern analysis

High molecular mass genomic DNA was extracted from sperm obtained from single individuals (Ghiglione et al., 1994). DNA was digested to completion with restriction enzymes and loaded at 5 μg per lane on 1% agarose gels.

Total RNA was prepared from embryos collected at the desired stages by low-speed centrifugation (Lepage and Gache, 1990), washed twice with MFSW and frozen in liquid nitrogen. RNA was loaded at 10 μg per lane on formaldehyde agarose gels.

Southern and northern blots at high stringency were carried out by standard methods (Sambrook et al., 1989). Probe G600 was the 633 bp fragment obtained by RT-PCR which corresponds to positions 571-1203 of the cDNA cloned as SU-G3. Three probes were derived by PCR from the cDNA SU-G3: G389 (429 bp, positions 1069-1497); G38 (395 bp, positions 4-398); and G39 (341 bp, positions 1524-1864). Probe G8 was a 320 bp fragment from position 423-472 in SU-GS8 and probe G9 a 798 bp fragment from position 1-798 in SU-GS9, plus a few nucleotides from the Bluescript polylinker in 5′. Fragments were ^{32}P-labeled by random priming.

Plasmid constructions

Sequences to be expressed were subcloned into the CS2+ expression vector (Turner and Weintraub, 1994). A fragment from plasmid SU-G3 containing 3 base pairs upstream of the ATG, the ATG, the entire GSK3 coding sequence and the stop codon was amplified by PCR with the Pfu polymerase (Stratagene). The 5′ and 3′ primers which contain a *Bam*HI and *Cla*I restriction site were CATTGGATCCAGCATGAGTGGAAGTGGAAGGCCAAGGACG and CTCAATCGATTCACTTGCTACTGGTTCCTCCGCTCGC. The amplified fragment was purified, digested by *Bam*HI and *Cla*I and cloned into vector CS2+ to give plasmid SU-G31.

A catalytically inactive form of SU-GSK3 was obtained by mutation of a conserved lysine residue (Pierce and Kimelman, 1995) which is indispensable for enzyme activity. A single nucleotide change at position 581 (in SU-G3) was introduced to convert K85 to an arginine residue. A large part of the coding sequence, from a *Bst*X1 site located a few bp upstream of the position to be mutated, to a *Bst*X1 site in the CS2+ polylinker, was amplified by PCR, using as 5′ and 3′ primers GGTTGATTCCAGTGATCTGGTCGCAATAAGG-AAAGTATTACAAGACAAGAGATTTAAGAATCG and CCGGG-CCCAATGCATTGGCGCCGC. The 5′ primer comprises the 5′ *Bst*X1 site and the mutated position. The amplified fragment was digested by *Bst*X1 and inserted into plasmid SU-G31 deleted from the wild-type fragment to give plasmid SU-G32. All constructs were verified by sequencing.

Constructs harboring various forms of the *Xenopus* GSK3β were generous gifts from D. Kimelman (University of Washington): wild-

type (GSK3β, plasmid XG73), kinase-dead (GSK3β K→R, plasmid XG114) and interrupted (frame-shift, plasmid XG92) (Pierce and Kimelman, 1995).

In vitro transcription and microinjection

All CS2+ plasmid constructs used for in vitro transcription were linearized with *Not*I. Capped RNAs were synthesized with the SP6 polymerase using the mRNA mMachine kit from Ambion and purified with a Microcon 50 (Amicon). RNAs were analyzed and quantified by gel electrophoresis and spectrophotometry. Before injection, RNAs were diluted to 0.2 to 2 μg/μl in DEPC-treated H_2O containing 10% glycerol.

Serial microinjection was based on the method designed by McMahon et al. (1985) for plasmid injection. About 2 pl of mRNA was injected into the cytoplasm of each unfertilized egg. For each experiment, 200 eggs (for morphological observations) or 400 eggs (for in situ hybridization or immunolabeling) were microinjected and the experiments were repeated 2-3 times. Embryos were washed and raised in MFSW containing 50 μg/ml penicillin and 50 μg/ml streptomycin. Embryos were collected at early blastula stages and either treated for fixation and labeling or transferred to small wells and raised in 1.5 ml MFSW containing antibiotics until control embryos reached the pluteus stage (48 hours). Morphology and labeling were monitored on a Zeiss Axiophot microscope. As controls, we used RNA coding for β-galactosidase or the *Xenopus* GSK3β RNA in which a frame-shift mutation interrupts the coding sequence (plasmid XG92).

In situ hybridization

Whole-mount in situ hybridization was carried out following a procedure adapted from Harland (1991). Single-strand RNA probes labeled with digoxigenin-UTP were synthesized as described (Lepage et al., 1992b). Sense and anti-sense probes used to detect *HE* transcripts were derived from plasmid 6p6.1 (Lepage and Gache, 1990) and those used to detect GSK3β transcripts were synthesized from plasmid pG600.

Immunolabelling

The hatching enzyme was labeled in whole-mount embryos using an affinity-purified anti-HE polyclonal antibody and an anti-rabbit Ig phosphatase-conjugated secondary antibody as described previously (Lepage et al., 1992a).

RESULTS

To investigate the role of GSK3β during early development of the sea urchin we followed an approach similar to that used for *Xenopus* GSK3β (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995, 1996). We first cloned the sea urchin GSK3β homolog and characterized its expression in the embryo. The cloned sea urchin GSK3β was mutated to produce a catalytically inactive form similar to the *Xenopus* kinase-dead GSK3β which has been shown to behave as a dominant-negative in *Xenopus* embryos (Pierce and Kimelman, 1995). Normal and mutated GSK3β from sea urchin (SU-GSK3β) and *Xenopus* (X-GSK3β) were produced from mRNAs synthesized in vitro and microinjected into eggs just before fertilization. The effects of overexpressing the normal and mutant GSK3β from both species could then be analyzed at the morphological and molecular level.

