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Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to $NiCl_2$

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Summary

Few treatments are known that perturb the dorsoventral axis of the sea urchin embryo. We report here that the dorsoventral polarity of the sea urchin embryo can be disrupted by treatment of embryos with $NiCl_2$. *Lytechinus variegatus* embryos treated with 0.5 mM $NiCl_2$ from fertilization until the early gastrula stage appear morphologically normal until the midgastrula stage, when they fail to acquire the overt dorsoventral polarity characteristic of untreated siblings. The ectoderm of normal embryos possesses two ventrolateral thickenings just above the vegetal plate region. In nickel-treated embryos, these become expanded as a circumferential belt around the vegetal plate. The ectoderm just ventral to the animal pole normally invaginates to form a stomodeum, which then fuses with the tip of the archenteron to produce the mouth. In nickel-treated embryos, the stomodeal invagination is expanded to become a circumferential constriction, and it eventually pinches off as the tip of the archenteron fuses with it to produce a mouth. Primary mesenchyme cells form a ring in the lateral ectoderm, but as many as a dozen spicule rudiments can form in a radial pattern. Dorsoventral differentiation of ectodermal tissues is profoundly perturbed: nickel-treated embryos underexpress transcripts of the dorsal (aboral) gene LvS1, they overexpress the ventral (oral) ectodermal gene product, EctoV, and the ciliated band is shifted to the vegetal margin of the embryo. Although some dorsoventral abnormalities are observed, animal-vegetal differentiation of the archenteron and associated structures seems largely normal, based on the localization of region-specific gene products. Gross differentiation of primary mesenchyme cells seems unaffected, since nickel-treated embryos possess the normal number of these cells. Furthermore, when all primary mesenchyme cells are removed from nickel-treated embryos, some secondary mesenchyme cells undergo the process of "conversion" (Ettensohn, C. A. and McClay, D. R. (1988) *Dev. Biol.* 125, 396-409), migrating to sites where the larval skeleton would ordinarily form and subsequently producing spicule rudiments. However, the skeletal pattern formed by the converted cells is completely radialized. Our data suggest that a major effect of $NiCl_2$ is to alter commitment of ectodermal cells along the dorsoventral axis. Among the consequences appears to be a disruption of pattern formation by mesenchyme cells.

Key words: sea urchin, dorsoventral axis, $NiCl_2$, nickel, *Lytechinus variegatus*, mesenchyme, skeleton.

Introduction

Elaboration of the embryonic body plan involves both the specification of the major axes of the embryo and the coordinated movements of cells and tissue sheets in response to preexisting axial information. In *Drosophila*, both the anteroposterior (A-P) and dorsoventral (D-V) axes are established thorough the interactions of localized maternal determinants in the egg with other regulatory molecules, many of which are transcription factors (Levine and Hoey, 1988; St. Johnston and Nüsslein-Volhard, 1992). In most organisms, however, the egg is only polarized along a single axis during oogenesis, and the other axes are established via cell-cell interactions during subsequent development (Davidson, 1990; Melton, 1991). For example, in *Xenopus*, the oocyte possesses an obvious animal-vegetal axis; following fertilization the egg is transformed and its contents are redistributed so that it now possesses bilateral symmetry (Gerhart et al., 1989; Danilchik and Denegre, 1991). Subsequent inductive signals from the vegetal hemisphere promote mesoderm formation in the marginal zone, and probably involve exchange of growth factors and the local expression of other cell signaling and regulatory molecules (Gerhart et al., 1989; Smith et al., 1989; Ruiz i Altaba and Melton, 1990; Christian et al., 1991; Melton, 1991).

The sea urchin embryo is another classical system for

studying how polarized cues along the embryonic axes affect subsequent differentiation and morphogenesis during gastrulation. Egg bisection experiments demonstrate that polarity along the animal-vegetal axis of the sea urchin egg is established prior to fertilization, since the fertilized vegetal half of the egg can give rise to a largely normal pluteus larva, whereas the fertilized animal half of the egg usually gives rise to a ciliated *Dauerblastula* (Hörstadius, 1939; Maruyama et al., 1985). The blastomere recombination experiments of Hörstadius demonstrated that animal-vegetal polarity imparts differing properties to tiers of cells along the animal-vegetal axis and that cell-cell interactions between cells from different positions along this axis can result in embryos that are remarkably normal in appearance (Hörstadius, 1973). More recent experiments have shown that the mesomeres generated at the fourth cleavage are susceptible to signals originating from the micromeres, which form at the vegetal pole of the embryo (Khaner and Wilt, 1990; Livingston and Wilt, 1990a,b), as well as signals from neighboring mesomeres (Henry et al., 1989). These and other experiments have led to proposals regarding inductive interactions between cells in the vegetal region and cells in overlying tiers (Hörstadius, 1973; Wilt, 1987; Davidson, 1989; Henry et al., 1989; Livingston and Wilt, 1990a).

In comparison with the animal-vegetal axis, less is known about mechanisms of determination of the dorsoventral (or oral-aboral) axis of the sea urchin embryo. By performing lineage tracer injections, Cameron et al. (1989) have presented evidence that the orientation of the dorsoventral axis can be statistically inferred in *S. purpuratus* as early as the 2-cell stage. Recently, other species have been examined by Henry et al. (1992). In some species, a consistent relationship exists between the orientation of the first cleavage plane and the dorsoventral axis; in others there is little correlation. These studies suggest that the mechanisms underlying dorsoventral axis specification involve processes independent of cleavage orientation. Cameron and coworkers have also shown through lineage tracer injections that by the 32-cell stage dorsal and ventral founder cells give rise to descendants that reliably lie either within dorsal or ventral ectoderm, but not across this boundary in *Strongylocentrotus purpuratus* (Cameron et al., 1987; 1990; reviewed in Cameron and Davidson, 1991). Although these studies address the specification of dorsal and ventral fates in terms of cell lineage, they do not address the time during which dorsal and ventral tissues become *committed*, i.e., at what time they are restricted in their developmental potency as they adopt a dorsal or ventral pathway of differentiation.

Treatments that perturb differentiation along the dorsoventral axis would be useful tools for investigating dorsoventral axis determination in the sea urchin embryo. A few chemical treatments have been reported to affect the dorsoventral axis of the sea urchin embryo, including 8-chloroxanthine (Hörstadius and Gustafson, 1954) and sodium dodecyl sulfate (Gustafson and Sävhagen, 1949). The mechanisms of action of these treatments are not known, but they exert their effects quite early in development, during the early cleavage stages. In addition to these agents, two brief reports indicated that nickel (II) chloride might perturb the dorsoventral axis (Rulon, 1953; Lallier, 1956). We have investigated these effects in more detail. We report here that the dorsoventral axis of the sea urchin embryo can indeed be disrupted by treating embryos with $NiCl_2$, that the period of sensitivity to nickel immediately precedes gastrulation, and that the resulting disruption of the dorsoventral axis has dramatic consequences for gene expression and pattern formation during gastrulation. Some of this work has appeared in abstract form elsewhere (Hardin et al., 1990).

Materials and methods

Procurement of embryos

Gametes of *Lytechinus variegatus* were obtained by intracoelomic injection of 0.5 M KCl, dejellied by passage thorough several layers of cheesecloth, and fertilized with dilute sperm. Embryos were reared in stirring cultures in artificial sea water (ASW) at 19-22°C.

Videomicroscopy and morphometrics

Time-lapse videomicroscopy of embryos was performed as described previously (Hardin, 1989). Tracings of larval skeletons were made from the video monitor onto acetate and the tracings were subsequently subjected to morphometric analysis using a Summagraphics MacTablet connected to a Macintosh II computer and software described previously (Hardin, 1989).

Removal of primary mesenchyme cells

Primary mesenchyme cells were removed from embryos treated with 0.5 mM $NiCl_2$ from fertilization onward using the techniques of Ettensohn and McClay (1988). Embryos were mounted into Kiehart chambers (Kiehart, 1982) in the presence of 0.5 mM $NiCl_2$, and primary mesenchyme cells were flushed from the blastocoel with a stream of ASW. Following the operation, embryos were removed from the chambers and transferred to depression slides containing ASW with 0.5 mM $NiCl_2$. At the early gastrula stage, the embryos were rinsed several times by serially transferring them to depression slides containing normal ASW.

Tissue-specific probes

A 0.9 kb cDNA corresponding to a portion of the coding region of the aboral ectoderm-specific gene LvS1, ligated via *Eco*RI into pBluescript (Wessel et al., 1989), and a 1.9 kb cDNA corresponding to a portion of the coding region of a polyubiquitin gene from *Lytechinus pictus*, ligated to pBluescript via *Pst*I linkers (Gong et al., 1991), were kindly provided by Dr G. Wessel. A monoclonal antibody (mAb) directed against the ciliated band (mAb 95) was provided as ascites fluid by Dr D. Adelson (Adelson, 1985), and diluted 1:20 in ASW prior to use. mAb Ic12 was purified from hybridoma supernatants using a protein A-sepharose column (Coffman and McClay, 1990). mAb Va7 (Coffman and McClay, 1990), mAb Vc7 (Wessel and McClay, 1985) and mAb Ig8 (Wray and McClay, 1988) were used as undiluted hybridoma supernatants. Rhodamine-phalloidin (Molecular Probes, Eugene, Oregon) was used at a dilution of 1:10 in ASW. Affinity-purified rabbit polyclonal antibodies against 5-hydroxytryptophan were generously provided by Dr J. Lauder, and were used at a dilution of 1:50 in ASW.

Immunoprecipitations

Immunoprecipitations were performed essentially according to Coffman and McClay (1990). Embryos were labeled for 4 hours

with 0.5 mCi/ml L-[^{35}S]methionine (Trans label, ICN; specific activity > 1000 Ci/mmol), washed in sea water and homogenized on ice in extraction buffer (1 M glycine, 0.1 M NaCl, 2 mM EGTA, 1% Triton X-100, pH 8.2). Cleared lysates were incubated with mAb Ic12 (20 μg of protein A-purified IgG) or mAb 95 (ascites fluid, diluted 1:20) overnight at 4°C. 50 μl protein A-agarose (Repligen) was then added and the mixture was incubated for 1 hour at 4°C. The resulting immunoprecipitates were washed, resuspended in SDS sample buffer and subjected to SDS-PAGE. Gels were processed for fluorography, dried and placed on film (XAR-omat, Kodak) at −70°C for 20-48 hours.

