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Altering cell fates in sea urchin embryos by overexpressing SpOtx, an orthodenticle-related protein

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SUMMARY

While many general features of cell fate specification in the sea urchin embryo are understood, specific factors associated with these events remain unidentified. SpOtx, an orthodenticle-related protein, has been implicated as a transcriptional activator of the aboral ectoderm-specific *Spec2a* gene. Here, we present evidence that SpOtx has the potential to alter cell fates. SpOtx was found in the cytoplasm of early cleavage stage embryos and was translocated into nuclei between the 60- and 120-cell stage, coincident with *Spec* gene activation. Eggs injected with SpOtx mRNA developed into epithelial balls of aboral ectoderm suggesting that SpOtx redirected nonaboral ectoderm cells to an aboral ectoderm fate. At least three distinct domains on SpOtx, the homeobox and regions in the N-terminal and C-terminal halves of the protein, were required for the morphological alterations. These same N-terminal and C-terminal regions were shown to be transactivation domains in a yeast transactivation assay, indicating that the biological effects of overexpressing SpOtx were due to its action as a transcription factor. Our results suggest that SpOtx is involved in aboral ectoderm differentiation by activating aboral ectoderm-specific genes and that modulating its expression can lead to changes in cell fate.

Key words: sea urchin, embryo development, homeobox protein, orthodenticle/Otx-genes, SpOtx

INTRODUCTION

In sea urchin embryos, initial cell fates are specified by a combination of invariant cleavages and cell-cell interactions. By the sixth cleavage, clusters of clonally derived founder cells have arisen that divide the embryo into five distinct territories: small micromeres, large micromeres, vegetal plate, and oral and aboral ectoderm. These territories are defined both by their lineages and by the expression of a variety of specific marker genes. Current evidence indicates that the large micromeres act as an organizing center for specifying cell fate in the early embryo. The micromeres, whose descendants produce skeletogenic mesenchyme cells, are thought to initiate a cascade of inductive events by first specifying the vegetal plate territory in the tier of cells immediately above them (Ransick and Davidson, 1993). Vegetal cell signaling is also important for ectoderm differentiation. Expression of aboral ectoderm-specific genes requires a signal from underlying vegetal cells in one species (*Lytechinus pictus*) and this is believed to be the case in another (*Strongylocentrotus purpuratus*) (Wikramanayake et al., 1995).

The above-mentioned signaling events are likely to modulate the activity of transcription factors required for lineage-specific gene expression (Davidson, 1989). Several sea urchin transcription factors and their mRNAs are present in the unfertilized egg and are distributed uniformly throughout the embryo during early development, including those believed to be involved in the restricted expression of lineage-specific genes (Gan et al., 1995; Wang et al., 1995a; Zeller et al., 1995).

We have been investigating factors that bind to DNA elements within the control region of the *S. purpuratus* aboral ectoderm-specific *Spec2a* gene (Gan et al., 1990a,b; Gan and Klein, 1993; Mao et al., 1994; Gan et al., 1995). *Spec2a* belongs to a small family of genes encoding intracellular calcium-binding proteins whose expression is activated shortly after the aboral ectoderm founder cells arise and whose mRNAs accumulate exclusively in aboral ectoderm cells (Hardin et al., 1988; Tomlinson and Klein, 1990). Aboral ectoderm-specific expression of *Spec2a* is conferred chiefly by an enhancer, which drives expression in aboral ectoderm and mesenchyme cells, and a negative element, which represses expression in mesenchyme cells (Gan et al., 1990a; Gan and Klein, 1993; Mao et al., 1994). The *Spec2a* enhancer contains multiple redundant DNA elements that bind to SpOtx, a homeobox-containing protein belonging to the orthodenticle (otd)/Otx family (Mao et al., 1994; Gan et al., 1995). These multiple Otx sites are essential for *Spec2a* expression and serve as the major source of positive control for *Spec* genes (Gan et al., 1990b; Mao et al., 1994). Thus, SpOtx is very likely to be involved in the activation of the *Spec2a* gene (Gan et al., 1995).

However, the presence of SpOtx mRNA in nonaboral ectoderm cells at the time that the *Spec2a* gene is activated indicates that SpOtx cannot be solely responsible for the aboral ectoderm-specific expression of *Spec2a*.

Orthodenticle-related proteins are an important class of transcriptional regulators that have been identified in several organisms. In *Drosophila*, otd is required for the formation of anterior head structures and a variety of other developmental processes (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Wieschaus et al., 1993; Royet and Finkelstein, 1995). In vertebrates, Otx1 and Otx2 are believed to play similar roles in head development (Simeone et al., 1992,1993; Bally-Cuif et al., 1995; Pannese et al., 1995). Otx2 knockout mice have deletions in their forebrain and midbrain regions due to defective anterior neuroectoderm specification (Acampora et al., 1995; Matsuo et al., 1995).

However, in contrast to flies and mice, the initial events of sea urchin embryogenesis do not rely on elaborating reiterative patterns along an embryonic axis but rather on the specification and differentiation of individual cell types (Davidson, 1991). In sea urchin embryos, SpOtx must function in events other than patterning the head since sea urchin larvae do not form heads. Based on its likely involvement in activating *Spec2a* expression, we hypothesize that SpOtx plays a key role in the differentiation of aboral ectoderm. If this idea is correct, SpOtx might be differentially activated in the aboral ectoderm territory, which would provide a mechanism for the specification process in spite of SpOtx's presence in other embryonic territories.

To learn more about the role of the otd/Otx proteins in development, we determined the intracellular location of SpOtx during the cleavage stages of *S. purpuratus*, when cell specification events are occurring. We found that SpOtx resided mostly in the cytoplasm of eggs and early cleavage stage embryos but that between the 60- and 120-cell stage, it translocated into nuclei coincident with *Spec2a* gene activation. Overexpressing SpOtx by injecting SpOtx mRNA into eggs caused dramatic alterations in embryo development, leading to embryoids that were virtual epithelial balls of aboral ectoderm. From these results, we suggest that SpOtx normally functions in the differentiation of aboral ectoderm and has the potential to redirect the fates of other cells when overexpressed.

MATERIALS AND METHODS

RNA injections into sea urchin eggs

S. purpuratus and *L. pictus* were purchased from Pacific Biomarine (Venice, CA) or Marinus (Long Beach, CA). Gametes were collected from gravid adults by intracoelomic injection of 0.5 M KCl. Embryos were cultured in artificial sea water (pH 8.0) at 15°C. Microinjection of sea urchin eggs was done as detailed in Gan et al. (1990a,b). RNAs were mixed with glycerol to a final concentration of 1-2 μg/μl. About 5-10 pl of this solution was injected into the egg cytoplasm.