Cloning and characterization of sea urchin GSK3β cDNAs

cDNAs encoding a conserved domain of GSK3 were cloned from sea urchin embryo by PCR-based methods. By using long degenerate oligonucleotide primers, we amplified a 600 bp fragment derived from GSK transcripts and we used this fragment as probe to screen a cDNA library from early embryonic stages. Three of the 4 clones isolated, SU-G3, SU-G8 and SU-G9, which appeared to have a complete or almost complete ORF were further characterized. The clones differ in size (SU-G3 and SU-G9 are about 6.5 kb long and SU-G8 is about 2.2 kb long) and their 5′ and 3′ ends. Their structure is shown in Fig. 1.

The 3 cDNAs have different 5′UTR, with some overlap between SU-G3 and SU-G9. As a consequence, the beginning of the ORFs are different over a short length (40-140 bp).

The short divergent sequences opening the ORFs are followed by a 1082 bp sequence which is almost identical in all clones. The very few base substitutions (<0.4%) are silent, giving strict identity at the amino acid level. This is well below the rate of polymorphic differences usually observed, even for different isolates from the same cDNA. The beginning of the common part of the ORF nearly coincides with the beginning of exon 3 (see Fig. 3B), an exon common to all transcripts of the *shaggy* gene in *Drosophila* (Ruel et al., 1993).

The 3′UTR of SU-G3 and SU-G9 are approximately the same length (about 4.5 kb), and are very similar, as judged by partial sequencing. SU-G8 ends abruptly with a series of A immediately adjacent to the coding sequence. This cDNA is much shorter than the transcript from which it derives (see below) and thus was produced through a cloning artefact or by copy of a mRNA containing interspersed polyA sequences (Davidson, 1986).

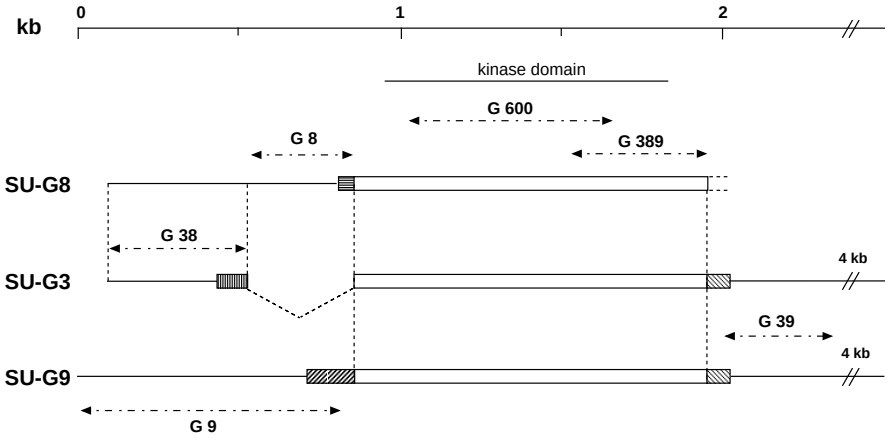

Fig. 1. Map of the cloned sea urchin GSK3β cDNAs. SU-G3 and SU-G9 are about 6.5 kb long. SU-G8 (2.2 kb) lacks the long 3′ UTR present in SU-G3 and SU-G9. The 5′UTR and the beginning of the coding sequence of SU-G9 are unique. The 5′ UTR of SU-G3 and SU-G8 start 2 nucleotide apart and are identical except for a 329 bp sequence which is absent in SU-G3 such that the beginning of the ORFs also differ. Untranslated regions are shown as thin lines, ORFs as boxes. The region of the ORF common to the 3 cDNAs is in white. Vertical dotted lines join extremities of identical regions. Double-headed horizontal arrows indicate positions from which probes were derived. Scale is in kilobase.

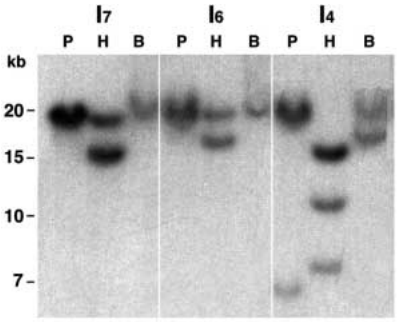

Fig. 2. Southern blot analysis of GSK3β genomic sequences. I7, I6 and I4 refer to 3 male individuals. (P), *Pst*I; (H), *Hin*dIII; (B), *Bam*HI. The probe used was the 429 bp fragment G389 amplified from the 3′ end of the coding sequence (see Fig. 1).

To understand the origin of these transcripts, we estimated the copy number of the GSK3 genes by Southern blot analysis. As the sea urchin genome displays a high polymorphism (Britten et al., 1978), genomic DNA was obtained from several individuals and digested with different restriction enzymes. The probe taken from the common coding region (probe 389, see Fig. 1) should identify all genes from which the cDNAs derive and thus should produce a complex pattern if several genes were involved. The simple patterns observed in Fig. 2 are consistent with a single copy gene displaying few restriction polymorphic forms.

Taken together these observations suggest that the 3 transcripts characterized derive from a single copy gene by alternative splicing and produce 3 different proteins. In *Drosophila*, the *shaggy* locus has been shown to give rise to 10 different transcripts and 5 different proteins (Siegfried et al., 1992; Ruel et al., 1993).

Sea urchin GSK3β

The 3 transcripts encode different proteins which are identical for about 90% of their length but differ by the length and sequence of their short N-terminal region. The N-terminal sequence of the SU-G3 protein is most similar to GSK3β from other organisms (Fig. 3A). The SU-G9 protein is longer by 18 amino acids and SU-G8 is shorter by 15 residues. The protein coded for by SU-G3, which will be referred to as SU-GSK3β, is predicted to be 414 amino acids long giving a M_r of 46300. Alignment of the SU-GSK3β sequence with those from vertebrates and *Drosophila* GSK3βs shows a very high conservation among species (Fig. 3A). SU-GSK3β has about 75% identity and 85% similarity with vertebrate GSK3β. In the kinase domain, which covers more than 2/3 of the protein, the sea urchin and human GSK3β share 87% identity and 94% similarity.