Immunocytochemistry and histochemistry

Embryos were processed in one of two ways. For immunofluorescent detection of Ecto V, Endo 1, 95 antigen and msp130 homologue, embryos were fixed in 3.7% formalin in ASW for 1 hour, rinsed twice in ASW, permeabilized in ice-cold acetone for 10 minutes, and rinsed three times in ASW. For rhodamine phalloidin staining and immunofluorescent detection of 5-hydroxytryptophan, embryos were fixed for 1 hour in 3.7% formalin, washed twice in ASW, permeabilized in 0.3% Triton X-100 in ASW for 1 hour and rinsed three times in ASW. Embryos were incubated in a solution containing antibody or phalloidin for 1-2 hours at room temperature and rinsed twice in ASW. For immunofluorescence, embryos were incubated in fluorescein-conjugated goat anti-mouse secondary antibody (Cappel; diluted 1:25) for 1 hour at room temperature and rinsed twice in ASW. For detection of alkaline phosphatase activity, 100 μl of dilute embryo suspension were incubated in 0.05% naphthol-MX phosphate, pH 8.6 (Sigma), plus 1-2 grains of Fast Blue RR salt (Sigma) in ASW, and the colored product was developed for 5 minutes.

RNA extraction and northern blots

4 ml of packed embryos were collected by centrifugation, washed twice in 1 M glycine, 0.1 M NaCl, 2 mM EGTA, pH 8.0 and collected again. The washed pellet was homogenized in 15 ml guanidinium isothiocyanate buffer (6.0 M guanidinium isothiocyanate, 5.0 mM sodium citrate, pH 7.0, 0.1 β-mercaptoethanol, 0.5% sarkosyl), 1 g CsCl was added per 2.5 ml of homogenate, the suspension was layered over 5.7 M CsCl in 0.1 M EDTA (pH 7.5), and centrifuged at 35,000 revs/minute overnight to pellet the RNA. The resulting pellet was resuspended in 50 mM sodium citrate, 5.0 mM EDTA, 1.0% SDS and extracted with chloroform/butanol (4:1). The organic phase was reextracted with citrate/EDTA/SDS and the RNA was precipitated from the pooled aqueous fractions in 0.3 M sodium acetate and 70% ethanol. The pellet was collected, resuspended in water, reprecipitated twice more and finally resuspended in water. 10 μg per lane of the resulting total RNA was processed and electrophoresed on a 1.0% formaldehyde-agarose gel using standard techniques (Sambrook et al., 1989). Gels were blotted onto nitrocellulose for 20-24 hours via capillary transfer using 20×SSC, air dried and vacuum baked at 80°C for 2-4 hours.

cDNA probe preparation

cDNA inserts were excised from purified plasmids using the appropriate restriction enzymes, purified from 1% agarose gels by electroelution, extracted with phenol/chloroform and chloroform, and precipitated in 0.3 M sodium acetate and 70% ethanol. 50-150 ng of each insert were labeled via the random priming method (Feinberg and Vogelstein, 1983) using kit reagents (Pharmacia) and ^{32}P-dCTP (Amersham; sp. act. 3000 Ci/mmol) to a routine specific activity of $0.5\text{-}2.0\times10^9$ μCi/ng of DNA.

Northern blot hybridizations

Baked blots were incubated in prehybridization solution (25 mM K_3PO_4, pH 7.4, 5×SSC, 5×Denhart's solution, 50 μg/ml denatured salmon sperm DNA, 50% formamide) overnight at 42°C, and hybridized for 16-24 hours at 42°C in 15 ml hybridization solution (same as prehybridization solution, plus 10% dextran sulfate) with 3×10^5 counts/minute of probe/ml of hybridization solution. Blots were washed at 65°C in decreasing concentrations of SSC + 0.1% SDS (Sambrook et al., 1989); the final two washes contained 0.2×SSC. Washed blots were wrapped in plastic wrap and placed on film at −70°C for 20-48 hours with a Cronex Lightning Plus intensifying screen (Dupont). Prior to rehybridization with the polyubiquitin probe, blots were stripped by washing twice in 0.05×SSC, 10 mM EDTA (pH 8.0) just below boiling for 15 minutes, rinsed in 0.01×SSC and placed into prehybridization solution.

Densitometry

Bands on autoradiograms exposed within the linear range of the film were quantified by scanning densitometry using a ScanMan 32 scanner (Logitech Corp., Freemont, CA), an Apple Macintosh computer and the public domain program NIH Image.

In situ hybridizations

Lytechinus variegatus embryos were fixed in either Black's fixative (1% K_2CrO_7, 2.5% glacial acetic acid, 10% sucrose, 12% formalin in ASW) or 2% glutaraldehyde in ASW for 1-2 hours, dehydrated and embedded in Paraplast as described (Angerer et al., 1987). Sections cut at 4 μm thickness were mounted on slides coated with poly-L-lysine, deparaffinized and rehydrated. Hydrated slides were treated with proteinase K and acetic anhydride as described (Angerer et al., 1987). ^{3}H-labeled sense and antisense transcripts were synthesized from the *LvS1* fragment using T3 and T7 RNA polymerases, respectively, using [^{3}H]UTP (40-60 Ci/mmole; Amersham) to a specific activity of $\sim1\times10^8$ disints/minute/μg. Labeled probes were hybridized to sections, washed and prepared for autoradiography as described (Angerer et al., 1987).

Results

Nickel treatment results in graded disruption of the dorsoventral axis

When *Lytechinus variegatus* embryos are treated with 0.5 mM $NiCl_2$ from fertilization thorough the early gastrula stage, they adopt a characteristic morphology, which only becomes apparent during gastrulation (Fig. 1). Primary mesenchyme cells ingress on schedule and the archenteron invaginates (Fig. 1A), but as gastrulation proceeds the normal overt manifestations of dorsoventral polarity do not appear. Primary mesenchyme cells do not aggregate in two ventrolateral clusters as they do in normal embryos. The usual flattening of the ectoderm on the ventral side, which gives rise to the typical 'prism' larva, fails to occur. The two ventrolateral thickenings in the ectoderm that normally appear at the gastrula stage also fail to appear; instead, the ventrolateral ectoderm is thickened as a radial belt (Fig. 1B,C). The archenteron elongates, but its tip does not attach to the ventral ectoderm near the animal pole as it does in normal embryos (Hardin and McClay, 1990); instead it attaches directly at the animal pole (Fig. 1C). In normal embryos, the coelomic pouches form as two bilateral outpocketings, with the left pouch usually giving rise to the echinus rudiment (an imaginal structure, which contributes

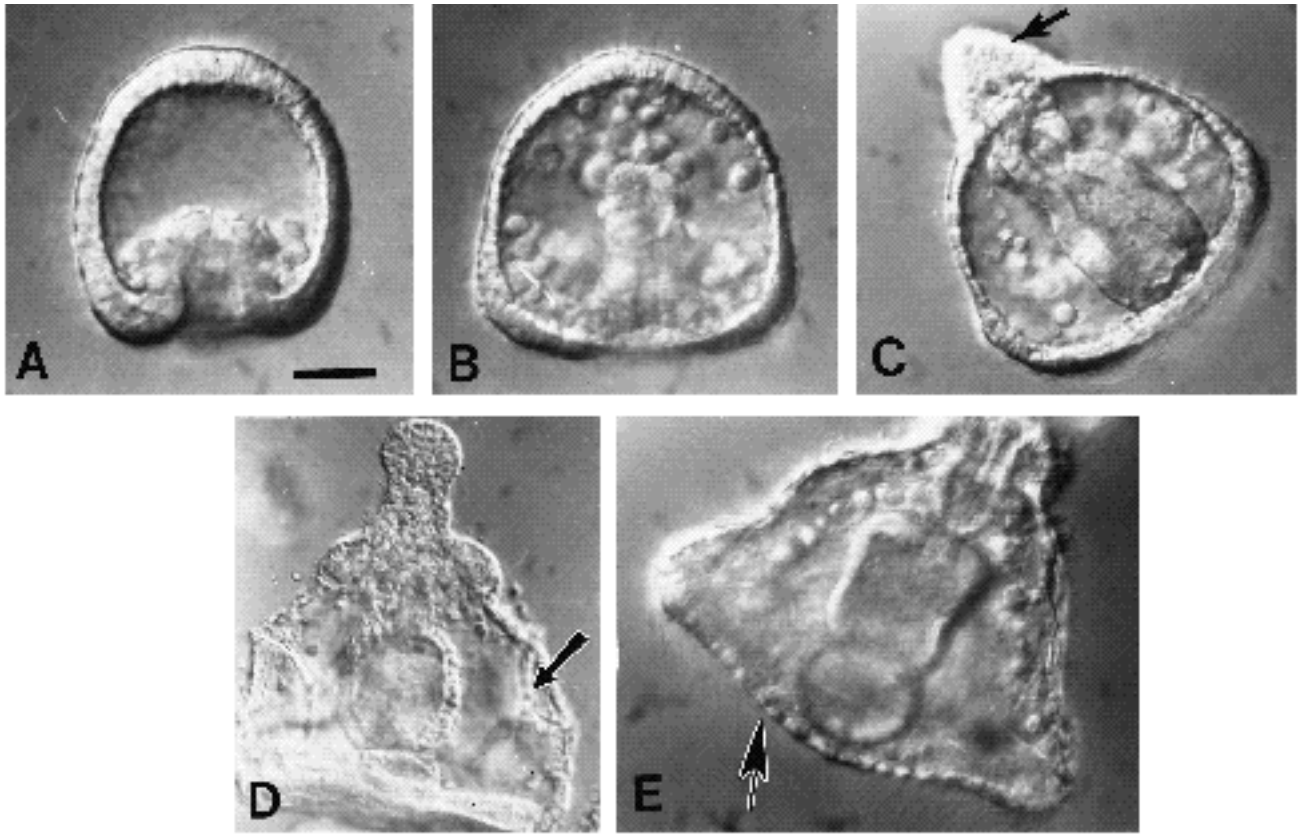

Fig. 1. Gastrulation in nickel-treated embryos. (A) Early gastrula, (B) late gastrula, (C) 'prism' stage embryo. Morphological differentiation of the gut occurs normally, but instead of the flattening of the ventral ectoderm and invagination of a stomodeum, a radially symmetric, cone-like constriction of the animal pole ectoderm occurs (large arrow), and an unpaired coelomic rudiment appears. (D) Early pluteus stage embryo. Note the formation of spicule rudiments (small arrow). (E) Advanced pluteus stage. Note the elongated cilia at the vegetal margin of the embryo (arrow). Bar = 25 μm.

to the juvenile urchin at metamorphosis: Gustafson and Wolpert, 1963; Czihak, 1971; Okazaki, 1975). In nickel-treated embryos, the coelomic pouch forms as a single, unpaired rudiment atop the archenteron (Fig. 1C). Following gastrulation, the ectoderm in the animal pole region begins to constrict, forming a cone-shaped 'proboscis' (Fig. 1D), which eventually pinches off as the tip of the archenteron fuses with it to produce a mouth (Fig. 1E). The primary mesenchyme cells form a skeleton, but instead of the usual two bilateral spicule rudiments, nickel-treated embryos can form as many as a dozen radially distributed spicules (see below). The ectoderm at the vegetal margin of the embryo possesses long cilia similar to those found in the ciliated band of normal embryos (Fig. 1E), a band of tissue that usually forms at the border between dorsal and ventral ectoderm (Czihak, 1971; Hörstadius, 1973; Cameron et al., 1990). Curiously, whereas normal embryos have numerous echinochrome-producing cells (Gibson and Burke, 1985), no pigment is produced in nickel-treated embryos.