RNA preparation

To generate DNA templates for in vitro RNA synthesis, SpOtx(K179Q) and SpHox-7 were linearized with *Bam*HI, pCAT1(18M) was digested with *Not*I and all the other SpOtx-derived constructs were digested with *Xba*I. SpOtx was linearized with *Xho*I for making SpOtx antisense mRNA. Large amounts of 5′ capped mRNA were synthesized by using the mMESSAGE mMACHINE kit (Ambion) as described in the instruction manual. All sense RNAs were synthesized by T7 RNA polymerase and the SpOtx antisense RNAs by T3 RNA polymerase. These RNAs were passed through a Sephadex G-50 column to separate the unincorporated cap analogue ($m^7G(5')ppp(5')G$) and free nucleotides. After precipitating with isopropanol, the RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated H_2O. The concentration was estimated by the FastCheck Nucleic Acid Quantification System (GIBCO, BRL).

Detection of cell-type-specific markers

Embryos were fixed with 4% paraformaldehyde buffered with phosphate-buffered saline (PBS), pH 7.4, for staining with the Spec1 antibody and with cold methanol for staining with Ecto V, Endo I and Meso I. An IgG fraction of the Spec1 antibody was preabsorbed with an acetone powder as described in Wikramanayake et al. (1995) and staining was done at a 1:500 dilution. The Ecto V antibody was used at a 1:10 dilution, and the Endo I and Meso I antibodies were used at a 1:250 dilution. Treatment with the Spec1 primary antibody was followed by a fluorescein-conjugated goat anti-rabbit antibody (Cappel), and the Ecto V, Endo I and Meso I monoclonal antibodies were followed by rhodamine-conjugated goat anti-mouse secondary antibodies (IgGAM, Cappel). Embryos were blocked in 3 mg/ml bovine serum albumin (BSA) and 0.2% Tween-20 in PBS, and the antibodies were diluted in the same buffer. The embryos were mounted in 90% glycerol and observed on a Nikon Diaphot-TMD inverted microscope equipped with differential interference contrast (DIC) and epifluorescence optics.

Detection of SpOtx

SpOtx was detected in embryos using the SpOtx-B polyclonal antibody as described in Gan et al. (1995) with a few modifications. An IgG fraction of the SpOtx-B serum was prepared and preabsorbed with *S. purpuratus* embryo extracts as previously described (Gan et al., 1995). Embryos were blocked with 3 mg/ml BSA in PBS/0.5× artificial sea water (ASW)/0.2% Tween-20 and for embryo staining the antibodies were diluted in the same buffer at 0.2 μg/ml. Treatment with primary antibodies was followed by Cy5 conjugated secondary antibodies (goat anti-rabbit IgG, Jackson Immunochemicals) at a 1:50 dilution. The embryos were then dehydrated through a graded series of ethanol and mounted in methyl salicylate (Summers et al., 1991).

Confocal microscopy

Sample slides were viewed on a Nikon Diaphot 200 inverted microscope attached to the scanning head of a Bio-Rad MRC 1000 laser scanning confocal microscope equipped with a krypton-argon laser. The Cy5 fluorescence was imaged using the 647 nm line with the laser set at 30% power. A complete Z-series of images through the embryos was collected at 2 μm intervals at a section thickness of approximately 2 μm using a 40× NA 1.2 fluor objective and further magnified 1.5-2.0 times. Confocal images were Kalmen averaged, overlaid with pseudocolor and stored as digitized Bio-Rad.PIC files on a Panasonic optical disc recorder. No further image processing was done. Selected PIC files were converted to TIF file format using Adobe Photoshop software and photographed using an LSR-digitized slide maker using Kodak Ectachrome Elite 100 film.

SpOtx overexpressing embryos were observed on the confocal microscope and the laser power was adjusted to eliminate any pixel saturation. This was achieved at 1% laser power. Control embryos were viewed at the same laser settings and at 1% laser power endogenous SpOtx was undetectable in uninjected embryos.

Embryo cell counts

The SpOtx mRNA-injected embryos were fixed with 4% paraformaldehyde in ASW. Stock solutions (1 mg/ml) of Hoechst 33342 were prepared with ASW. Fixed embryos were transferred to ASW with 20 μg/ml Hoechst 33342 and stained for 15 minutes. For

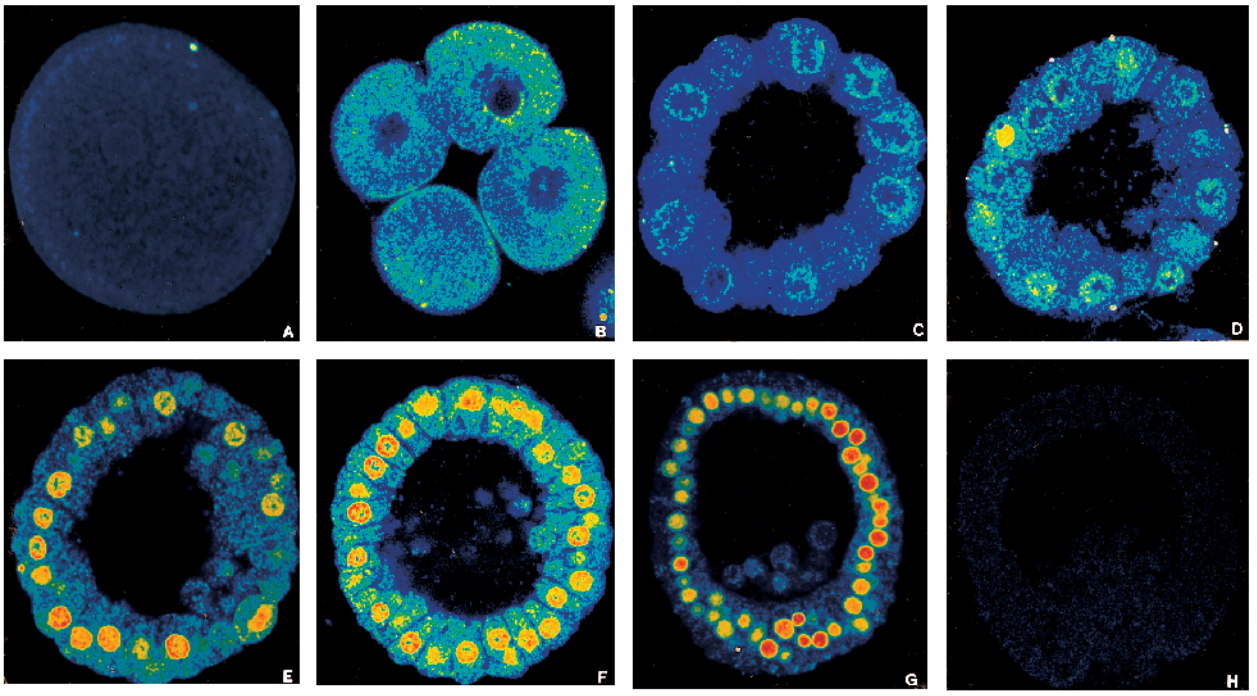


Fig. 1. Localization of SpOtx in *S. purpuratus* eggs and embryos. Paraformaldehyde-fixed eggs (A), 4-cell stage (B), 60-cell stage (C), 120-cell stage (D), 12 hour (E), 15 hour (F) and 24 hour (G) embryos were incubated with SpOtx antibody and analyzed by indirect immunofluorescence using confocal microscopy. Background fluorescence of a 24 hour embryo incubated with nonimmune serum is shown in H.

total cell counts, the stained embryos were slightly compressed and viewed on the microscope with UV illumination. Eight differentially focused images for each embryo were photographed. Each fluorescent-labeled nucleus was then marked and counted.