GSK3β gene expression during early development

Several probes with different specificities derived from the 3 GSK3β clones were used to follow changes in transcript level during development. Probes G389 and G600 derived from a coding region common to all clones and should detect all GSK3β transcripts, while the other probes corresponded to regions unique to one, or shared by two of the three clones as indicated (Fig. 1). A crude estimate of the transcript prevalences, made by comparing exposure times of gels shown in Fig. 4 and by semi-quantitative measurement of signal intensity of each probes hybridized to egg RNA (not shown), indicated relative prevalences of about 5% for SU-G8 type, 20-25% for SU-G9 type and 75-80% for other types (essentially if not exclusively of the SU-G3 type, since they are identified by both probes G38 and G39).

All probes detect transcripts of about 6.5 kb. This indicates that clones SU-G3 and SU-G9 were nearly full-length cDNAs, while the SU-G8 cDNA was truncated.

GSK3β transcripts are present in the unfertilized egg, and the maternal level is maintained during early cleavage stages. The transcript level then declines rapidly at the blastula stage and remains low at all stages from hatching blastula to pluteus

A

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H  M--SGRPRTTSFAESCKPVQQPSAFGSMKVSRDKDGSKVTTVVATPGQGPDRPQEVSYTDTKVIGNGSFGVVYQAKLCDS   80
X  M--SGRPRTTSFAESCKPVQQPSSFGSMKVSRDKDGSKVTTVVATPGQGPDRQQEVTYTDTKVIGNGSFGVVYQAKLCDT
S  MSGSGRPRTTSFADANRGS--TSSYGGLKVSRDKDSSKITSVTATEGPPPDRTKEISYTDTRVIGNGSFGVVYQARMVDS
D  M--SGRPRTSSFAEGNKQSP-SLVLGGVKTCS-RDGSKITTVVATPGQGTDRVQEVSYTDTKVIGNGSFGVVFQAKLCDT
   *  ******.***.  .        * .* .  .* **.*.* ** *   ** .*..****.**********.**.. *.

H  GELVAIKKVLQDKRFKNRELQIMRKLDHCNIVRLRYFFYSSGEKKDEVYLNLVLDYVPETVYRVARHYSRAKQTLPVIYV  160
X  GELVAIKKVLQDKRFKNRELQIMRKLDHCNIVRLRYFFYSSGEKKDEVYLNLVLDYVPETVYRVARHYSRAKQALPIIYV
S  SDLVAIKKVLQDKRFKNRELQIMRRLDHHNIVKLKYFFYSSGEKKDEVFLNLVLEYVPETVYRVARHYSKAKQTIANLYV
D  GELVAIKKVLQDRRFKNRELQIMRKLEHCNIVKLLYFFYSSGEKRDEVFLNLVLEYIPETVYKVARQYAKTKQTIPINFI
     .**********.***********.*.* ***.* *********.***.*****.*.*****.***.*...**..   ..

H  KLYMYQLFRSLAYIHSFGICHRDIKPQNLLLDPDTAVLKLCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSI  240
X  KLYMYQLFRSLAYIHSFGICHRDIKPQNLLLDPETAVLKLCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSI
S  KLYMYQLFRSLAYIHSMGICHRDIKPQNLLLNPETAVLKLCDFGSAKVLVRGEPNVSYICSRYYRAPELIFGATDYTCDI
D  RLYMYQLFRSLAYIHSLGICHRDIKPQNLLLDPETAVLKLCDFGSAKQLLHGEPNVSYICSRYYRAPELIFGAINYTTKI
   .*************** ************** *.************* *..********************  **. *

H  DVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFPQIKAHPWTKVFRPRTPPEAIALCS  320
X  DVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFPQIKAHPWTKVFRARTPPEAIALCS
S  DVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPSRDQIKEMNPNYTEFKFPQIKPHPWNKVFRARTQPEAIQLCS
D  DVWSAGCILAELLLGQPIFPGDSGVDQLVEVIKVLGTPTREQIREMNPNYTEFKFPQIKSHPWQKVFRIRTPTEAINLVS
   *******.**********************.*******.*.**.*************** *** **** **  *** * *

H  RLLEYTPTARLTPLEACAHSFFDELRDP-NVKHPNGRDTPALFNFTTQELSSNPPLATILIPPHARIQAAAS--TPTNAT  400
X  RLLEYTPTSRLTPLDACAHSFFDELRDP-NLKLPNGREFPALFNFTTQELSSNPSLSSILIPAHARNQAAVS--TTSNTT
S  RLLEYTPKSRIKPLDACAHQFFNELREP-GIKLPNGRELPPLFNFTSSELAIKQSLRTALIPPHISQNSSS-----S--T
D  LLLEYTPSARITPLKACAHPFFDELRMEGNHTLPNGRDMPPLFNFTEHELSIQPSLVPQLLPKHLQNASGPGGNRPSAGG
    ****** .*. ** **** ** ***    .   ****. * *****  **.    *   *.* *    .       .

