

## The expression of the *C. elegans* labial-like *Hox* gene *ceh-13* during early embryogenesis relies on cell fate and on anteroposterior cell polarity

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### SUMMARY

Clusters of homeobox-containing *HOM-C/hox* genes determine the morphology of animal body plans and body parts and are thought to mediate positional information. Here, we describe the onset of embryonic expression of *ceh-13*, the *Caenorhabditis elegans* orthologue of the *Drosophila labial* gene, which is the earliest gene of the *C. elegans Hox* gene cluster to be activated in *C. elegans* development. At the beginning of gastrulation, *ceh-13* is asymmetrically expressed in posterior daughters of anteroposterior divisions, first in the posterior daughter of the intestinal precursor cell E and then in all posterior daughters of the

AB descendants ABxxx. In this paper, we present evidence that supports position-independent activation of *ceh-13* during early *C. elegans* embryogenesis, which integrates cell fate determinants and cell polarity cues. Our findings imply that mechanisms other than cell-extrinsic anteroposterior positional signals play an important role in the activation and regulation of the *C. elegans Hox* gene *ceh-13*.

Key words: homeobox, asymmetry, *Caenorhabditis elegans*, polarity, intestine

### INTRODUCTION

The underlying principle of every developmental program is that single cells divide to give rise to daughter cells with distinct developmental fates. Differences between daughter cells can originate from differences in the daughter cells' environment or can be generated by unequal segregation of cell fate determinants during oriented cell division. The development of the nematode *Caenorhabditis elegans* follows a remarkably reproducible lineage pattern (Sulston et al., 1983; Sulston and Horvitz, 1977), whereby oriented asymmetric cleavages play essential roles (reviewed by Guo and Kemphues, 1996). Oriented asymmetric divisions produce cells in appropriate spatial relationships that differ in size, cell cycle timing, cell fate and cleavage spindle orientation (Sulston et al., 1983). Many of these asymmetric cleavages are externally triggered. At the 4-cell stage, for example, the P<sub>2</sub> cell induces the EMS cell both to become polarized to give rise to distinct daughters MS and E (intestinal precursor) and to direct its division axis for a correct partitioning of developmental potential (Schierenberg, 1987; Goldstein, 1992, 1993, 1995a,b; Bossinger and Schierenberg, 1996).

*C. elegans* has a repertoire of developmental regulatory genes that is remarkably similar to that found in other animals with less determinant cell lineages. These genes include the homeobox-containing *HOM-C/Hox* gene family that are found

in a wide variety of organisms. They determine the animal body plan and are thought to mediate positional information. *C. elegans* has a cluster of four evolutionarily conserved *HOM-C/Hox* genes (Bürglin et al., 1991; Bürglin and Ruvkun, 1993; Kenyon and Wang, 1991; Wang et al., 1993; Wilson et al., 1994; Hunter and Kenyon, 1995) which, during development, are expressed in consecutive domains along the anteroposterior body axis (Wang et al., 1993; Clark et al., 1993; Wittmann et al., unpublished data).

In this paper, we describe the early expression of *ceh-13*, the orthologue of the *Drosophila labial* gene (Schaller et al., 1990; Sharkey et al., 1997; Wittmann et al., unpublished data). *ceh-13* is the earliest gene of the *C. elegans Hox* gene cluster to be activated in development. Its protein product first appears in the 26-cell-stage embryos in the Ep cell and soon afterwards also in the posterior daughters of anteroposterior cell divisions in the AB lineage. *ceh-13* has a dynamic expression pattern, which shows the typical characteristics of a labial gene (Wittmann et al., unpublished data). Using *skn-1* and *pop-1* mutant backgrounds that change cell fates, as well as by performing trypan blue experiments to block cell-cell interactions, we show that *ceh-13* expression in Ep relies on the intestinal fate and is independent of the position of the cell in the embryo. This finding is in agreement with the recent data on *mab-5* (Cowing and Kenyon, 1996) and suggests that the expression pattern of the *C. elegans Hox* genes must be established by a

complex lineage-based molecular mechanism that differs from that operating in *Drosophila* and vertebrates, where *HOM-C/Hox* gene expression is strongly influenced by the position of a nucleus or a cell in the embryo. Furthermore, we show that the onset of early *ceh-13* expression depends on asymmetric cell divisions along the anteroposterior axis, thus providing strong evidence for an animal-wide system directing anteroposterior cellular polarity during embryonic development.

## MATERIALS AND METHODS

### Worm strains and culture methods

*C. elegans* strains were cultured using standard conditions (Brenner, 1974). The following mutations and rearrangements were used in this analysis:

LG I: *pop-1(zu189)*, *lin-17(n671)*, *hT1(I;V)*

LG II: *rol-6(n1276e187)*, *swIs1[rol-6(su1006) + ceh-13::GFP]*

LG IV: *skn-1(zn67)*, *nT1(IV;V)*

LG V: *him-5(e1490)*

The *pop-1(zu189)* mutation was given to us by Dr. J. Priess, Seattle (USA) and the other strains used in this study were provided by the *Caenorhabditis* Genetics Center at the University of Minnesota, USA. The strain FR307 is of the genotype *swEx307[rol-6(su1006) + ceh-13::GFP]; rol-6(n1276e187)II*.