RT-PCR analysis

Total RNA was isolated from 23 to 56 control and mRNA-injected embryos at different developmental stages using a single-step method (Chomczynski and Sacchi, 1987). The extracted RNAs were suspended in 12 μl of DEPC-treated H_2O. 10 μl of RNA per reaction was used to synthesize cDNA using SuperScript reverse transcriptase (GIBCO, BRL) and random hexamers. 5 % of the RT reaction was then used for PCR. PCR contained 0.6 μM concentrations of appropriate primers, 1 μCi of [a^{32}P]dCTP and 1.5 mM $MgCl_2$. PCR amplification conditions were 94°C for 1 minute, 58°C for 1 minute and 72°C for 40 seconds. All reactions were performed in the linear range of amplification. One tenth of the products were resolved on native polyacrylamide gels (6%).

Plasmid constructions

The original SpOtx cDNA was isolated from *S. purpuratus* (Gan et al., 1995) and used as template for PCR to generate the different SpOtx deletions and mutations shown in Fig. 3. Each of these constructs was made by subsequently subcloning two PCR fragments into the appropriate enzyme sites of pBlueScript IISK+ (Stratagene). For example, the lower primer 196-L (5′-ATTGGATCCGTTTTGTTGCTGT-3′) was coupled with M13-20mer (Stratagene) to amplify a SpOtx subfragment containing the full 5′-UTR and 196 N-terminal codons, and the fragment was digested with *Bam*HI/*Eco*RI and then subcloned into the corresponding sites of pBlueScript IISK+ and termed SpOtx(1-196). The upper primer 239-U (5′-TCGGGATCCGTCTCATCGAG-3′) was paired with the Reverse primer (Stratagene) to generate a subfragment containing 3′-UTR and C-terminal codons 239 to 371, and this fragment was digested with *Bam*HI/*Xba*I and then subcloned into the corresponding sites of SpOtx(1-196) to create SpOtx(Δ197-238). All constructs were confirmed by partial sequencing at the junctions. The size of the in vitro transcription/translation products of these constructs was verified by SDS-PAGE.

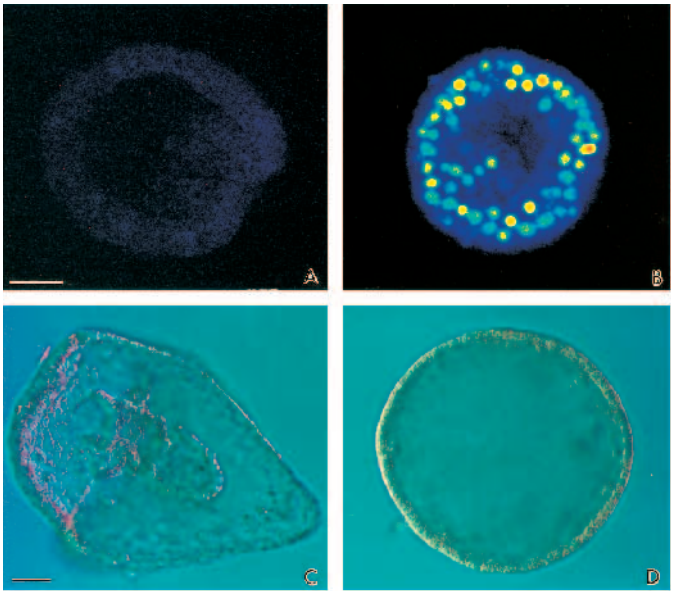


Fig. 2. Morphological effects of overexpressing SpOtx in *S. purpuratus* embryos. (A,B) Confocal images of 24 hour uninjected control (A) and SpOtx mRNA-injected (B) embryos stained with SpOtx antibody. Note that these images were obtained at 1% laser power, while the images in Fig. 1 were at 30% laser power (see Materials and Methods for details). (C) DIC image of an uninjected control 72 hour pluteus stage embryo. (D) DIC image of SpOtx mRNA-injected embryo at 72 hours after fertilization. (Bar A,B, 25 μm; C,D, 25 μm)

Mapping SpOtx transactivation domains in yeast

To map SpOtx transactivation domains in yeast, pAS1 (Durfee et al., 1993) was used to generate Gal(1-147)-SpOtx fusion constructs. The SpOtx cDNA was used as template with appropriate PCR primers to generate the fusion constructs depicted in Fig. 6. All pAS1-SpOtx fusion constructs were transformed into yeast strain HF7c (Feilotter et al., 1994). Transformants were plated on yeast drop-out medium lacking tryptophan. The quantitative analysis for β-galactosidase was carried out as described in Durfee et al. (1993).

RESULTS

Translocation of SpOtx from the cytoplasm to the nucleus during early development

In an earlier study, we showed that SpOtx is in nuclei of all cells of mesenchyme blastula and pluteus stage embryos with the possible exception of primary mesenchyme cells (Gan et al., 1995). If SpOtx is involved in the temporal activation or spatially restricted expression of *Spec2a*, it should be present and active in the nuclei of aboral ectoderm cells at the late cleavage stage, the time when *Spec2a* is first activated. Immunoblot analysis shows that SpOtx is present in unfertilized eggs and that levels accumulate several fold to the blastula stage and then drop precipitously during later development (C.-K. C. and W. H. K., unpublished results). We used indirect immunofluorescence and confocal microscopy to investigate the subcellular location of SpOtx in the early stages of *S. purpuratus* development.

Antibodies against the SpOtx homeobox and 36 amino acid residues on the N-terminal side of the homeobox showed a specific reaction to both cytoplasmic and nuclear forms of SpOtx as seen by the absence of signal when nonimmune serum was used (Fig. 1H) or when antibodies were first preabsorbed with soluble recombinant SpOtx (not shown). Optical sections through fertilized eggs showed low but detectable amounts of SpOtx distributed throughout the cytoplasm (Fig. 1A). In 4-cell stage embryos (2.5-3 hours after fertilization), SpOtx labeling was much more intense than in eggs and again most of the label was found in the cytoplasm (Fig. 1B). Nuclei appeared devoid of label, although in many sections a perinuclear ring was observed (Fig. 1B). By the 60-cell stage (7 hours after fertilization), the labeling pattern had changed. Much of the signal was perinuclear, although cytoplasmic labeling was still observable (Fig. 1C). In these sections, it appeared that SpOtx was localized in some cells to the inner side of the nuclear-cytoplasmic interface while the center of the nucleus was generally not labeled (Fig. 1C). At the 120-cell stage (8 hours after fertilization), several cells within the same embryo showed intense SpOtx labeling throughout the nucleus while, in other cells, SpOtx continued to be cytoplasmic or perinuclear (Fig. 1D). Three-dimensional reconstructions of optical sections through single embryos indicated that the nuclear labeling at this stage did not correspond to a distinct embryonic cell type or region (not shown).