To determine whether or not the disruption seen with 0.5 mM $NiCl_2$ was part of a graded series of defects, such as that seen in the anteroposterior axis of *Xenopus* (Gerhart et al., 1989; Kao and Elinson, 1988) or the dorsoventral axis of *Drosophila* (Anderson, 1987), embryos were treated with various concentrations of $NiCl_2$ from fertilization through the early gastrula stage. Typical results are shown in Fig. 2 for the concentrations tested. Below 0.5 mM $NiCl_2$, the severity of disruption of the dorsoventral axis steadily diminishes, until at a concentration of 0.05 mM $NiCl_2$ there are little or no observable defects as judged by external morphology (Fig. 2). At intermediate concentrations, there is a graded broadening of the oral hood and associated structures (Fig. 2B-D), with a concomitant shortening of the two long, postoral skeletal rods. As the concentration is raised, a bulbous constriction appears in the oral hood, which is quite similar to that seen in fully radialized embryos, the postoral rods shorten and the angle that they make with each other broadens, and supernumerary spicules appear (Fig. 2C,D). At a concentration of 0.25 mM $NiCl_2$, the bilaterality of the embryos virtually disappears (Fig. 2D) and is completely abolished at 0.5-1.0 mM $NiCl_2$ (Fig. 2E,F). At concentrations higher than 2.0 mM (Fig. 2G), metabolic poisoning seems to take place: the archenteron fails to elongate and motility of primary and secondary mesenchyme cells is suppressed (Fig. 2H).

The period of sensitivity to nickel immediately precedes gastrulation

To determine the period of sensitivity of *Lytechinus varie* -

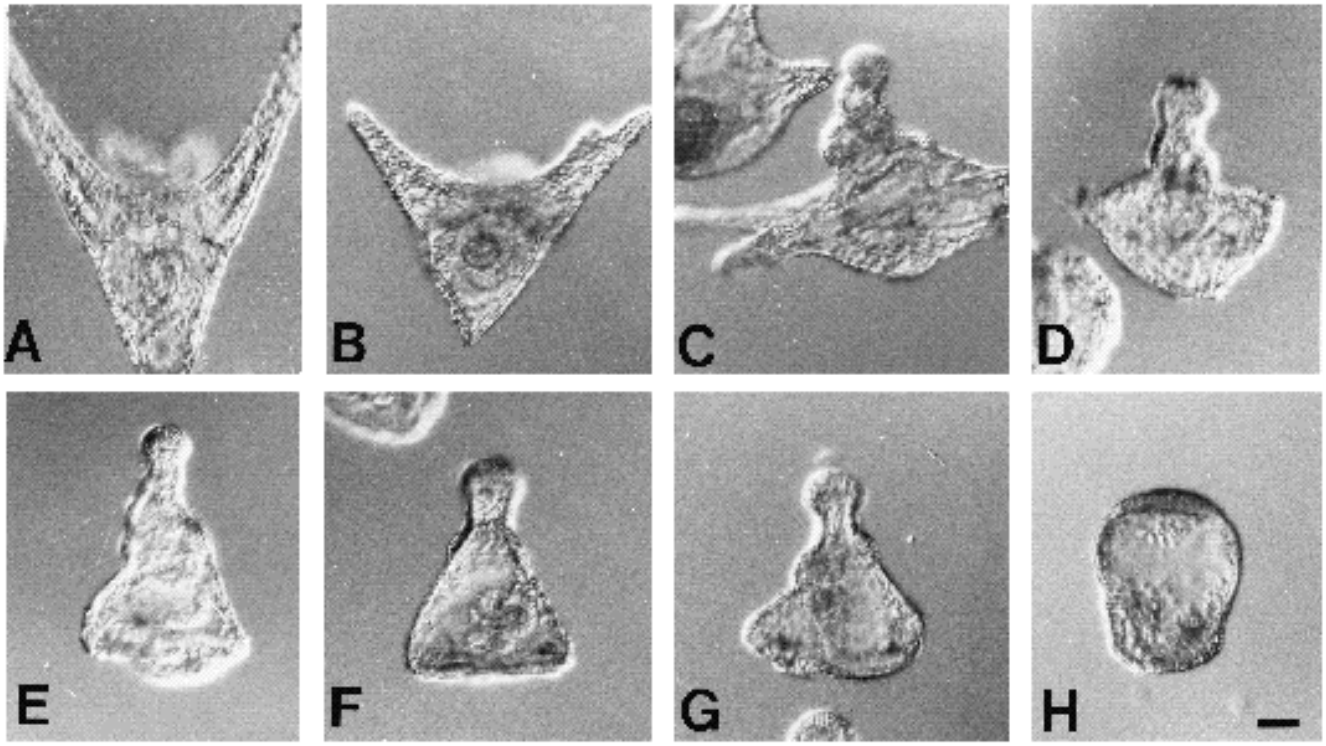

Fig. 2. Increasing doses of $NiCl_2$ produce graded effects on the dorsoventral axis. Embryos were treated from fertilization through the early gastrula stage. (A) 0 mM, (B) 0.05 mM, (C) 0.1 mM, (D) 0.2 mM, (E) 0.5 mM, (F) 1.0 mM, (G) 2.0 mM, (H) 5.0 mM. Bar = 25 μm.

gatus embryos to $NiCl_2$, embryos were pulsed with 0.5 mM $NiCl_2$ for various periods of time. The duration of the pulsed applications and their effects on larval morphology are summarized in schematic fashion in Fig. 3. A graded disruption of the dorsoventral axis was obtained if the duration of treatment extended for all or part of the period between the hatched blastula and early gastrula stages (Fig. 3). As with the graded concentration series, the severity of disruption of the dorsoventral axis steadily diminished as the total time of treatment during this sensitive period was decreased. In particular, $NiCl_2$ had no effect if it was removed by the hatched blastula stage, a result quite different from that obtained with lithium chloride, the classic 'vegetalizing' agent (Bäckström and Gustafson, 1954). Furthermore, the sensitive period extended quite late into development; not until the early gastrula stage did embryos become completely refractory to its effects. The morphology of the partially radialized embryos obtained by pulsed application of 0.5 mM $NiCl_2$ is quite similar to that obtained with suboptimal doses of $NiCl_2$.

Nickel does not disrupt animal-vegetal differentiation of the archenteron

A number of antigenic and histochemical markers are available to assay for differentiation of endodermal and mesodermal cells associated with the archenteron, and the results indicate that differentiation of these cell populations along the animal-vegetal axis is in many ways normal in nickel-treated embryos. The esophageal muscle bands, which surround the foregut and are responsible for its contractions during feeding, stain preferentially with rhodamine phalloidin at the pluteus stage (Harris, 1986). These muscle bands form in nickel-treated embryos, although the bilateral extensions of these bands present in the normal pluteus (Fig. 4A,B) are absent in nickel-treated embryos (Fig. 4C,D). The esophageal muscles are functional in radialized larvae; peristaltic contractions can be observed at regular intervals in the foreguts of such embryos. Fig. 4E-H shows normal and nickel-treated embryos stained for the Endo 1 antigen, a midgut and hindgut marker (Wessel and McClay, 1985). The boundary of expression, between midgut and foregut, is sharp in both cases. A histochemical assay for alkaline phosphatase activity, which also localizes to the midgut and hindgut, gives similar results (Fig. 4I,J). The Ecto V antigen, which localizes to the oral ectoderm and foregut after mouth fusion takes place, is also expressed in the correct location in the foregut (see below). In summary, these results indicate that nickel does not affect the correct animal-vegetal positioning of structures associated with the archenteron, although it does affect the symmetry of those structures that are ordinarily bilateral.