H  AASDANTGDRGQTNNAASASASNST
X  STSDSNTGERGSTNNAASASASNSS
S  APHDATEPSTSATASSASGGTSSK
D  AASIAASGSASVSSTGSGASVEGSAQPQSQGTAAAAGSGSGGATAGTGGASAGGPGSGNNSSSGGASGAPSAVAAGGANA
   .  .      .  .
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B

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                                                    ▼
SU-G8                                    MAAMHVSHQRRSQHGDKDSSKITSV
                                                    ::::::::::
SU-G3                          MSGSGRPRTTSFADANRGSTSSYGGLKVSRDKDSSKITSV
                                                    ::::::::::
SU-G9    MCTVERSSSGEWSNHRRPVSSAYSKIVMPRISSTKILPPSTSSSKSMRDKDSSKITSV
                                                    .: :::::.:
sgg e3                                              XRDGSKITTV
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Fig. 3. Similarities between GSK3β from sea urchin and other species. (A) Sequences from human (H), *Xenopus* (X), sea urchin (S), and *Drosophila* (D), were aligned using Clustal W. (the *Drosophila* sequence has 38 additional residues at the C-terminal end). Positions with identical (*) or similar (.) residues in the 4 sequences are indicated. The nucleotide sequence of the sea urchin GSK3β cDNA (clone SU-G3) will appear in the EMBL, GenBank and DDJB Sequence Databases under the accession number AJ222641. (B) N-terminal sequences of the proteins coded by the 3 cDNAs isolated. Identity at the nucleotide level begins within the codon marked by an arrowhead. Sequence of the first amino acids from exon 3 of the *Drosophila shaggy* protein is included for comparison.

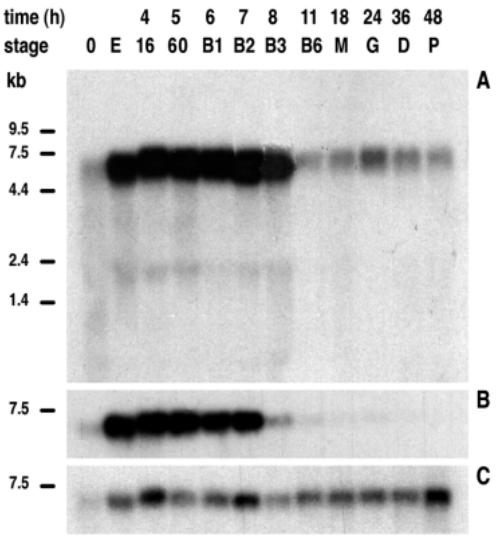

Fig. 4. Expression of SU-GSK3β transcripts during development. Northern blot analysis of total RNA prepared from embryos at the indicated stages. Blots were probed with (A) probe G600 amplified from the kinase domain. Identical results were obtained with probe G389; (B) probe G9, specific for cDNAs of the SU-G9 type; (C) probe G8, specific for cDNAs of the SU-G8 type. Blots were exposed for 5 hours (A), 22 hours (B) and 120 hours (C). O, ovaries; E, unfertilized egg; 16, 16-cell stage; 60, 60-cell stage; B1-B6, blastula stages; M, mesenchyme blastula; G, gastrula; D, prism; P, pluteus.

(Fig. 4A). SU-G3 (which is largely prevalent) and SU-G9 (Fig. 4B) both showed this expression profile, while SU-G8 transcripts were found to be expressed at a very low but almost constant level throughout development (Fig. 4C).

Whole-mount in situ hybridization using probe G600, which detects all types of transcripts, gave strong labeling in unfertilized eggs and early stages (Fig. 5). The signal decreased at late blastula and was faint at the gastrula stage, in agreement with northern blot data. Transcripts did not display any obvious localization in unfertilized eggs and did not become restricted to any particular lineage or area in early embryos. At this level of analysis, GSK3β transcripts appeared to be uniformly distributed within the embryo, at least at all stages before hatching.

Kinase-dead mutants of GSK3β vegetalize the embryo

mRNAs coding for kinase-dead GSK3β were injected into unfertilized eggs at doses varying from about 0.4 pg to 4 pg per egg (4×10^5 to 4×10^6 copies). Doses below 0.4 pg had no visible effect on embryo morphology (Fig. 6A). Around the threshold value of 0.4 pg, most embryos (>95%) were unaffected, but a few had an abnormal pluteus-like shape (Fig. 6B). At about 0.8 pg, injected embryos displayed a range of phenotypes, shown in order of increasing alteration in Fig. 6C-H and I-L. At 2 pg and up to 4 pg, nearly all embryos (>95%) displayed the same extreme phenotype (Fig. 6H,L). The phenotypes were more homogeneous and more extreme with SU-GSK3β than with X-GSK3β, suggesting that in the sea urchin embryo, the mutant form derived from the endogeneous GSK3β transcript may be slightly more efficient than the *Xenopus* one. In both cases, the intermediate phenotypes displayed the same main features. The digestive tract was greatly enlarged and was formed by exogastrulation, reflecting overdevelopment of the endoderm at the expense of the ectoderm. Spicules had abnormal shapes and were strongly reduced in most cases. These are classic features of the vegetalized morphology obtained by treatment with vegetalizing agents such as lithium. The most strongly affected embryos (Fig. 6H,L) consisted of a hollow sphere of thick epithelium, with a small invagination, one or two clusters of pigmented cells and a few other cells within the central cavity. Spicules were totally absent. This phenotype probably represents an almost complete vegetalization, the ectoderm being nearly completely absent. Such extreme phenotypes have never been observed following lithium treatment, probably because high concentrations of lithium are toxic. Except for this extreme form, the series of increasingly abnormal phenotypes closely resembled that obtained with increasing concentrations of lithium. These results suggest that GSK3β is the target for lithium in the sea urchin embryo, with the kinase-dead GSK3β having a dominant-negative effect that mimics lithium inhibition.

Animalization by misexpression of wild-type GSK3β

A series of experiments similar to those described above was carried out with the wild-type GSK3β. Microinjection of about 1 pg per egg of either sea urchin or *Xenopus* GSK3β RNAs produced embryos with a narrow range of altered phenotypes (Fig. 7).