These early patterns of SpOtx localization probably represented the gradual translocation of SpOtx into the nucleus. This became obvious by the early blastula stage (12 hours after fertilization), when much of SpOtx staining was nuclear (Fig. 1E). At blastula stages, 15 and 24 hours after fertilization, SpOtx was in the nuclei of most cells of the embryo except primary mesenchyme cells (Fig. 1G). This is consistent with our earlier analysis showing an absence of SpOtx mRNA in primary mesenchyme cells (Gan et al., 1995). The nuclear translocation of SpOtx is concordant with the time of *Spec2a* gene activation and activation of other aboral ectoderm-specific genes (Hickey et al., 1987; Tomlinson and Klein, 1990; Gagnon et al., 1992).

Morphological alterations in SpOtx mRNA-injected embryos

To examine the role that SpOtx plays in early development, we initially designed experiments to perturb the embryo by inducing expression of various forms of SpOtx, specifically ones that might disrupt SpOtx function. Unexpectedly, we obtained dramatic results by simply overexpressing the wild-type SpOtx protein. Injections of large amounts of SpOtx mRNA into *S. purpuratus* or *L. pictus* eggs led to increased levels of SpOtx protein. Immunostaining SpOtx mRNA-injected embryos with SpOtx antibodies showed a large enhancement of SpOtx in the nuclei of all cells relative to uninjected controls 24 hours after fertilization (Fig. 2A,B). Injection of SpOtx mRNA was thus an effective method for overexpressing SpOtx in embryos.

The injections yielded highly reproducible morphological changes (Fig. 2C,D for *S. purpuratus*; Fig. 3A, left panel for *L. pictus*). Injected eggs went through the cleavage divisions at about the same rate as eggs injected with control RNAs, but by the mesenchyme blastula stage, they lacked the thickened region corresponding to the vegetal plate. In addition, primary mesenchyme cells failed to ingress into the blastocoel. The embryoids did not gastrulate nor did they form a stomodeum or apical tuft and, by 3 days after fertilization, they had developed into uniform, thin, ciliated epithelial spheres with blastocoels devoid of cells (Fig. 2D). These structures were strikingly different from the classical animalized embryoids formed by culturing isolated animal halves or treating embryos with zinc ions (Nemer et al., 1985; Wikramanayake et al., 1995): such animalized embryos are distinctly polarized and display prominent apical tufts. The SpOtx-injected embryoids did not appear to be developmentally arrested and were active swimmers, capable of surviving for at least 5 days in this peculiar state.

To determine if the effects that we observed were specific for SpOtx mRNA, we injected several control RNAs at similar concentrations, including SpOtx antisense RNA and mRNAs for the sea urchin homeobox-containing protein SpHox-7 and bacterial chloramphenicol acetyl transferase (CAT). These controls resulted in embryos that gastrulated, formed stomodea, and produced guts and skeletons; no embryoids like those shown in Fig. 2D were ever observed.

The amount of SpOtx mRNA injected was difficult to determine precisely, but efficient production of the epithelial balls required approximately 5-10 pg of RNA, which is 20-40% of the total mRNA in the embryo. With certain batches of *S. purpuratus* or *L. pictus* eggs, 30-50% of the injected eggs survived the injection and more than 80% of the surviving embryos formed these ball-like structures. With other egg batches, survival rates were much lower; but at these RNA concentrations, the percentage of survivors that formed epithelial balls was always high.

When 25-50% as much SpOtx mRNA was injected, partial effects were observed which could be ranked in order of

severity (Fig. 3A). At 2 days after fertilization, some embryoids had small clumps of cells at the vegetal pole corresponding to small amounts of presumptive endoderm and a thickening at the opposite pole (Fig. 3A, middle left panel). If these embryoids were left in culture for longer than 3 days, many eventually formed archenterons that attached to the oral surface. Another common embryoid class had a shortened archenteron that attached to the oral face, resulting in a disk-shaped structure (Fig. 3A, middle right panel). Although we did not attempt to precisely quantify the morphological severity with SpOtx mRNA levels, it was clear from many experiments that increases in the amount of SpOtx mRNA correlated with increases in the severity of the morphological effects.

Characterization of cell types in SpOtx mRNA-injected embryoids

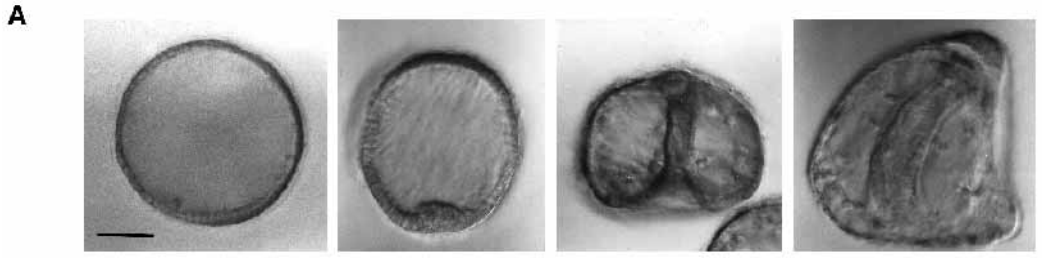
Gross inspection of the epithelial balls suggested that they were composed entirely of ciliated squamous epithelial cells, not unlike those found in aboral ectoderm. To establish the identity of these cells, we probed the embryoids with a set of cell-type-specific antibodies using indirect immunofluorescence. All cells of 3-day embryoids showed intense labeling with the Spec1 antibody (a marker for aboral ectoderm; Wikramanayake et al., 1995), while control embryos cultured for the same time gave only aboral ectoderm staining, as expected (Fig. 4A,E versus B,F). In contrast, staining with antibodies against Ecto V (oral ectoderm; Coffman and McClay, 1990; Fig. 4C,G versus D,H), Meso I (primary mesenchyme cells; Wessel and McClay, 1985; Fig. 4I,J) and Endo I (endoderm; Wessel and McClay, 1985; Fig. 4K,L) produced undetectable or very weak signals compared to uninjected controls.

Progenitors of nonaboral ectoderm cells may have been respecified to an aboral ectoderm fate or they may have arrested early in development. To determine which, we counted cells in the SpOtx-injected embryoids at different times during development. Cell counts of embryoids cultured for 22, 48 and 72 hours yielded averages of 318 (s.d.=1, *n*=3), 416 (s.d.= 25, *n*=3) and 925 cells (s.d.=44, *n*=3) respectively. Although embryos injected with large amounts of RNA were developmentally retarded, these averages were in the same range as cell numbers found in normal embryos at the corresponding times (350 cells at 22 hours, 650 at 48 hours and 1500 at 72 hours; Davidson, 1986). Since nonaboral ectoderm cells make up a large fraction of cells of the embryo during these times and aboral ectoderm cells divide very slowly (Kingsley et al., 1993), these results suggested that descendants of nonaboral ectoderm progenitor cells contributed to the cells in the embryoid.