Nickel perturbs ectodermal differentiation

To investigate the effects of nickel on the dorsoventral axis of the sea urchin embryo at the molecular level, we have used three probes to assay for differentiation of the ectoderm in *L. variegatus*: (1) LvS1 cDNA, which is specific for dorsal (aboral) ectoderm (Wessel et al., 1989); (2) monoclonal antibodies Va7 and Ic12, which recognize the EctoV antigen, a ventral (oral) specific marker protein (Coffman and McClay, 1990); and (3) monoclonal antibody UH2-95, which is specific for the ciliated band (Adelson,

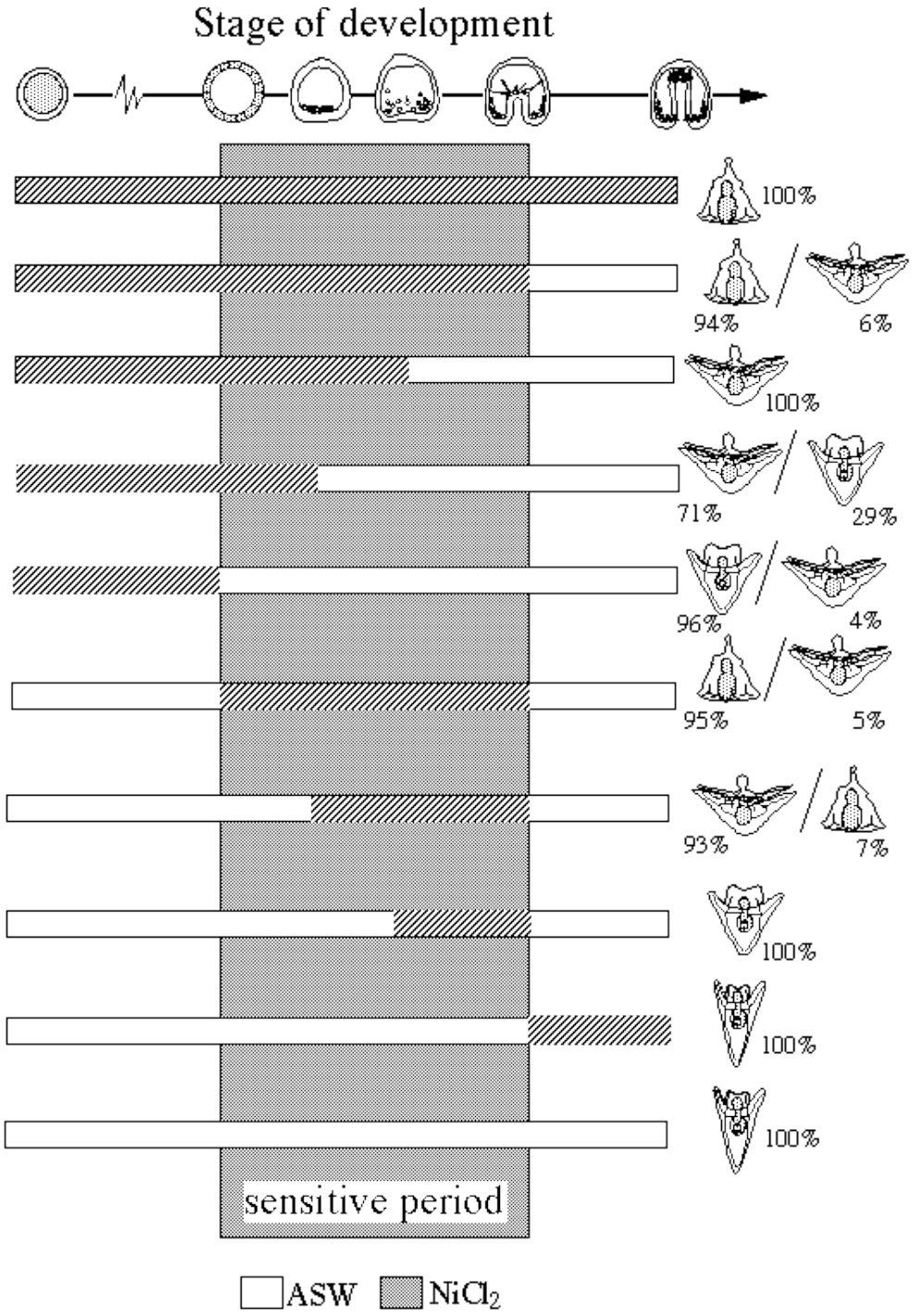

Fig. 3. Pulsed treatments of embryos with 0.5 mM $NiCl_2$. The period of treatment is indicated in each case, as are the percentages of each of the following phenotypes (shown schematically in the diagram): (1) fully radialized embryos, (2) partially radialized embryos with two longer postoral arms, (3) bilaterally symmetric embryos with enlarged oral hoods and broadened postoral arms, and (4) normal embryos. $n = 60$ embryos for each treatment period.

1985). In the normal embryo, the boundaries of expression of LvS1 message and the Ecto V antigen are non overlapping and complementary; LvS1 mRNA is restricted to the dorsal ectoderm, while the Ecto V antigen is restricted to the ventral ectoderm. The 95 antigen localizes to a belt of material at the boundary between these two major ectodermal territories. Thus, by using these three probes, it is possible to assay for the spatial extent of various regions of the ectoderm in nickel-treated embryos.

The overall levels of expression of dorsal and ventral markers are altered in response to $NiCl_2$. Fig. 5A shows the results of immunoprecipitations (Ecto V, 95 antigen) and northern blots (LvS1) performed with nickel-treated and normal embryos. In the case of the ventral marker, Ecto V, considerably more immunoprecipitable material is synthesized by nickel-treated versus normal embryos. In contrast, the globally distributed extracellular matrix protein echinonectin (Alliegro et al., 1988), which binds non-specifically to agarose and thus provides an internal control, is expressed at equal levels in the two types of embryo. Immunoprecipitation of the 95 antigen likewise reveals no difference in the level of expression of this molecule in nickel-treated and normal embryos. Conversely, the level of LvS1 mRNA is markedly reduced in nickel-treated

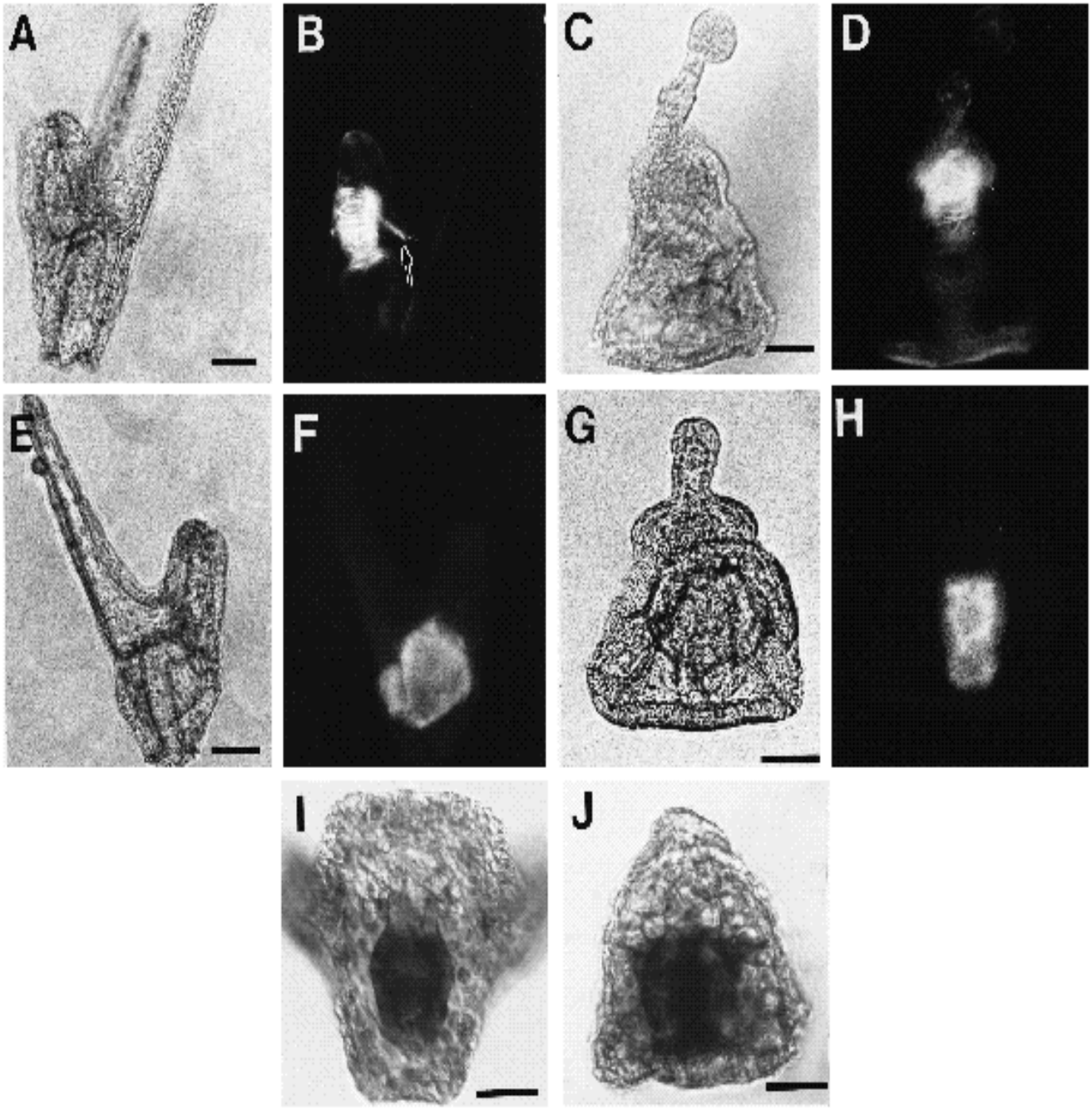

Fig. 4. Differentiation of the archenteron and associated structures in nickel-treated embryos. (A-D) Bright-field and epifluorescence views of normal (A,B) and nickel-treated (C,D) embryos stained with rhodamine phalloidin to reveal the esophageal muscle bands. Note one of the bilateral extensions of the muscle bands in the normal pluteus (B, arrow). (E-H) Bright-field and epifluorescence views of normal (E,F) and nickel-treated (G,H) embryos immunostained for the Endo 1 antigen. (I,J) Normal (I) and nickel-treated (J) embryos stained for alkaline phosphatase activity. Bar = 25 μm.

embryos as compared with controls. This underexpression is in contrast to the equal levels of polyubiquitin message expressed by the two types of embryo. These results are summarized by the densitometry data presented in Fig. 5B. The ventral marker Ecto V is expressed at fivefold higher levels than in controls, whereas the dorsal marker LvS1 is expressed at twofold to threefold lower levels. Echinonectin, polyubiquitin message and 95 antigen are expressed at equal levels by both groups.