Embryos overexpressing GSK3β did not gastrulate. In a very few cases (<1%), embryos had the overall shape of a gastrula with apparently normal spicules but no archenteron (Fig. 7B). In most cases the morphology was much simpler (Fig. 7C-D). Embryos were spherical, sometimes slightly flattened, and frequently had very long cilia. The epithelium was thickened on one side, indicating morphological polarity. Only single,

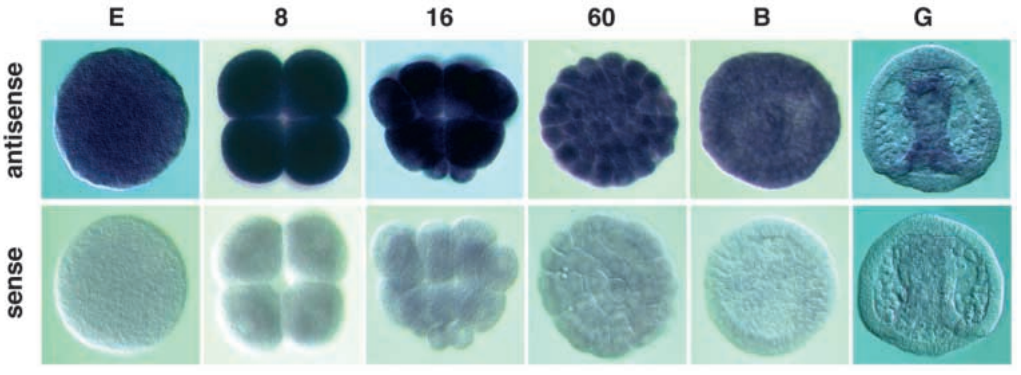

Fig. 5. Distribution of the SU-GSK3β transcripts during development. Whole embryos were hybridized with sense and antisense probe G600. E, unfertilized egg; 8, 8-cell stage; 16, 16-cell stage; 60, 60-cell stage; B, 8-hour blastula stage; G, gastrula.

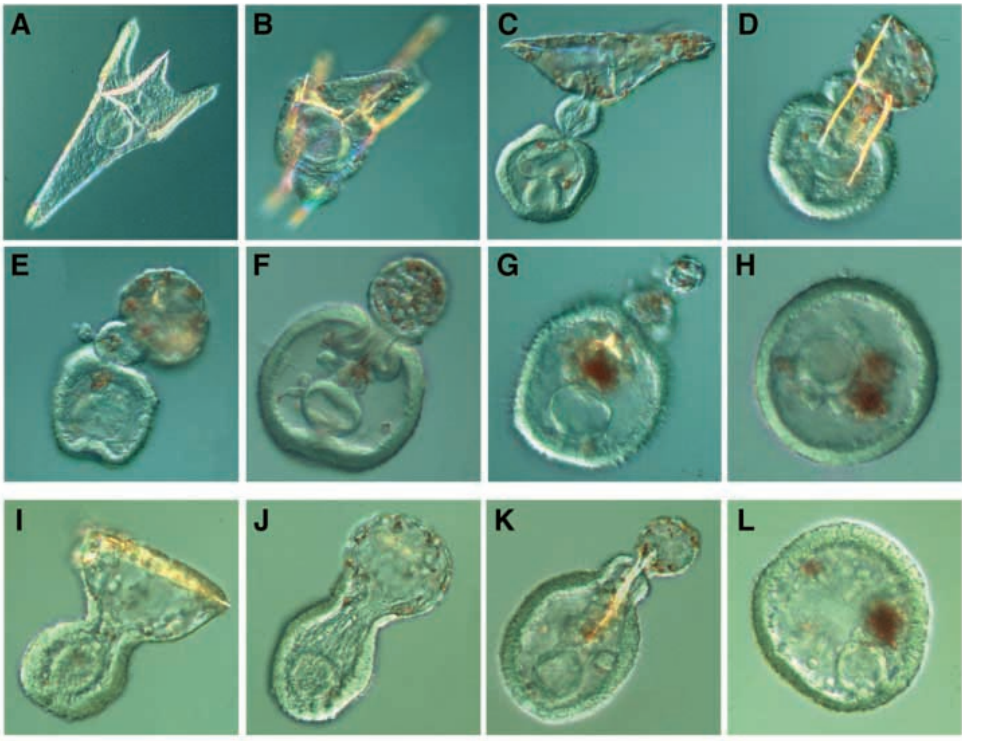

Fig. 6. Morphology of 48 hour embryos expressing kd-GSK3β. (A) Control embryo; (B-H) embryos expressing X-kd-GSK3β; (I-L) embryos expressing SU-kd-GSK3β. In each series, representative embryos from 5 groups of 100 embryos (X-kd-GSK3β) and 2 groups of 100 injected embryos (SU-kd-GSK3β) are shown in order of increasing vegetalized phenotype.

small abnormal spicules or, more often, no spicules were seen. Very few or no cells were present inside the blastocoel. These features characterize the animalized phenotype, which can be obtained by treatment with a variety of chemical agents, including zinc.

Wild-type and mutant forms of GSK3β alter *HE* expression domain

The hatching enzyme gene (*HE*) is the earliest known strictly zygotic gene to be activated following fertilization and it is expressed only transiently during cleavage (Lepage and Gache, 1990). *HE* expression is restricted to an area which corresponds roughly to the presumptive ectoderm (Lepage et al., 1992b). The boundary of the *HE* expression domain, which is perpendicular to the AV axis, is shifted towards the animal pole by treatment with lithium (Ghiglione et al., 1993). In embryos in which the kinase-dead GSK3β or the wild-type GSK3β were misexpressed, monitoring *HE* gene expression thus allowed patterning changes along the AV axis to be analyzed very early, well before the development of any morphological abnormalities.