The fate of nonaboral ectoderm cells in SpOtx mRNA-injected embryoids

Following the early development of SpOtx-injected eggs proved difficult because, in a given batch of injected eggs, it was never certain which embryos would survive to produce epithelial balls. We found that large amounts of injected RNA retarded cleavage and, in many cases, the first two or three cleavages were abnormal. However, by 36 hours after fertilization, we could predict which embryoids would manifest epithelial balls and which would produce partial effects. We used RT-PCR to monitor the expression of various cell-type-specific marker genes at 38, 72 and 96 hours of development. Fig. 5A shows a series of RT-PCR measurements under conditions where the amount of PCR product was proportional to the amount of input RNA. In Fig. 5B, these experiments were standardized to ubiquitin RNA, which was assumed to be unaffected by injection of SpOtx mRNA and the results are presented as the percentage of the control value at each time point.

Injected embryoids showed six times more SpOtx mRNA at 38 hours after fertilization than uninjected controls (Fig. 5A,B). Exogenous SpOtx mRNA levels gradually decreased



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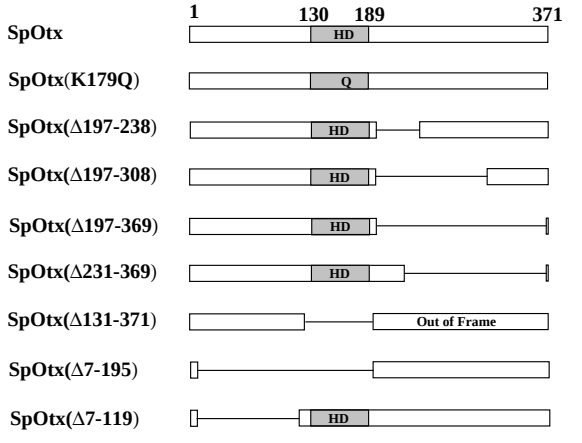

	Epithelial Ball	Partial & Disk-Shaped	Normal Pluteus
SpOtx	369 (75.6%)	117 (24.0%)	2 (0.4%)
SpOtx(K179Q)	7 (18.0%)	32 (82.0%)	0
SpOtx(Δ197-238)	205 (67.0%)	95 (31.0%)	6 (2.0%)
SpOtx(Δ197-308)	1 (1.5%)	41 (59.4%)	27 (39.1%)
SpOtx(Δ197-369)	0	5 (2.0%)	250 (98.0%)
SpOtx(Δ231-369)	5 (1.5%)	257 (75.6%)	78 (22.9%)
SpOtx(Δ131-371)	0	8 (10.3%)	70 (89.7%)
SpOtx(Δ7-195)	0	5 (2.4%)	200 (97.6%)
SpOtx(Δ7-119)	29 (14.2%)	128 (62.4%)	48 (23.4%)

Fig. 3. Effects of mutating or deleting portions of SpOtx on biological activity in *L. pictus* embryos. (A) Representative embryos at 48 hours of development injected with SpOtx mRNA; the embryo on the right is an uninjected control. (Bar, 50 μm). (B) Injections of different forms of SpOtx. HD is the homeodomain; Q refers to the lysine to glutamine mutation at position 50 of the homeobox. The percent of embryos analyzed is indicated in parentheses next to the number of embryos. Thin lines represent deleted DNA.

relative to controls in later development (Fig. 5A,B). Spec1 mRNA (aboral ectoderm) levels were 60% of control levels at 38 hours but then increased to four and three times control levels at 72 and 96 hours respectively. In uninjected embryos, Spec1 mRNA accumulates to peak levels by 48 hours and levels subsequently decrease by half by 72 hours (Hardin et al., 1988). The results in Fig. 5 indicated that, in injected embryos, Spec1 continued to accumulate between 38 and 72 hours. These results suggested that the differentiation of aboral ectoderm was delayed by several hours. Eventually, however, Spec1 was overexpressed relative to controls, perhaps due to more aboral ectoderm cells in the injected embryoids than in control embryos and to enhanced transcription rates mediated by higher levels of SpOtx.

Spec2a mRNA levels were lower than controls at 38 and 72 hours but, by 96 hours, levels were 120% of uninjected control embryos (Fig. 5A,B). Spec2 mRNAs are known to accumulate more slowly and to a lesser extent than Spec1 (Hardin et al., 1988). Thus, the time course for the accumulation of the Spec mRNAs appeared to be stretched out in SpOtx-injected embryos. This was also the case for CyIIIa actin mRNA, another marker specific for aboral ectoderm (Coffman and Davidson, 1994; Fig. 5A,B). It was likely that this retardation was the result of the large amounts of RNA introduced into the eggs.

We observed very different results when we probed for Endo16 (endoderm; Nocente-McGrath et al., 1989) and SM50 (primary mesenchyme cells; Benson et al., 1987) mRNAs. Cell-type-specific activation of both of these genes takes place early in their differentiation programs, and Endo16 and SM50 are thus sensitive markers for following the fate of endoderm and primary mesenchyme cells (Ransick and Davidson, 1993; Makabe et al., 1995). Both Endo16 and SM50 mRNAs were detectable at 38 hours in SpOtx mRNA-injected embryos, but levels were less than one-third of control values (Fig. 5A,B). A portion of Endo16 and SM50 expression at 38 hours was perhaps due to embryoids that would have shown partial effects at later times. Most important, unlike the Spec and CyIIIa actin mRNAs, Endo16 and SM50 mRNAs accumulated to very low levels relative to controls at 72 and 96 hours. These