Immunofluorescence and in situ hybridization reveal that these changes in expression of dorsal and ventral genes and gene products are due to changes in the spatial extent of their expression, rather than to simple changes in overall synthetic rates. Fig. 6A-D shows whole mount immunostaining of normal and nickel-treated embryos using antibodies directed against the Ecto V antigen. By the pluteus stage, ectodermal expression of Ecto V is restricted to the oral region of the ventral ectoderm (Fig. 6A,B). In nickel-

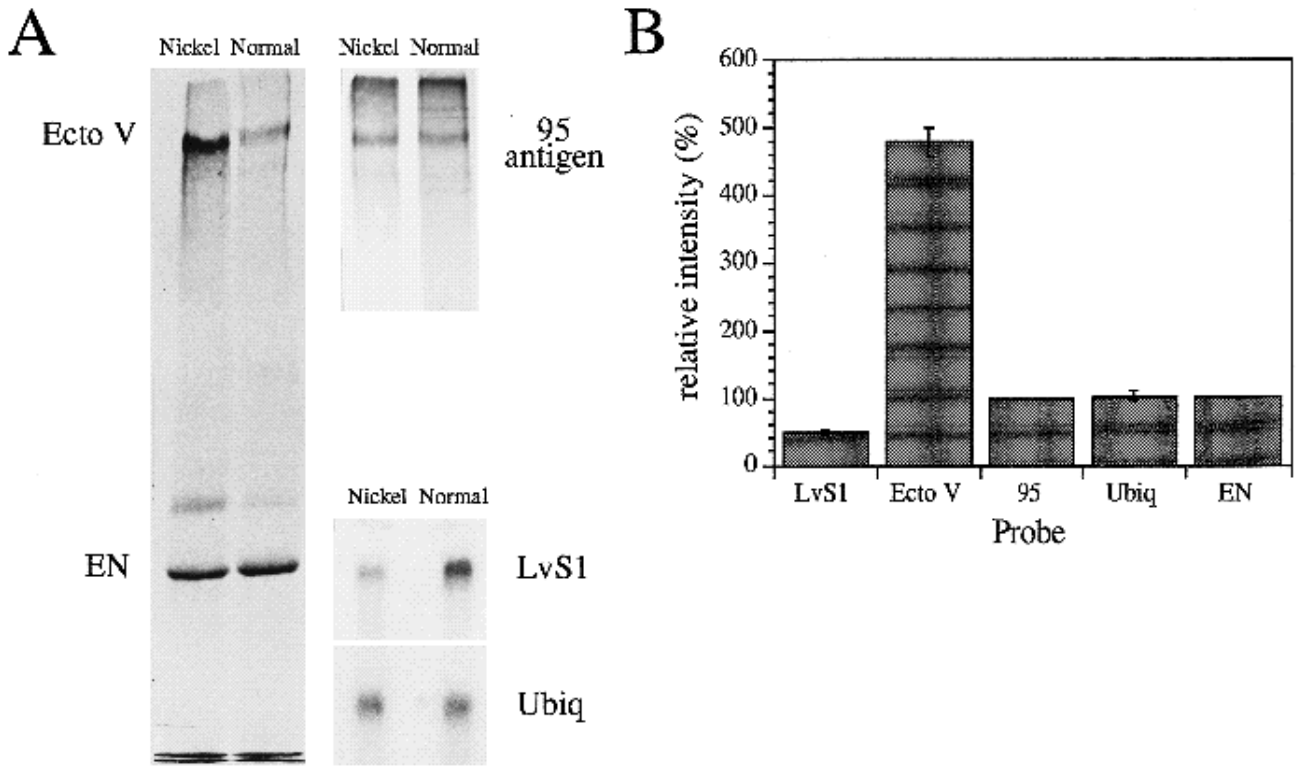

Fig. 5. (A) Expression of ectodermal genes and gene products in normal and nickel-treated embryos. Ecto V and 95 antigen expression was assayed by immunoprecipitation of ^{35}S-labeled proteins from equal volumes of pelleted embryos. Echinonectin protein, which binds non-specifically to the agarose beads used in the immunoprecipitation, is also indicated (EN). Levels of LvS1 transcripts were assayed by hybridization of a ^{32}P-labeled LvS1 cDNA to a northern blot of 10 µg of total RNA from normal and nickel-treated embryos per lane. The same blot was subsequently washed and reprobed with a polyubiquitin cDNA (Ubiq). (B) Relative levels of transcripts and proteins in nickel-treated embryos as compared to controls. Densitometry results are expressed for nickel-treated embryos as a percentage of control levels in replicate experiments (mean ± s.e.m.).

treated embryos, Ecto V is expressed radially, and the total area of expression is increased (Fig. 6C,D). By the pluteus stage, ectodermal expression of LvS1 is restricted to the dorsal (aboral) ectoderm in normal embryos (Fig. 6E,F). Sections of nickel-treated embryos taken from the same slide show little or no expression of LvS1, although in some cases signal is detected in the lateral ectoderm (Fig. 6G,H). In normal embryos, expression of the 95 antigen is restricted to the ciliated band region, which is at the margin of the oral ectoderm in normal embryos (Fig. 6I,J). In nickel-treated embryos, the 95 antigen is expressed radially at the vegetal margin of the embryo, shifting its position so that it no longer overlaps the domain of Ecto V expression (Fig. 6K,L). Taken together, these results indicate that nickel treatment results in changes in the position of the three major regions of ectoderm in *Lytechinus variegatus*; in addition, the sizes of the dorsal and ventral domains also appear to change.

Since the disruption of the dorsoventral axis as judged by morphological criteria occurs in a graded fashion, it was of interest to examine partially radialized embryos using the three ectodermal probes. Fig. 7 shows the expression patterns of LvS1 mRNA, Ecto V and the 95 antigen in such embryos. The ventral antigen, Ecto V, shows an expanded domain of expression compared with controls (Fig. 7A,B). In transverse section, it is clear that partially radialized embryos are somewhat flattened on one side, reminiscent of the ventral flattening of normal embryos. The ectoderm opposite this region expresses LvS1 message in a pattern complementary to that of Ecto V (Fig. 7C,D). The ciliated band, which normally forms a contiguous belt, becomes separated into two domains, one associated with the enlarged oral hood, and the other associated with the shortened arms of the pluteus stage larva (Fig. 7E,F); in progressively more radialized larvae, the region associated with the oral hood shrinks, until only the belt of material at the vegetal margin of the embryo remains positive for the 95 antigen (see above).

Nickel treatment disrupts pattern formation by primary mesenchyme cells

The patterning of primary mesenchyme cells in the sea urchin embryo has been thought to be generated by the interaction of these cells with the underlying ectoderm and its associated basal lamina, with the final pattern arising from the interplay between behaviors intrinsic to the primary mesenchyme cells and information imparted by the ectoderm (Gustafson and Wolpert, 1961; Okazaki et al., 1962; Harkey and Whiteley, 1980; Ettensohn and McClay, 1986; Solursh, 1986; Ettensohn, 1990). Since the location and amount of dorsal and ventral tissues is disrupted in nickel-treated embryos, pattern formation relating to the

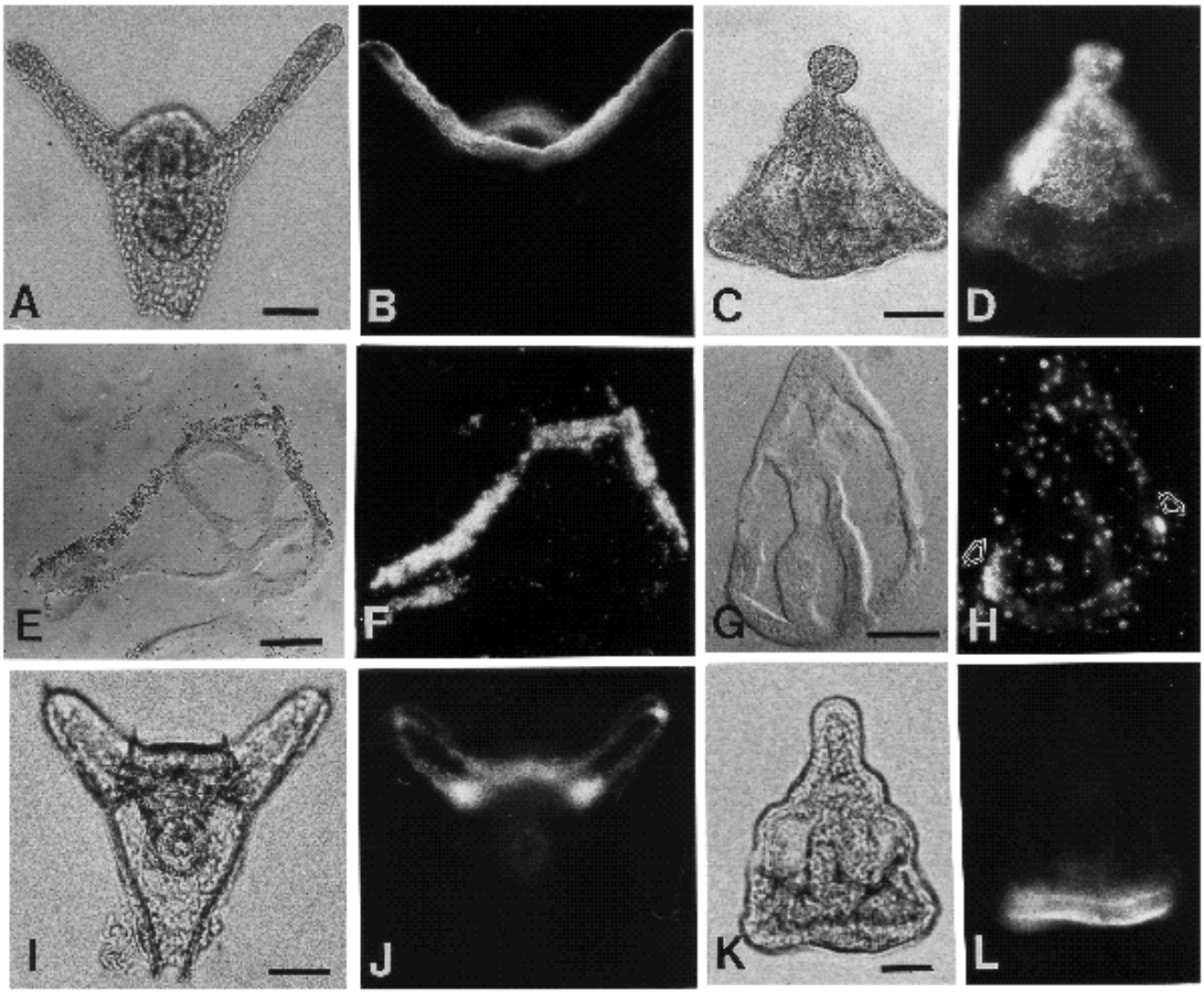

Fig. 6. Spatial expression of ectodermal probes in normal and nickel-treated embryos. (A-D) Bright-field and epifluorescence views of normal (A,B) and nickel-treated (C,D) embryos immunostained for the Ecto V antigen. (E-H) Differential interference contrast and dark-field views of sections processed for in situ hybridization and probed with LvS1 antisense RNA. Sense transcripts gave no detectable signal (data not shown). (E,F) Normal pluteus, (G,H) nickel-treated pluteus stage embryo. LvS1 expression is strongly reduced, although in some cases signal is detectable (arrows). (I-L) Bright-field and epifluorescence views of normal (I,J) and nickel-treated (K,L) embryos immunostained for the ciliated band marker, 95 antigen. Bars = 25 μm.