In embryos injected with kd-GSK3β (*Xenopus* or sea urchin), *HE* expression at the blastula stage was repressed (Fig. 8). At low doses (0.6 pg) the *HE* domain was strongly reduced, being limited to a few cells or to a small group of cells (Fig. 8C,D). The residual domain is usually well delimited but sometimes irregular and faintly labeled. At doses (≥1 pg) which produce a uniform and extreme vegetalization the expression of *HE* was undetectable in most embryos (>98%) or limited to 2-3 cells (Fig. 8E,F). These results are consistent with the vegetalized phenotype of 48-hour embryos expressing kd-GSK3β. Both the observed phenotypes and the reduction of the *HE* expression territory in embryos injected with kd-GSK3β resemble those seen with lithium (Ghiglione et al., 1993), but are even stronger since they can repress *HE* to an undetectable level.

Misexpression of the wild-type GSK also modified *HE* spatial expression but in the opposite direction (Fig. 9). In >98% cases, the *HE* domain was enlarged. In some embryos (20%) an unlabelled area was clearly visible, but was always smaller than the normal non-expressing region (Fig. 9B). In most embryos, no unlabeled area could be seen (Fig. 9C). Because of some irregularities (dividing cells, perturbation at the microinjection site), we cannot exclude that single cells or

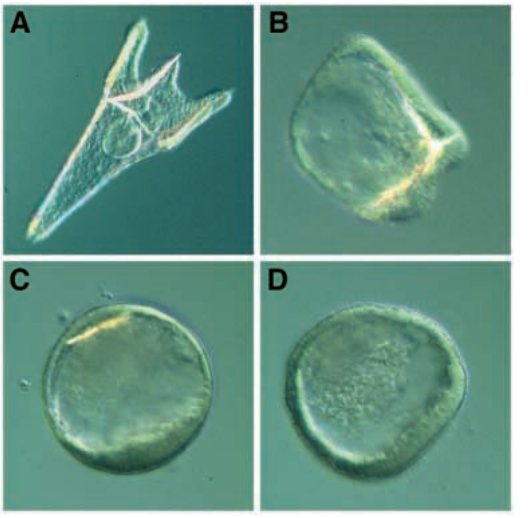

Fig. 7. Morphology of 48 hour embryos expressing wt-GSK3β. (A) Control embryo; (B-D) embryos injected with wt-GSK3β RNA from sea urchin or *Xenopus*. Representative embryos from 2 groups of 200 injected embryos.

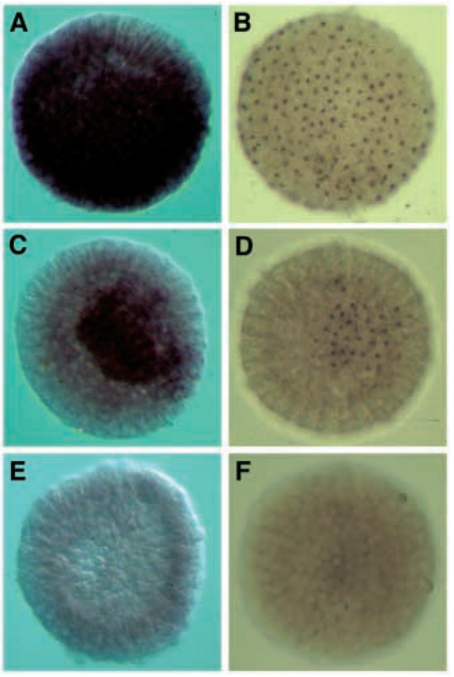

Fig. 8. Expression of the *HE* gene at the blastula stage in embryos overexpressing kd-GSK3β. (A,C,E) Whole-mount in situ hybridization using a probe derived from the *HE* cDNA. (B,D,F) Whole-mount immunolocalization using a polyclonal anti-HE antibody. (A,B) Animal pole views of control embryos; (C-F), injected embryos. Embryos in C and D are probably seen from the animal side. Representative embryos from 5 groups of 400 injected embryos.

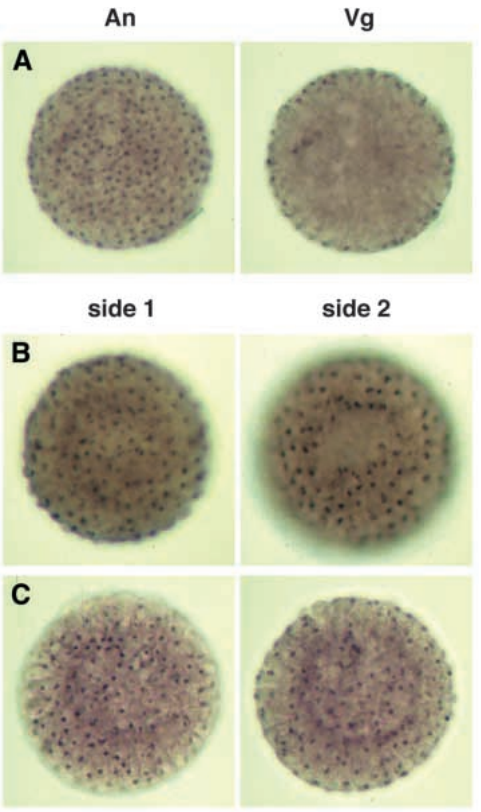

Fig. 9. Expression of the *HE* gene at the blastula stage in embryos overexpressing wt-GSK3β. Immunolocalization of the HE protein as in Fig. 8. Paired views of the 2 sides of each embryo are shown. (A) Control embryo seen from the animal pole (An) and the vegetal pole (Vg). (B,C) Injected embryos seen from 2 opposite sides (side 1 and 2). For the embryo in B, side 2 probably corresponds to the vegetal side since a small patch of cells not expressing *HE* remains. Representative embryos from 3 groups of 400 injected embryos.

very few cells did not express *HE* in embryos overexpressing GSK3β, but the *HE* domain appeared to cover the whole surface of the blastula.

The enlargement in the *HE* expression territory parallels the change in morphology provoked by overexpression of wt-GSK3β, and suggests that wt-GSK3β produces a true animalization, resulting from an extension of the presumptive ectoderm territory.