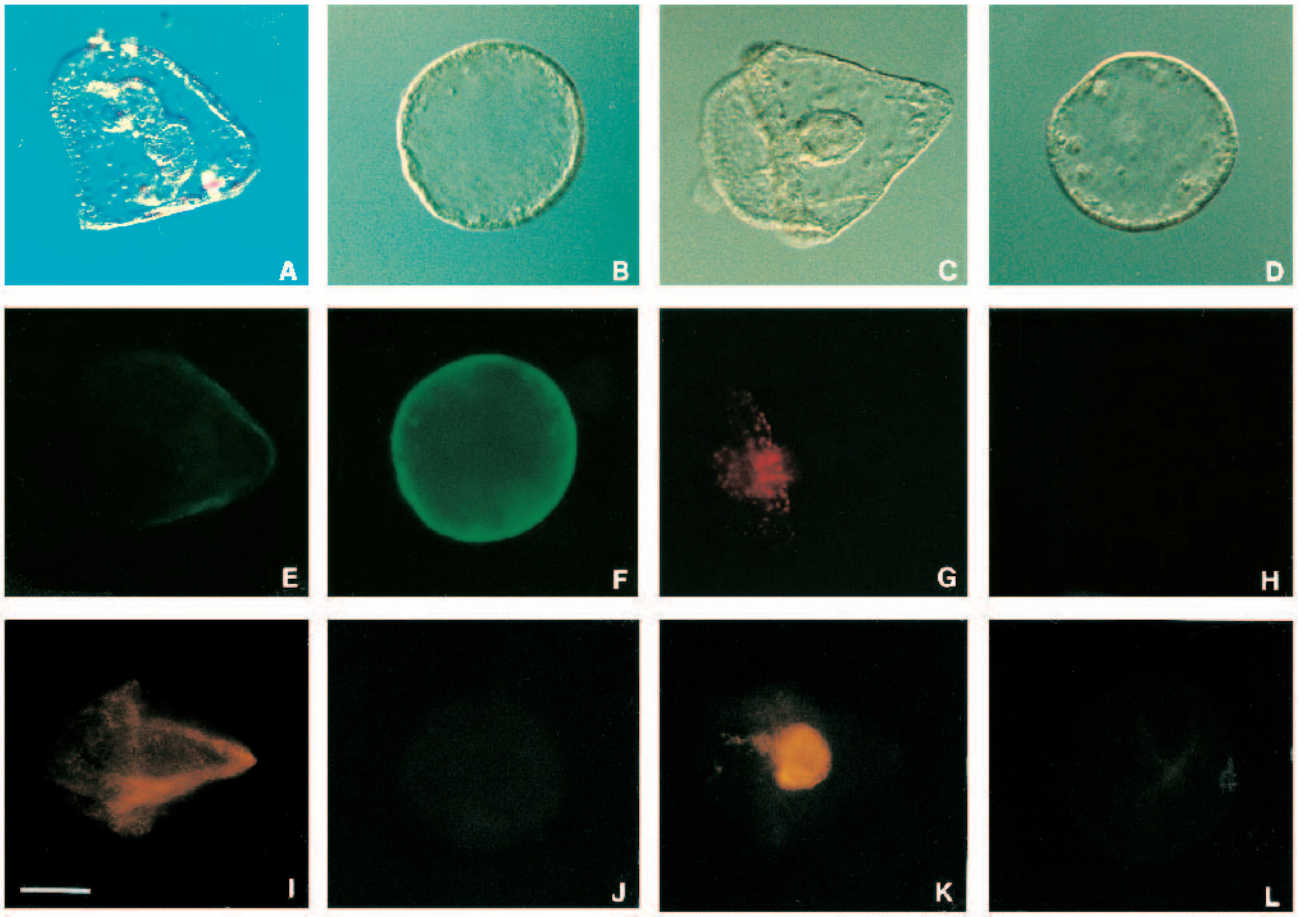

Fig. 4. Expression of cell-type-specific proteins in SpOtx mRNA-injected *S. purpuratus* embryos. Spec1 expression in SpOtx mRNA-injected and control embryos (A,B,E,F). (A,E) DIC and epifluorescent images of 3 day control embryo stained with Spec1 antibody. (B,F) DIC and epifluorescent images of 3 day SpOtx mRNA-injected embryos stained with Spec1 antibody. Conditions for staining and exposure times for photography were identical for both the control and injected embryos. Ecto V expression in SpOtx mRNA-injected and control embryos (C,D,G,H). (C,G) DIC and epifluorescent images of control embryos stained with Ecto V antibody. (D,H) DIC and epifluorescent images of SpOtx mRNA-injected embryos stained with Ecto V antibody. (I,J) Epifluorescent images of control and SpOtx mRNA-injected embryos stained with Meso I antibody, respectively. (K,L) Epifluorescent images of control and SpOtx mRNA-injected embryos stained with Endo I antibody, respectively. (Bar, 50 μm).

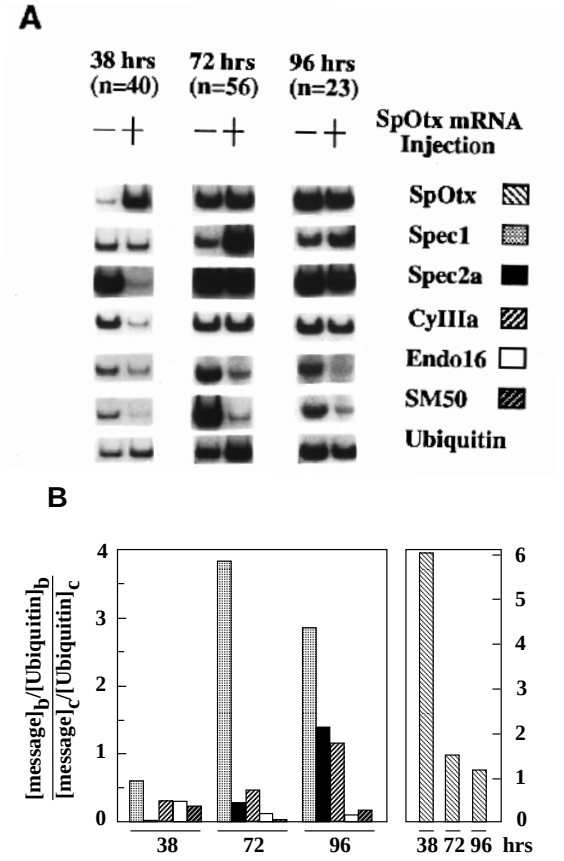

Fig. 5. RT-PCR analysis of various mRNAs in control and SpOtx mRNA-injected *S. purpuratus* embryos. (A) RT-PCR reaction products. (–) and (+) refer to uninjected and SpOtx-injected embryos. (B) Histogram showing the ratio of RT-PCR product from SpOtx-injected embryos to control embryos. The data are normalized to the ubiquitin control. $[message]_b/[ubiquitin]_b$ and $[message]_c/[ubiquitin]_c$ refer to RNA from SpOtx-injected (b) and uninjected (c) embryos.

results indicated that, while low levels of endoderm and primary mesenchyme cell gene expression might have occurred during the first day or two after fertilization, expression was greatly attenuated throughout development in the SpOtx-injected embryoids.

Domains in SpOtx required for biological activity

We suspected that the SpOtx homeobox was essential for producing the morphological effects, but subsequent experiments showed that other domains on SpOtx were also required. For these experiments, we generated a series of SpOtx mRNAs with mutations and deletions in different regions along the molecule. Because *L. pictus* eggs developed the same range of defects as *S. purpuratus* when injected with SpOtx mRNA (Fig. 3A) and this species gave higher survival rates, *L. pictus* embryos were used for these experiments.