larval skeleton would be predicted to be perturbed as well. Fig. 8 shows that pattern formation by primary mesenchyme cells is severely disrupted in nickel-treated embryos. Primary mesenchyme cells can be identified using monoclonal antibody Ig8, which recognizes the primary mesenchyme-specific *Lytechinus variegatus* homologue of the msp130 protein (Leaf et al., 1987; Wray and McClay, 1988). Ig8-positive cells localize in a ring at the vegetal margin of the embryo (Fig. 8A). Polarization microscopy reveals that as many as a dozen small, triradiate spicule rudiments can form in a radial arrangement in nickel-treated embryos (Fig. 8B). As they grow in length, these rudiments usually, although not always, adopt the correct orientation (i.e., two of the three extensions point vegetally and laterally, while the third points towards the animal pole), but their morphology and size are altered dramatically, resulting in many more roughly identical spicules of shorter length (Fig. 8C,D). In particular, most of the side branches are absent in nickel-treated embryos, although disorganized branches can be seen in more mature skeletons from nickel-treated embryos (data not shown). This pattern is in stark contrast to the normal one, in which two bilateral spicule rudiments elongate to form the various portions of the skeleton (Fig. 8E).

That the observed defects in skeletal pattern do not reflect gross changes in the differentiation of primary mesenchyme cells is indicated by several independent assays. First, the number of primary mesenchyme cells can be assessed by using the monoclonal antibody Ig8. The number of primary mesenchyme cells in nickel-treated plutei is 62.6 ± 0.7 (mean ± s.e.m., n = 14 embryos), which is statistically indistinguishable at the 99% confidence level from the number in normal plutei (61.6 ± 0.6, n = 15; cf. Ettensohn and McClay, 1988). In addition, morphometric analysis indi-

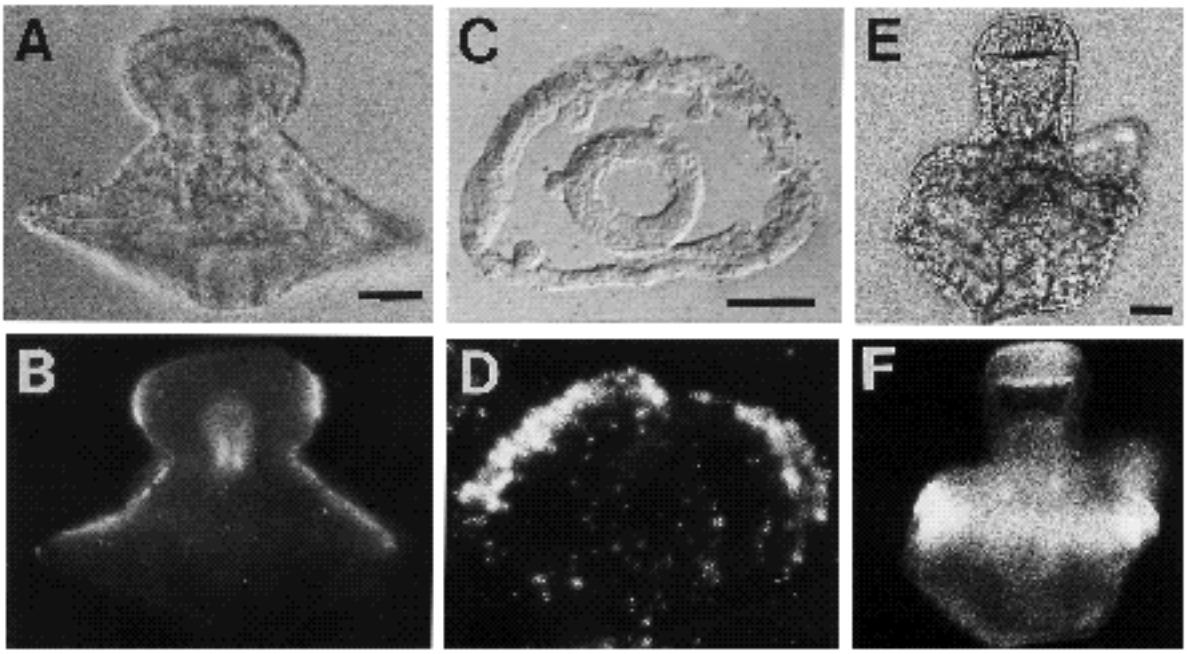

Fig. 7. Expression of ectodermal markers in partially radialized embryos. (A-B) Bright-field and epifluorescence views of a partially radialized pluteus immunostained for the Ecto V antigen. (C-D) Differential interference contrast and dark-field views of a transverse section thorough a partially radialized embryo processed for in situ hybridization and probed with LvS1 antisense RNA. (E,F) Bright-field and epifluorescence views of a partially radialized pluteus immunostained for the ciliated band marker, 95 antigen. Bars = 25 μm.

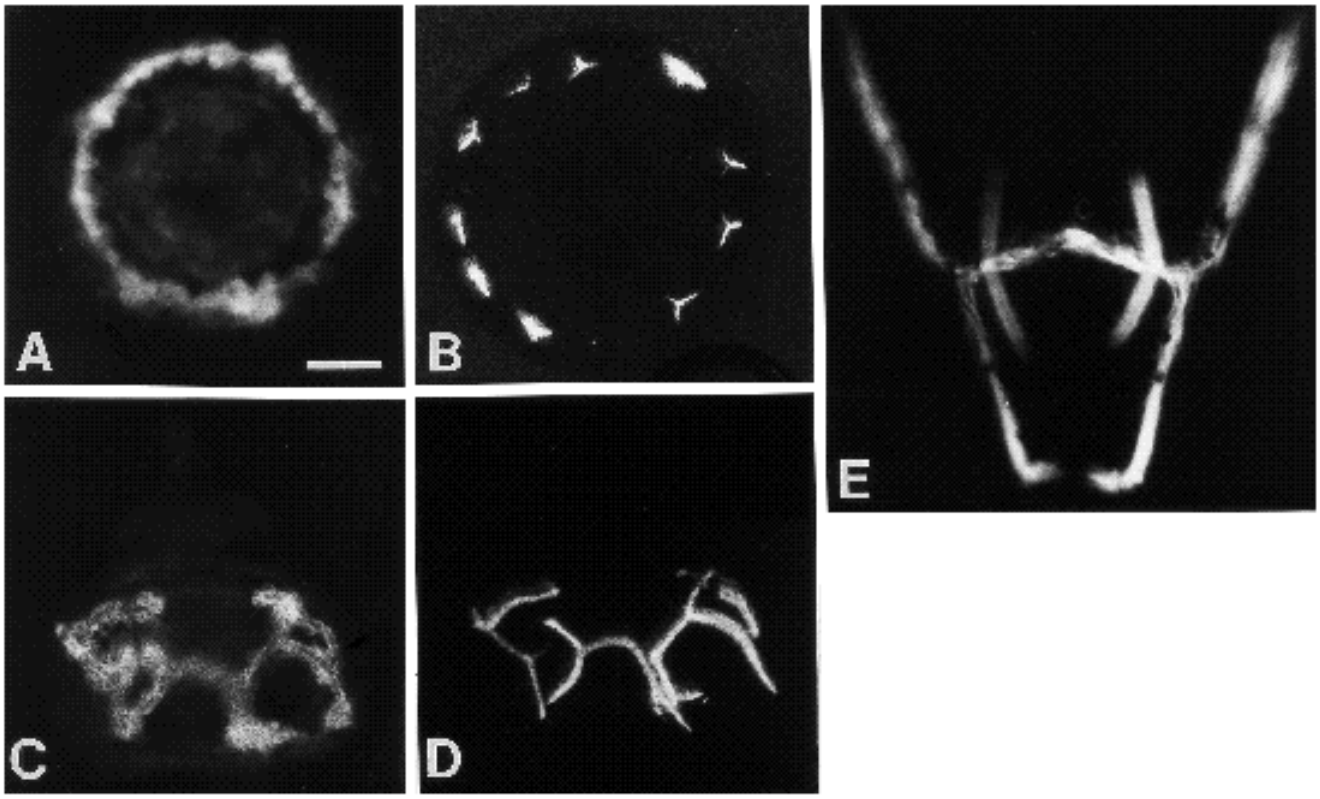

Fig. 8. Disruption of pattern formation in nickel-treated embryos. (A) Immunofluorescent localization of primary mesenchyme cells in a nickel-treated 'prism' stage embryo using monoclonal antibody Ig8, as viewed from the vegetal pole. (B) 'Prism' stage embryo viewed from the vegetal pole by polarization microscopy. (C) A nickel-treated pluteus-stage embryo, immunostained to reveal the pattern of primary mesenchyme cells. (D) The same embryo viewed using polarization optics. (E) Normal pluteus viewed using polarization optics. Bar = 25 μm.