DISCUSSION

We have described the cloning of cDNAs coding for sea urchin homologs of GSK3β/shaggy proteins. The GSK3β gene expresses maternal transcripts which persist during embryogenesis. These transcripts appear to be uniformly distributed in the egg and in the embryo during cleavage and blastula stages. GSK3β proteins are thus potentially present throughout embryos at all stages.

Normal or mutated GSK3β from RNAs microinjected into the egg were found to perturb the development of the sea urchin embryo along the AV axis (see Fig. 10). Expression of the mutated kd-GSK3β, predicted to have a dominant-negative activity (He et al., 1995; Pierce and Kimelman, 1995), vegetalized the embryo and decreased the size of the *HE* domain. These effects are similar to, but could be more extreme than those obtained by lithium treatment: in embryos expressing kd-GSK3β, the development of the ectoderm could be completely suppressed and the *HE* gene repressed even at the animal pole. Overexpression of active wild-type GSK3β animalized the embryos and caused the expression territory of *HE* to extend to cover all or nearly all cells of the blastula. Zinc treatment also produces larvae with an animalized phenotype, however zinc does not appear to provoke changes in cell fate, but rather to cause a reversible developmental arrest with some features of animalization (Nemer et al., 1985). In agreement with this, we have shown previously that zinc treatment does not affect *HE* spatial expression (Ghiglione et al., 1993). In contrast, the extension of the domain of *HE* expression and the morphological alteration seen following overexpression of GSK3β suggest that the progeny of blastomeres from the vegetal half have been converted to ectoderm. GSK3β thus probably provokes a true animalization by enlarging the ectoderm presumptive territory.

The extension of the *HE* domain seen in animalized embryos is consistent with our analysis of the regulation of this gene showing that it can potentially be expressed in all regions of the embryo but is normally negatively controlled in the vegetal-most area. Transgenes controlled by a long *HE* promoter (3 kb) display correct spatial restriction, while ones with a promoter shortened to about 500 bp are expressed outside the normal *HE*

territory (Ghiglione et al., 1997). Thus, the upstream region probably contains regulatory elements directly or indirectly dependent on the GSK3β pathway.

By manipulating the global level of GSK3β activity in the embryo, we have been able to shift the border of the *HE* domain fully in both directions (towards the animal or vegetal pole) and to generate morphological defects ranging from the most extreme animalized to the most extreme vegetalized phenotypes described classically and beyond. These results suggest that gene expression and cell fate along the AV axis are sensitive to GSK3β activity level. GSK3β actively represses vegetal fate and promotes *HE* expression and very likely some aspects of ectoderm differentiation. β-catenin, a downstream component of the Wnt pathway, also affects axis patterning when misexpressed (F. E. F. unpublished results; Wikramanayake and Klein, personal communication) and has been found to be localized to the nucleus of vegetal blastomeres during cleavage stages (Miller and Moon, 1996; Logan and McClay, 1997). These observations suggest that downstream components of the Wnt pathway participate in the establishment and/or patterning of the AV axis during normal development.

The opposite effects of wt-GSK3β and kd-GSK3β in sea urchin embryos show strong similarities with those reported in *Xenopus* embryos. In *Xenopus*, GSK3β and the Wnt pathway play an early role in the formation of a dorsalizing center and later participate in patterning the mesoderm (Heasman, 1997; Moon et al., 1997). Ectopic expression of GSK3β microinjected into embryos interferes with the first function: kd-GSK3β injected in ventral blastomeres causes formation of a secondary set of dorsal structures while exogenous expression of wt-GSK3β in dorsal blastomeres ventralizes the embryo (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995, 1996) indicating that endogenous GSK3β actively represses dorsal fate. GSK3β acts by controlling the stability of β-catenin. GSK3β is involved in the enrichment of β-catenin in dorsal cytoplasm leading to its accumulation in the nuclei of dorsal blastomeres (Wylie et al., 1996; Schneider et al., 1996; Larabell et al., 1997). How GSK3β is regulated during this early phase is not known. All components of the Wnt pathway, wnts, dsh, APC, β-catenin, TCF/Lef, and dominant-negative GSK3β, can induce formation of ectopic embryonic axes (Heasman, 1997; Moon et al., 1997). GSK3β and downstream components are maternally required: dominant-negative GSK3β expressed in ventral blastomeres induce formation of an ectopic axis, dominant-negative TCF/Lef expressed in dorsal blastomeres ventralizes the embryo, and reduction of the maternal pool of β-catenin with antisense oligonucleotides prevent the endogeneous dorsal axis forming (Heasman et al., 1994; He et al., 1995; Pierce and Kimelman, 1995; Behrens et al., 1996; Molenaar et al. 1996). In contrast, dominant-negative forms of wnts and dsh have no effect, suggesting that components upstream of GSK3β are not required for endogeneous axis formation (Hoppler et al., 1996; Sokol, 1996). In addition, the known maternal wnts have only weak axis inducing activity and are not localized appropriately for a dorsalizing function (Christian et al., 1991; Christian and Moon, 1993). Furthermore, expression of zygotic genes such as *siamois* in dorsal-vegetal cells is autonomous (Lemaire et al., 1995). Thus, it has been suggested that dorsalization might be triggered by an unknown maternal Wnt bound to the surface of the blastomeres, or alternatively by a ligand-independent, cell-autonomous activation of an intracellular component of the pathway (Hoppler et al., 1996; Yost et al., 1996; Fagotto et al., 1997; Leyns et al., 1997).