A mutation that changed the lysine in position 50 in the recognition helix of the homeobox to a glutamine [SpOtx(K179Q)] should result in a protein that cannot bind to the consensus Otx target site (TAATCC/T) but should bind to DNA elements recognized by several other classes of homeobox genes (Hanes and Brent, 1991). At concentrations at which wild-type SpOtx mRNA led to 76% of the surviving embryos developing as epithelial balls, SpOtx(K179Q) mRNA yielded less severe effects (Fig. 3B). While a small but significant fraction of embryoids exhibited the most drastic effect (18%), most of the embryos displayed partial effects (82%) (Fig. 3B). This experiment was repeated with both *L. pictus* and *S. purpuratus* eggs and we always observed weaker effects with the mutant RNA. These results implied that replacing the lysine-179 with glutamine attenuated but did not abolish the biological activity of SpOtx.

A series of C-terminal deletions showed that a region in the carboxyl terminal half of the protein was required for activity. Deletion of amino acids 197-238 [SpOtx(Δ197-238)] did not alter the morphological effects, but a deletion of the entire C-terminal end of the protein [SpOtx(Δ197-369)] eliminated activity (Fig. 3B). Deletions of amino acids 197-308 [SpOtx(Δ197-308)] and 231-369 [SpOtx(Δ231-369)] yielded partial effects. These results indicated that the region containing amino acids 238 to 369 was essential for biological activity.

A deletion that eliminated the homeobox and C-terminal half of SpOtx [SpOtx(Δ131-371)] abolished activity as was the case when the homeobox and N-terminal half of the protein were deleted [SpOtx(Δ7-195)] (Fig. 3B). However, a deletion that removed only the N-terminal half of SpOtx [SpOtx(Δ7-119)] produced mostly partial effects, indicating that this region by itself was required for full activity.

The deletion analysis revealed that at least three distinct domains on SpOtx, the homeobox and regions in the N-terminal and C-terminal halves of the protein, were required for the observed morphological effects on sea urchin development.

Correlation of SpOtx transcriptional activation domains with biological activity

If SpOtx functions by activating transcription, transactivation domains should exist within the molecule and might play a role in the morphological effects that were observed when SpOtx was overexpressed in sea urchin embryos. We mapped the putative transactivation domains in *Saccharomyces cerevisiae* by fusing portions of SpOtx with the Gal4-DNA-binding domain (Gal4-DBD) and analyzing the ability of these fusion proteins to activate a *lacZ* reporter gene driven by a CyC1 minimal promoter containing three Gal4-DNA-binding sites. Fig. 6 shows that one region in the C-terminal half of SpOtx contained a transactivation domain, between amino acids 270 and 371. In addition, a region in the N-terminal half of the protein between amino acids 1 and 130 also had strong activity. These transactivation domains mapped to the same regions shown to be required for the morphological effects observed in SpOtx mRNA-injected embryos. The results suggested that the observed biological effects of overexpressing SpOtx were due to its action as a transcriptional activator.

DISCUSSION

Two separate lines of evidence suggest that SpOtx is involved in aboral ectoderm differentiation during sea urchin embryogenesis. First, SpOtx is likely to be the in vivo activator of the aboral ectoderm-specific *Spec2a* gene and probably the other *Spec* gene family members as well. An enhancer within the *Spec2a* transcriptional control region contains multiple DNA elements with the consensus sequence TAATCC/T and these

are essential for transcriptional activity in aboral ectoderm cells (Mao et al., 1994). SpOtx is a prominent protein in early blastula nuclear extracts and binds with high affinity to the TAATCC/T sites (Gan et al., 1995). In our study, SpOtx translocated to the nucleus of most cells at the late cleavage stage, when *Spec* genes are first activated (Tomlinson and Klein, 1990; Gagnon et al., 1992). Moreover, SpOtx had strong transactivation domains on either side of the homeobox, implying that it functions in sea urchin embryos as a transcriptional activator. Thus, SpOtx is probably a key transcription factor controlling the temporal activation of *Spec2a* and other *Spec* genes. However, because SpOtx is present in the nuclei of cells other than aboral ectoderm cells at this time, it cannot by itself be responsible for aboral ectoderm-specific expression.

The second line of evidence arguing for a role for SpOtx in aboral ectoderm differentiation is the strong gain-of-function activity observed upon overexpressing its mRNA. The embryoids resulting from overexpressing SpOtx mRNA were composed entirely of aboral ectoderm cells. We believe that excess SpOtx redirects the fate of nonaboral ectoderm cells by ectopic activation of the aboral ectoderm differentiation program.

SpOtx expression and intracellular localization in early development

Both SpOtx mRNA and protein are present in the unfertilized sea urchin egg and both accumulate several-fold above their maternal levels by the end of the cleavage stage (Gan et al., 1995; Fig. 1). Since the vast majority of SpOtx protein molecules are in the cytoplasm at this time, our results suggest that SpOtx does not function as a transcription factor in the first few hours after the egg is fertilized. This would seem to rule out a role for SpOtx in initial specification events and suggests rather that the early events of cleavage act to translocate SpOtx into the nucleus where it then participates in activating genes at the early blastula stage and later.

New synthesis should account for some portion of cytoplasmic SpOtx at this time, since SpOtx mRNA is accumulating and is presumably active in translation. However, newly synthesized SpOtx would not be expected to accumulate to high levels in the cytoplasm since it should rapidly translocate into the nucleus. At present, we do not know whether SpOtx is actively retained in the cytoplasm or requires a nuclear translocation mechanism that is lacking at this early stage. In *Xenopus laevis*, a cytoplasmic retention mechanism involving a phosphorylation/dephosphorylation event prevents xnf7 from translocating to the nucleus until the gastrula stage (Li et al., 1994). It is not yet clear if similar retention mechanisms are associated with SpOtx.

The buildup of SpOtx at the cytoplasmic/nuclear interface during the early cleavage cycles suggests that once the translocation process begins, a rate-limiting step associated with the nuclear membrane prevents immediate transport into the nucleus. In this regard, there are two arginine/lysine-rich stretches along the SpOtx sequence that could serve as nuclear localization signals, although we have not determined whether these are required for nuclear transport.

SpOtx and aboral ectoderm differentiation

Since SpOtx is normally in the nuclei of cells whose descendants will give rise to oral ectoderm and endoderm as well as aboral ectoderm, why should overexpression lead solely to aboral ectoderm differentiation? It is possible that large amounts of SpOtx mRNA or protein are more toxic to nonaboral ectoderm cells. It is known that aboral ectoderm cells divide more slowly than other cells (Kingsley et al., 1993) and excess SpOtx might be lethal to actively dividing cells. While we cannot rule out such a possibility, it seems unlikely since we did not observe necrotic cells within the SpOtx-injected embryoids and we found as many cells in the embryoids as in the normal embryo at comparative times in development.

We favor the hypothesis that excess SpOtx results in widespread gain of function in cells where it is not normally active. As has been generally hypothesized for sea urchin territorial specification (Davidson, 1989), cell-cell signaling may prevent SpOtx from functioning in nonaboral ectoderm territories.