### Immunolocalization

Anti-CEH-13 was used as a primary antibody at a working dilution of 1:40 to 1:100 (Wittmann et al., unpublished data). The secondary antibodies, anti-rabbit IgG FITC conjugate (Sigma F-0382), were incubated overnight at 4°C with fixed worms in the following proportions: 150 µl of antibody for 200 µl of compact worms and 200 µl of antibody buffer A (AbA; Finney and Ruvkun, 1990). After this purification step, they were used at a working dilution of 1:10. Embryos were immunostained on poly-lysine slides as described (Bowerman et al., 1993; Okkema and Fire, 1994) or, for better results, by a non-freeze-crack large-scale method. For this, embryos were collected by hypochlorite treatment (Lewis and Fleming, 1995), rinsed at least twice in M9 and pure water. They were resuspended in a fixation solution containing freshly solubilized 1-2% paraformaldehyde, 80 mM KCl, 20 mM NaCl, 10 mM EGTA, 5 mM spermidine HCl, 30 mM sodium Pipes pH 7.4 and 50% methanol, immediately frozen in dry-ice/ethanol or liquid nitrogen. Embryos were thawed on ice, incubated on ice for 1 hour, washed once with 100 mM Tris-HCl pH 7.4, 1% Triton X-100, 1 mM EDTA and blocked for half an hour in AbB (Antibody buffer B; Finney and Ruvkun, 1990). Incubation with primary antibodies was conducted overnight at room temperature in AbA + goat serum (DAKO X907; at least in a 1:2000 dilution). Embryos were washed in 3-4 changes of AbB (Finney and Ruvkun, 1990) at room temperature before incubation with the secondary antibodies. Embryos were mounted on 0.5% poly-lysine slides or on agarose pads (2% agarose in 50mM Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>) in a 1:1 ratio of 100 mM Tris pH 9.5 and Vectashield (Vector Inc. Burlingame, CA; H-1000) + 2 mg/ml DAPI. Fluorescence was enhanced using 50 to 100 W mercury bulbs and specific filters: 450-490 nm for FITC and 365 nm for DAPI.

### *C. elegans* germline transformation and in vivo observation

The translational *ceh-13::gfp* fusion gene (Chalfie et al., 1994; Heim et al., 1995) used in this study was constructed by inserting the *PstI* fragment derived from pPDpst37 (Wittmann et al., unpublished data) into the vector pPD95.69 (provided by A. Fire). This construct encodes a CEH-13::GFP fusion protein. Injection procedure, genome integration and transgenic homozygote selection were performed as

described for the β-galactosidase fusion (Mello and Fire, 1995; Wittmann et al., unpublished data). Of the stable lines, FR301 was chosen for the integration of the transforming DNA into a chromosomal locus. *swEx301[rol-6(su1006) + ceh-13::gfp]; rol-6(n1276e187)II* worms were treated with 1500 rads of gamma radiation and their F<sub>3</sub> progeny screened for the presence of 100% Rol-6 individuals, resulting in the isolation of the integration *swIs1[rol-6(su1006) + ceh-13::gfp]* in the strain FR308. FR308 hermaphrodites were out-crossed to N2 males 5 times (FR317) and the site of integration was mapped to LG II. To visualize the CEH-13::GFP, worms were excited with a 450-490 nm light source (50 to 100 W mercury bulb).

### Whole-mount in situ hybridization

mRNA in situ hybridization was executed as described (Seydoux and Fire, 1995) with a prolongation of the detection step to overnight. The two 356 bp and 839 bp long antisense probes were generated on gel-purified *EcoRI* inserts from the *ceh-13* cDNA clone Bar23C with the primers 5002 (5'-TTTCGTCTATCACCTTC-3') and 5006 (5'-GATG-GAGGTGGAAGAGTG-3'), respectively. As control, the sense probe was produced with the primer 3270 (5'-GCTCATCCATCCAACCTAC-3') and a 650 bp long *SacI* fragment from Bar23C (length of the probe: 536 b). Another negative control was performed with a pBS plasmid probe, which was produced by amplifying *NaeI* linearized plasmid with a T7 primer.

### Blastomere culture and analysis

AB and P<sub>1</sub> blastomeres were isolated from the N2 strain and from FR307 (*swEx307[rol-6(su1006) + ceh-13::gfp]; rol-6(n1276e187)II*); 2/6AB blastomeres and 6/8 P<sub>1</sub> blastomeres were from N2 and were examined using the anti-CEH-13 antibody; the rest were examined for GFP as above. Methods for removing eggshells and vitelline membranes and for culturing *C. elegans* cells have been described (Edgar, 1995). Isolated cells were identified on the basis of size; for example, in the 2-cell-stage AB is larger than P<sub>1</sub>. Fixation and immunostaining was performed according to Goldstein (1995b).