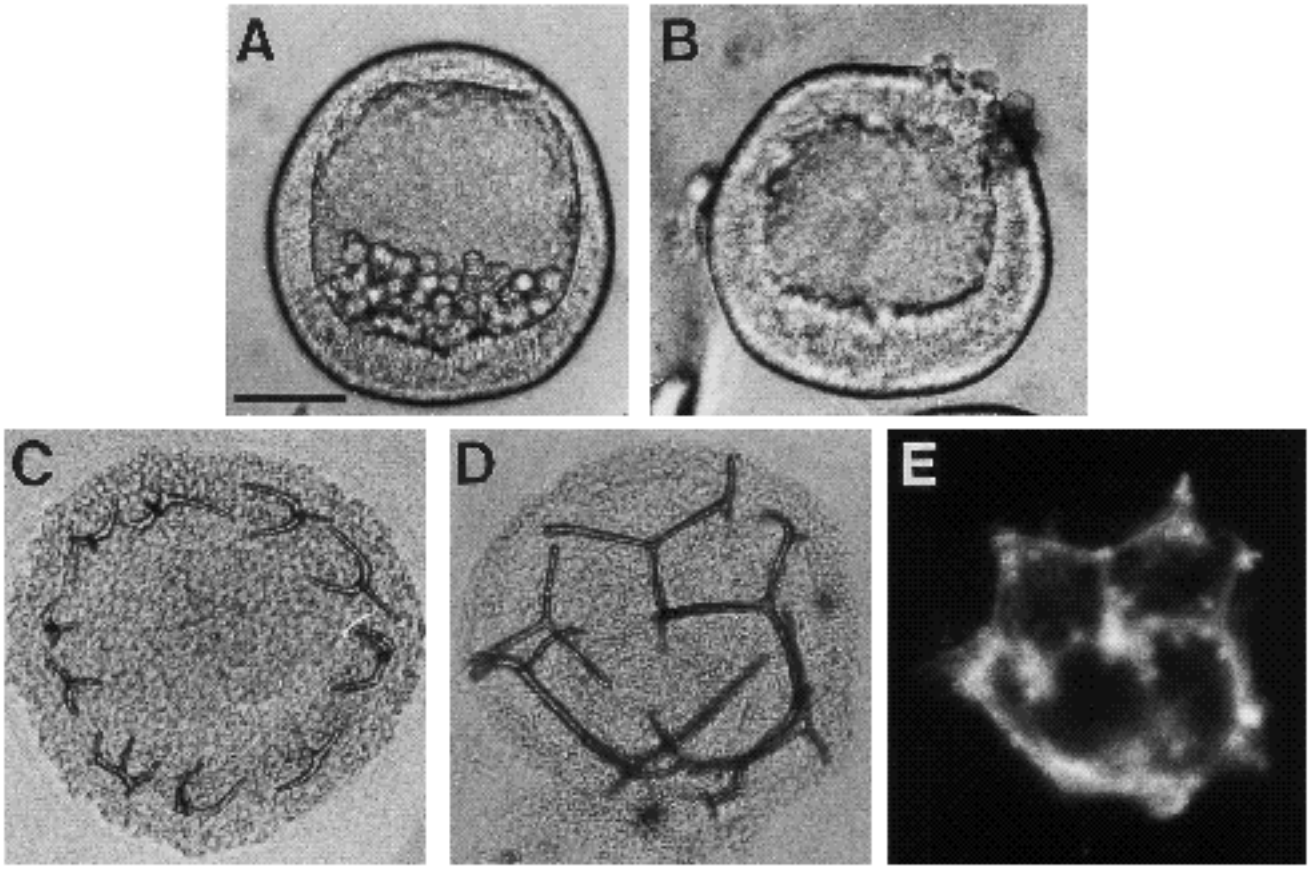

Fig. 9. 'Conversion' of secondary mesenchyme cells in nickel-treated embryos. (A) A nickel-treated mesenchyme blastula prior to removal of primary mesenchyme cells, (B) a nickel-treated embryo following removal of primary mesenchyme cells, (C) operated embryo 8 hour after removal of primary mesenchyme cells, (D) operated embryo 26 hour after removal of primary mesenchyme cells and (E) the same embryo immunostained with monoclonal antibody Ig8. Bar = 25 μm.

cates that the total length of spicule material produced by nickel-treated plutei is actually slightly less than that in normal plutei: nickel-treated plutei produce 1059.4 ± 43.0 μm of spicule per embryo (mean ± s.e.m., n = 11 embryos), whereas normal plutei produce 1261.6 ± 26.2 μm (n = 11). Thus the greatly increased number of spicule rudiments is not simply due to overproduction of spicule material, but does reflect a pattern defect.

Finally, primary mesenchyme cells in nickel-treated embryos participate in normal cell-cell interactions with secondary mesenchyme cells. When all primary mesenchyme cells are removed from a normal mesenchyme blastula, after a delay of several hours, a population of secondary mesenchyme cells migrates away from the tip of the archenteron to the sites where primary mesenchyme cells would ordinarily aggregate. These cells then express the msp130 homologue, and go on to produce an essentially normal skeleton (Ettensohn and McClay, 1988). Such 'conversion' indicates that under normal circumstances primary mesenchyme cells somehow suppress this response in secondary mesenchyme cells via cell-cell interactions (Ettensohn and McClay, 1988). All primary mesenchyme cells can be removed from a nickel-treated embryo as well (Fig. 9A,B), and then the embryo can be washed free of the nickel at the early gastrula stage to allow for subsequent development. The results of such an experiment are shown in Fig. 9C-E. After a delay of 4-8 hours, secondary mesenchyme cells also undergo the conversion response, secreting the msp130 homologue on their cell surfaces and producing a skeleton. Furthermore, the conversion response is quantitatively similar to the response in normal embryos. The number of msp130-positive cells is 61.5 ± 1.5 (mean ± s.e.m., n = 6 embryos), which is very similar to the result obtained with normal embryos when all primary mesenchyme cells are removed (cf. Ettensohn and McClay, 1988). These results indicate that cell-cell interactions between primary and secondary mesenchyme cells are not seriously impaired. However, as with the primary mesenchyme cells, the pattern of the resulting skeleton is severely affected. The converted cells localize near the vegetal margin of the embryo as they do in normal embryos, but instead of the bilateral skeleton produced by converted cells in untreated embryos, the skeleton is completely radialized (Fig. 9C-E).

Nickel treatment alters mouth formation

In normal embryos, secondary mesenchyme cells send out long filopodial protrusions in cycles of extension, attachment and retraction, until they eventually make stable contacts with an anatomically defined site in the ventral ectoderm near the animal pole which will later form the mouth of the larva (Hardin and McClay, 1990). Time-lapse

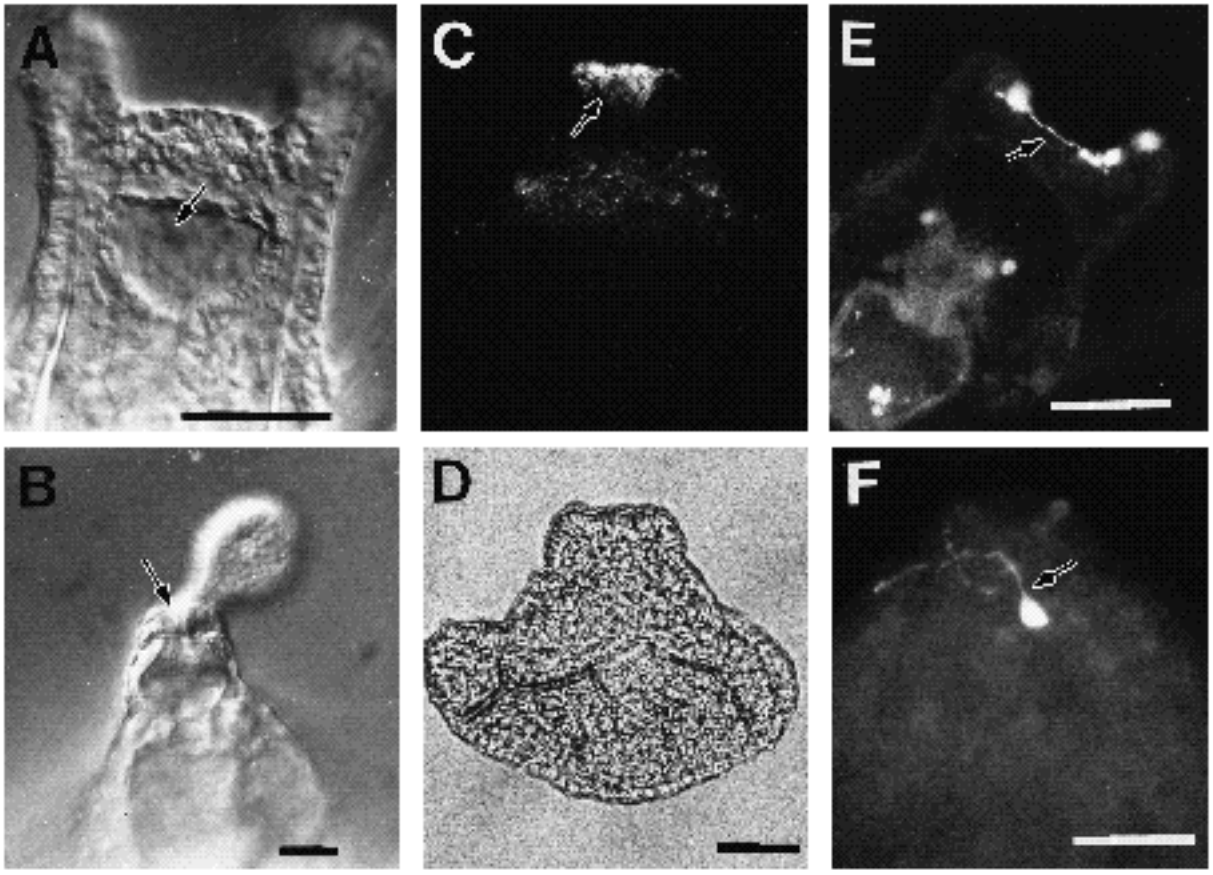

Fig. 10. Alterations in mouth formation in nickel-treated embryos. (A) Normal mouth formation. The tip of the archenteron fuses with the ectoderm in the oral hood region to produce an oral opening (arrow). (B) Mouth formation in an embryo treated with 0.5 mM $NiCl_2$. The tip of the 'proboscis' detaches to produce a mouth (arrow). (C,D) Epifluorescence and bright-field views of a living, advanced radialized larva immunostained for the Ecto V antigen. The mouth region specifically stains with the antibody (arrow). (E,F) Epifluorescence micrographs of normal and nickel-treated embryos immunostained for 5-hydroxytryptophan. (E) Normal embryo. The serotonergic nerve net in the oral hood is clearly visible (arrow). (F) An advanced nickel-treated larva. Serotonergic cells are present, but they are not organized (arrow). Bar = 25 μm.

videomicroscopy reveals that secondary mesenchyme cells in nickel-treated embryos show similar activity, but rather than localizing to a discrete region in the ventral ectoderm, they appear to attach to a cone or ring of ectoderm near the animal pole (data not shown). Eventually, the archenteron elongates across the blastocoel and its tip attaches to the animal pole. As in normal embryos (Fig. 10A), fusion of the ectoderm and the tip of the archenteron eventually takes place, with the result that a functional oral opening is produced (Fig. 10B). Frequent peristaltic contractions occur in the foregut and cilia actively sweep small particles into the lumen of the gut. After mouth fusion takes place, the Ecto V antigen can be detected by immunofluorescence in the mouths of living nickel-treated embryos, as in normal embryos (Coffman and McClay, 1990), but the mouth does not form in the same location as it would normally (Fig. 10C,D). In addition to these defects in mouth formation, some structures associated with the oral region fail to form properly. In the normal pluteus, a serotonergic nerve net appears in the oral hood at the pluteus stage (Bisgrove and Burke, 1986), forming a highly organized array of nerve cells (Fig. 10E). In nickel-treated embryos, an occasional serotonergic cell can be found near the mouth, but the neurites of such cells appear to be randomly extended and they display no ordered localization into a discrete nerve net (Fig. 10F).