In the sea urchin embryo, the morphological alterations provoked by manipulation of the GSK3β level reflect essentially an imbalance between the 2 major tissue types, ectoderm and endoderm and thus an incorrect positioning of the boundary between the presumptive ectoderm and endoderm territories along the AV axis. It has been proposed that cell specification along the AV axis occurs essentially between the 16- and 60-cell stage by sequential inductive interactions triggered from the vegetal pole by the micromeres and progressing upward from tier to tier (Davidson, 1989). However, the revised fate map proposed by Logan and McClay (1997) shows that the ectoderm-endoderm boundary does not coincide with early cleavage planes (see Fig. 10) and is not defined before the end of cleavage. This implies that signaling between cell tiers at the 16- to 60-cell stages cannot define the ectoderm-endoderm boundary. Nevertheless, micromere signaling does induce the vegetal plate (Ransick and Davidson, 1993, 1995) and complete vegetal plate specification may require permanent contact between micromere progeny and the overlaying cells beyond 6th cleavage (Ransick and Davidson, 1995), almost until the late allocation to presumptive ectoderm

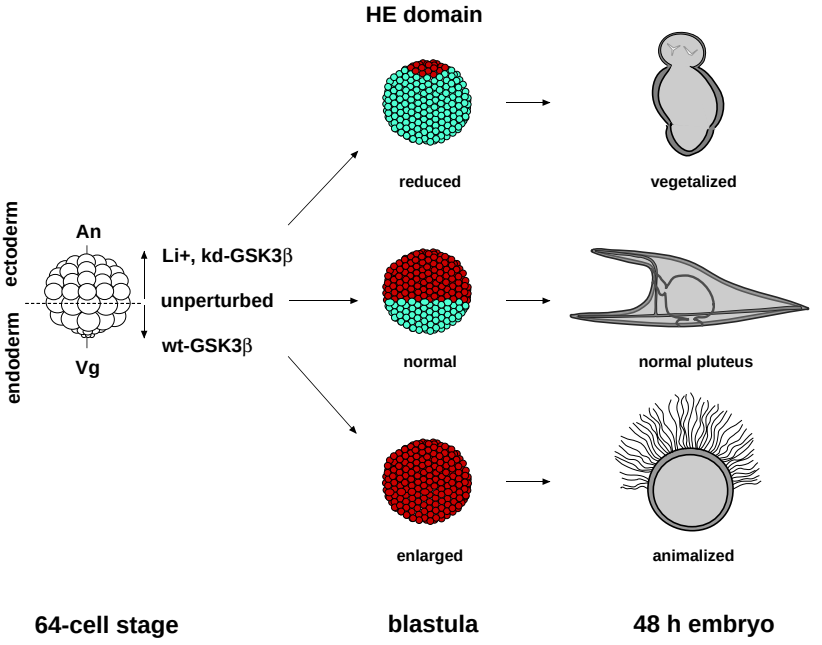

Fig. 10. Alteration of sea urchin development by lithium treatment and misexpression of GSK3β. 60-cell stage embryo: ectoderm comprises oral and aboral ectoderm; endoderm refers here to endoderm and mesoderm (primary and secondary mesenchyme). Red, cells expressing *HE*; green, cells not expressing *HE*.

or endoderm territories (Logan and McClay, 1997). Thus misexpression of GSK3β might interfere with a complex series of interactions involved in the progressive positioning of the ectoderm-endoderm boundary.

GSK3β levels also control the expression of a very early gene, the *HE* gene, which is spatially restricted along the AV axis. While the *HE* domain is close or identical to the presumptive ectoderm territory, the *HE* gene is almost completely turned off by the time the boundary between the presumptive ectoderm and endoderm territories is fixed (Lepage and Gache, 1990). Control of *HE* expression appears to be cell autonomous: transcription follows the same time course and reaches almost the same level in dissociated blastomeres and in intact embryos, with the ratio of expressing to non-expressing cells remaining nearly unchanged in isolated blastomeres (Ghiglione et al., 1993). Furthermore, exogenous micromeres implanted at various positions in recipient embryos at the 4-cell or 8-cell stages, while able to induce secondary archenterons, do not affect *HE* expression, suggesting that during normal development, *HE* is not controlled by signals emanating from the micromeres (Ghiglione et al., 1996). The 3 main features of *HE* expression – extreme precocity, autonomy, simple restriction along the AV axis – suggest that the *HE* expression domain is dependent on a maternal prepattern, without excluding late refinement. This idea is reinforced by the observation that lithium treatment restricts *HE* expression even in embryoids derived from animal halves isolated at the 8-cell stage or dissected from unfertilized eggs, and thus in the absence of the vegetal half (Ghiglione et al., 1996). Recently, Logan and McClay (1997) reported that the translocation of β-catenin to the nuclei of vegetal blastomeres is independent of micromere signals, which correlates with the independence of *HE* from micromeres and the implication of GSK3β in patterning the AV axis. Therefore, alteration of the *HE* spatial pattern by misexpression of GSK3β suggests that GSK3β and the Wnt pathway may interfere with maternal cues, or the interpretation of such cues, which control a major division along the AV axis. In this case, as in *Xenopus*, it remains to be determined whether a maternal Wnt is present, whether only downstream components of the Wnt pathway are involved or whether GSK3β is controlled by other transduction pathways as has been reported in some other organisms (Welsh et al., 1996).

While both *Xenopus* and sea urchin embryos use the same signaling pathway to define early territories, spatial and temporal connections between maternal and zygotic events appear to differ. In the *Xenopus* embryo, analysis of early axial patterning events is complicated by the displacement of dorsal determinants during the cortical rotation without which no D-V axis forms. In the sea urchin embryo, the main axis of patterning is identical to the primary maternal AV axis. Furthermore, in *Xenopus*, embryonic patterning is initiated in the virtual absence of transcription, whereas in sea urchin, transcription starts soon after fertilization.

The Wnt pathway has so far been implicated in patterning the main embryonic axis only in vertebrates. Sea urchins belong to a phylum which diverged shortly after the protostome-deuterostome separation. Our finding that GSK3β is an essential element in patterning the main axis of the sea urchin embryo suggests that the Wnt pathway, or components of this pathway, is likely to be of fundamental importance during the first steps of axial patterning in deuterostomes.

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