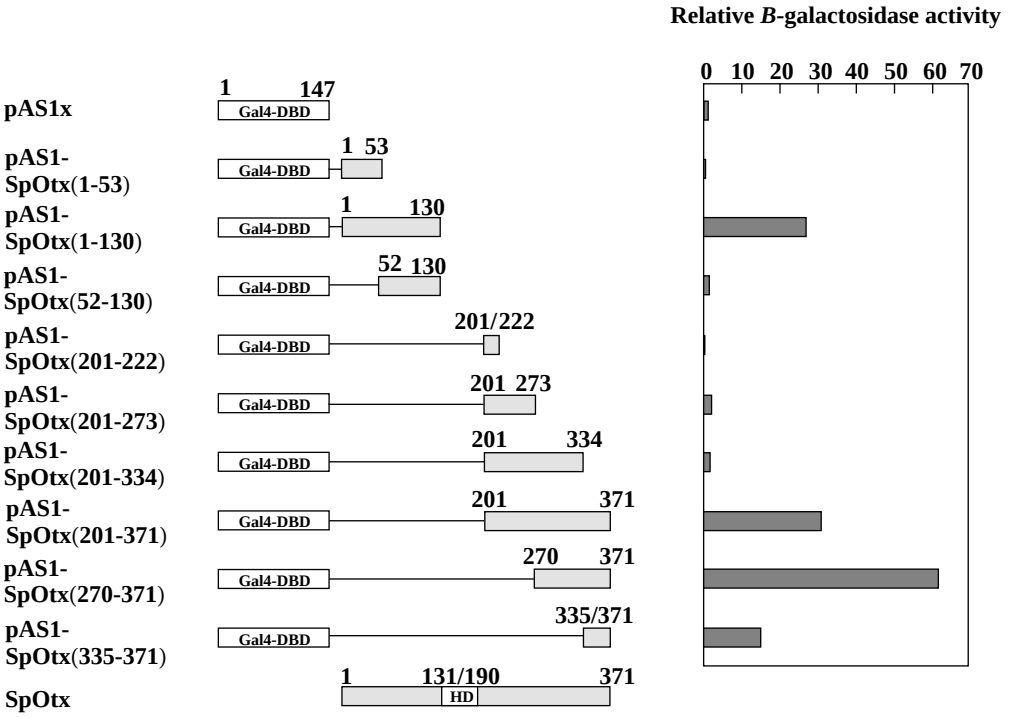

Fig. 6. Analysis of transcriptional activation domains within SpOtx using Gal4-DBD-SpOtx fusions in yeast. HD refers to the SpOtx homeodomain from amino acids 131-190.

Excess SpOtx may overcome these signaling mechanisms. This could be due to interference with post-translational modification events or interactions with other proteins required for DNA binding or transcriptional activation. In each of these cases, the overexpressed SpOtx would essentially inactivate a limiting component, either an enzyme or interacting protein involved in modulating SpOtx function. The lysine-to-glutamine mutation at positon 179, which resulted in partial effects, might have competed for an interacting protein in nonaboral ectoderm cells, causing the ectopic activation of endogenous SpOtx.

SpOtx appears to activate the entire aboral ectoderm differentiation program and yet it is unlikely to be required for the expression of all aboral ectoderm-specific genes. The *CyIIIa* actin gene control region has some 20 DNA elements associated with its transcription, but none are target sites for SpOtx (Theze et al., 1990). This is also the case for another aboral ectoderm-specific actin gene, *CyIIIb* (Niemeyer and Flytzanis, 1993). Our RT-PCR analysis showed that, after 4 days of development, Spec1, Spec2a and CyIIIa actin mRNAs all accumulated in SpOtx-injected embryos to higher levels than in controls. This suggests that all aboral ectoderm-specific genes are expressed in the cells of the SpOtx-injected embryoid. If this is so, excess SpOtx must be directly or indirectly responsible for the activation of these genes.

A TAATCC site found in the promoter of the *SpHe* hatching enzyme gene was recently shown to bind to SpOtx from blastula nuclear extracts (Wei et al., 1995). This gene is expressed at early stages in ectoderm cell progenitors. While the function of the site is not yet defined, it could be a functional SpOtx site associated with the activation of *SpHe* in aboral ectoderm cells.

SpOtx function in later development

We have suggested that SpOtx has at least two functions, the early function discussed here and a late function associated with gene activation in proliferating cell types (Gan et al., 1995). In this regard, there are two distinct SpOtx mRNAs, one accumulating earlier than the other, which might serve different functions (Gan et al., 1995). After the mesenchyme blastula stage, SpOtx transcripts decrease three to four fold, become enriched in oral ectoderm and endoderm cells and depleted in the aboral ectoderm (Gan et al., 1995). This type of spatial expression pattern occurs for many sea urchin genes and reflects an accumulation of RNAs and proteins in regions that are active in cell proliferation and growth (Kingsley et al., 1993).

After the early blastula stage, signals associated with initial cell fate specification events are unlikely to operate. Thus, mechanisms that might differentially modulate SpOtx activity in different cell types would gradually be lost and SpOtx would be active in any cell where concentrations were high enough for it to bind to its DNA target sites. Recently, a TAATCC site that binds to SpOtx from blastula nuclear extracts, has been found in the control region of *Endo16*, a gene that is expressed starting at the late blastula stage (Yuh et al., 1994). While a functional role for this site remains to be determined, it suggests a later function for SpOtx in the activation of genes in proliferating cell types.

After the blastula stage, *Spec* and other aboral ectoderm-specific genes have greatly reduced rates of transcription (Tomlinson and Klein, 1990; Gagnon et al., 1992). At least in the case of the *Spec* genes, this could be due to decreased levels of SpOtx in aboral ectoderm cells and the onset of active repression mechanisms in nonaboral ectoderm cells. Such late repression mechanisms have been reported for the *CyIIIa* gene (Wang et al., 1995b). The gradual transition of SpOtx from an early function in aboral ectoderm cells to a late function in oral ectoderm and endoderm cells may explain an observation where *Spec* genes were shown to be transcriptionally active in nonectodermal cell types at the early gastrula stage but not at later developmental stages (Gagnon et al., 1992). As active SpOtx accumulates in nonectodermal cell types, it might spuriously activate *Spec* genes until subsequent repression mechanisms become available.

SpOtx and other orthodenticle-related proteins

In addition to virtual identity in their homeoboxes, SpOtx shares similarity with the mouse Otx1 and Otx2 in several stretches within their C termini (Simeone et al., 1992; Gan et al., 1995). These similarities probably reflect the conservation of transactivation domains. Nevertheless, SpOtx has doubtless been recruited to activate a separate set of genes from those targeted by Otx1, Otx2 and Otd. In flies and mice, orthodenticle-related proteins may have similar sets of target genes, particularly those associated with head development (Finkelstein and Boncinelli, 1994). However, the role of SpOtx in sea urchin development appears very different from those attributed to the mouse and *Drosophila* Otd/Otx proteins. This illustrates the evolutionary flexibility associated with orthodenticle-related proteins as transcription factors. The generation of new target genes for Otd/Otx proteins over evolutionary time by creating TAATCC/T motifs in the promoters of non-Otd/Otx-regulated genes could readily lead to novel regulatory patterns.

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