Discussion

Dorsoventral tissues are not fully committed until the gastrula stage in the sea urchin embryo

The early sea urchin embryo develops in a relatively simple manner via the establishment of five major tissue 'territories'. These territories, defined by both cell lineages and patterns of gene expression, arise as clonal descendants of lineage founder cells through consistent cleavage patterns (Cameron and Davidson, 1991). Lineage-specific patterns of gene expression presumably arise thorough the binding of trans-acting DNA-binding proteins to tissue-specific regulatory elements in these lineage-specific genes (reviewed by Davidson, 1989). For such events to lead to tissue-specific gene expression, the amounts of these proteins and/or their ability to bind to target DNA must vary in dif-

ferent regions of the embryo (Davidson, 1989). How such differences arise in time and space requires a fuller understanding of dorsoventral axis specification in the sea urchin embryo.

Two different embryological approaches have been taken to attempt to understand how cells differentiate along the dorsoventral axis. The first involves examination of lineage relationships between cells in various territories of the embryo. Cameron and coworkers have shown through lineage tracer injections that dorsal and ventral lineages are distinct by the 32-cell stage in *Strongylocentrotus purpuratus* (Cameron et al., 1987; 1990). However, issues of cell commitment must also be addressed experimentally. Using this second approach, early experiments involving compression, ligation and aspiration of embryos all indicated that cells in the early embryo possess a tremendous amount of developmental plasticity regarding their differentiation along the dorsoventral axis (reviews: Hörstadius, 1973; Wilt, 1987; Davidson, 1989).

Less is known about how such plasticity is gradually restricted prior to the appearance of the definitive dorsoventral axis at gastrulation. However, our results indicate that the dorsoventral axis of the sea urchin embryo can be perturbed until immediately prior to its apparent fixation at gastrulation. This result is consistent with the experiments of Jenkinson (1911), Hörstadius (1936), and Hörstadius and Wolsky (1936) on the differentiation of bilaterally symmetric structures in isolated fragments of embryos. They found a progressive loss of the ability of bisected embryos to regulate the full spectrum of dorsoventral structures, until at the early gastrula stage such ability was completely lost.

It is curious that the sensitive period for other chemical treatments (e.g. non-ionic detergents, metabolic poisons) precedes the sensitive period for nickel. In the case of 8-chloroxanthine, even unfertilized eggs can be treated with the chemical and effects can be observed on dorsoventral polarity (Hörstadius and Gustafson, 1954). However, dorsoventral axis determination is clearly a multistep process in other organisms such as *Drosophila* (Akam, 1987; Levine, 1988; Rushlow and Arora, 1990; Heitzler and Simpson, 1991; St. Johnston and Nüsslein-Volhard, 1992) and *Xenopus* (Gerhart et al., 1989; Ruiz i Altaba and Melton, 1990; Melton, 1991), and so it is not surprising that different chemical agents might affect different steps in this process in the sea urchin embryo. That nickel can still perturb the dorsoventral axis of the sea urchin embryo when added at the late mesenchyme blastula stage suggests that the ion is affecting a late phase of dorsoventral axis specification.

Nickel appears to shift ectoderm cells toward ventral fates

Based on both morphological evidence and results using available tissue-specific probes, the number of cells committed to a ventral pathway of differentiation seems to be increased with a corresponding decrease in the number of dorsal cells. This conclusion is based on the following observations: (1) embryos that are partially radialized show a progressive enlargement of oral structures (e.g., the oral hood); (2) such embryos also display an increase in both the domain and level of expression of the ventral-specific marker, Ecto V; and (3) they show a simultaneous loss of expression of the dorsal marker, LvS1. These results are summarized and interpreted schematically in Fig. 11. We have chosen to maintain the relationship between the three tissue boundaries (i.e., ciliated band still lies between dorsal and ventral ectoderm). However, based on our data, we cannot conclusively assign precise tissue boundaries. In particular, the assignment of positions to the ciliated band and the remaining dorsal/aboral ectoderm are somewhat ambiguous, given the uncertainties in interpreting the planes of section of in situ hybridizations in those cases where a detectable signal was present with LvS1. Because only one probe currently exists for each of the major ectodermal tissues in *Lytechinus variegatus*, it is also possible that, when other probes become available, the disruption of ectodermal differentiation induced by nickel may turn out to be more complicated. Finally, it is already known that homologous ectodermal genes are regulated in somewhat different ways in different species of sea urchin (Franks et al., 1988; Wessel et al., 1989), so the effects that we have observed in *Lytechinus variegatus* may not be identical with those observed in other species in the future.

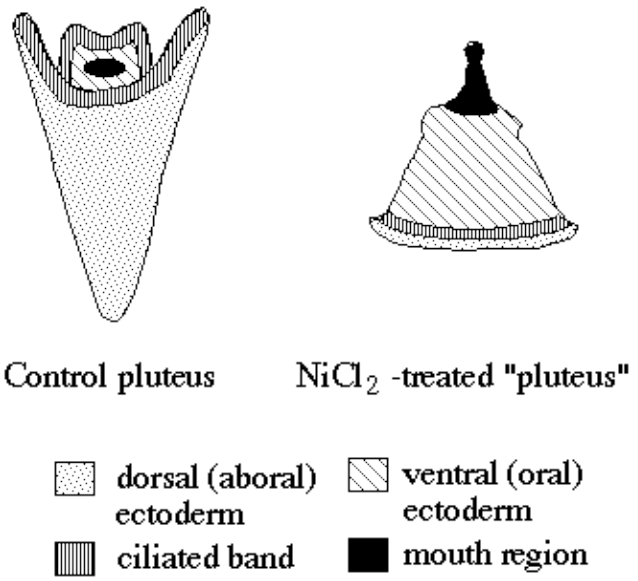

Fig. 11. A schematic interpretation of the results of this study regarding the effects of nickel treatment on ectodermal differentiation. The regions committed to various types of ectodermal tissue are altered in location and/or spatial extent in nickel-treated embryos, resulting in an over commitment of cells to an oral/ventral pathway of differentiation at the expense of dorsal/aboral tissue. The ciliated band, which normally forms at the margin between dorsal and ventral ectoderm, is concomitantly shifted in position. Based on our data, we cannot conclusively assign precise positions to the ciliated band and the remaining domain of dorsal/aboral ectoderm (see text).

Although a major effect of nickel treatment is the disruption of normal ectodermal differentiation, it should be noted that other effects are apparent, including the failure of pigment cells to differentiate morphologically, and some abnormalities in the formation of bilateral mesodermal structures (e.g., the coelomic pouches and extensions of the esophageal muscle bands). It is not known whether the changes in mesodermal tissues result directly from effects

on the cells they comprise, or whether they result from the disruption of ectodermal cues required by the mesoderm. Since no markers of dorsoventral or right-left polarity within the mesoderm are currently available, this question remains open.

The molecular mechanisms by which nickel exerts its effects are not known. In other systems, higher concentrations of Ni^{2+} can perturb a variety of biological processes, including conductance of calcium channels, transitions between b- and z- forms of DNA, and gap junctional communication (Sunderman, 1989). Nickel has also been known for some time to be a carcinogen in vertebrates, indicating that at some level it has effects on vertebrate gene expression (Sunderman, 1989). Although it is clear that nickel does have profound effects on gene expression in the sea urchin embryo, it far from clear what signals mediate these effects. There has been a report that another inorganic calcium channel blocker, cobalt, produces radialized sand dollar embryos (Rulon, 1953), but these results cannot be reproduced in sea urchin embryos (Lallier, 1956), so what role calcium may play in the effects observed with nickel is unclear.

Nickel as a potential tool for studying pattern formation by mesenchyme cells

The ectoderm is presumed to play an important role in specifying the intricate pattern that primary mesenchyme cells adopt during gastrulation (Gustafson and Wolpert, 1963, 1967; Okazaki, 1975; Harkey and Whiteley, 1980; Ettensohn and McClay, 1986; Solursh, 1986; Wilt, 1987; Ettensohn, 1990; Hardin, 1990), as well as the attachment of secondary mesenchyme cells to the animal pole at the end of gastrulation (Hardin and McClay, 1990). However, it has been difficult to alter the distribution of various types of ectodermal tissue in the sea urchin embryo. One exception is lithium chloride, which is known to shift the fates of cells along the animal-vegetal axis (see, for example, Runnström, 1935; Child, 1940; Hörstadius, 1973; Livingston and Wilt, 1990b). Okazaki et al. (1962) observed that primary mesenchyme cells localize at sites in the ectoderm where the epithelial cells are thickened. When this belt of cells is shifted along the animal-vegetal axis by lithium chloride, primary mesenchyme cells localize to the shifted ectoderm (Gustafson and Wolpert, 1961; Okazaki et al., 1962). These data suggest that regionally specific information in the ectoderm helps to specify the pattern of primary mesenchyme cells. In light of its effects on ectodermal differentiation, nickel may be a promising tool for studying how ectodermal differentiation influences mesenchymal pattern. By transplanting nickel-treated primary mesenchyme cells into normal host embryos and normal mesenchyme cells into nickel-treated hosts, it should be possible to determine whether the pattern defects that we have observed are due predominantly to disruption of ectodermal differentiation or also involve direct effects on mesenchyme cells. We are currently performing such experiments, which promise to shed additional light on how mesenchyme and ectoderm interact during sea urchin gastrulation (Armstrong et al., 1992, manuscript in preparation). How ectodermal differentiation normally occurs, and how it leads to the appearance of localized information required for pattern formation during gastrulation remain central questions for further investigation.

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