### Blastomere identification and lineage analysis

Ep and ABxxxp (see Results and Fig. 1) were identified as *ceh-13*-expressing cells by reconstructing immunolocalized 28-cell-stage embryos. Embryos were photographed (see below for image processing) and comparison of these pictures with the original embryos and hand-made models (Sulston et al., 1983) allowed the final reconstruction. For detailed cell lineage analysis in wild-type embryos and in mutant backgrounds, CEH-13::GFP-expressing embryos were mounted in egg salts on 2% agarose pads, sealed with nail polish and submitted to manual 4-D recording. Specific cells were traced until selected time-points.

### Trypan blue incubation, blastomere removal, cytochalasin D block and blastomere removal

Eggs were dissected out of gravid adults with a scalpel in a drop of distilled water on a microscope slide. Uncleaved zygotes were identified under the dissecting microscope and transferred with a drawn-out glass mouth pipette to a second microscope slide, carrying either a thin layer of 3-5% agar in H<sub>2</sub>O as a cushion (blastomere removal) or a poly-lysine layer (cytochalasin D blocks, blastomere removal). The eggs were covered with a coverslip and the edges sealed with Vaseline (Sulston and Horvitz, 1977). Manipulations were performed under Nomarski optics using a ×100 oil immersion objective. The impermeable eggshell and underlying vitelline membrane were perforated with brief pulses of a N<sub>2</sub>-pumped, microscope coupled dye laser (Lambda Physics, Göttingen; Schierenberg, 1984) by using the laser dye Rhodamine 6G. Penetration was performed in cell culture media containing 15% fetal calf serum (Edgar, 1995) with 14 mg/ml trypan blue (Sigma Nr 961). This concentration reliably blocked early cellular interactions without showing toxic effects (Bossinger and

Schierenberg, 1996). To neutralize the inhibitive effect of trypan blue in cleavage-blocking experiments and after blastomere isolation, 40% instead of 15% FCS was used (Bossinger and Schierenberg, 1996). Cell division of 1-, 2- and 4-cell stages were inhibited by adding 5 µg/ml cytochalasin D to the cell culture medium (Bossinger and Schierenberg, 1992). For the blastomere isolation experiments, cells were extruded through a laser-induced hole in the eggshell as described in Schierenberg and Wood (1985).

### Image processing

Figs 1A-D and 3 are the result of Fuji PROVIA 400 slides taken with an Olympus OM 4-Ti camera mounted on a Zeiss Axioplan microscope. These pictures were either digitalized (Fig. 1A-D) or exposed on photography paper using the Cibachrome procedure (Fig. 3). Fig. 4 was assembled from 6-10 second-integrated fluorescent signals. The image analysis system consisted of a Hamamatsu chilled CCD C5985 camera mounted on the Zeiss Axioplan microscope (118.11 pixels/cm), a Hamamatsu chilled CCD driver C6391 and a Hamamatsu image processor Argus-20. Figs 1E, 5 and 7 are frame-grabbed pictures (28.346 pixels/cm) from video recordings, using a Panasonic WV-BC700 video camera (without infrared filter) and a time-lapse video recorder (Panasonic, AG-6720-E), linked to a Leica DM IRBE inverted microscope. The sensitivity of the Panasonic camera was virtually enhanced (up to  $\times 32$ ) with the Panasonic WV-CU204 camera control unit (Bossinger and Schierenberg, 1992) and a Hamamatsu image processor Argus-10. The frame-grabbing was realized using the Movie Machine II (Fast Multimedia AG) videocard and the Movie TV II (release 1.02, version 2.02) software in the Windows environment. Final image processing was performed in the MacIntosh environment, using the Adobe Photoshop 3.0 software (US patent 5146346). A final resolution of 300 dpi was chosen. Manipulations, such as contrast enhancement, color balance refinement, color curve adjustment or filtering were always applied to the total surface of the picture for avoiding any modifications of the original signal(s). Confocal microscopy was performed on a Biorad confocal microscope and images were processed in Adobe Photoshop as above.

## RESULTS

### Early embryonic *ceh-13* activation occurs asymmetrically in different cell lineages

By using specific anti-CEH-13 antibodies, weak CEH-13 expression was first detected in 26-cell-stage embryos in the intestinal precursor cell Ep. At the 28-cell stage, the nucleus of Ep was brightly stained (Fig. 1A,C). Nuclear CEH-13 also appeared in posterior-ventral ABxxxp cells and cytoplasmatic staining was detectable in anterior-dorsal ABxxxp cells (Fig. 1A-D). ABxxxp cells showed higher CEH-13 levels than ABxxap cells. During the next round of division, the CEH-13 protein was inherited by the daughters of Ep and ABxxxp cells, and became nuclear in all cells. The antibody staining pattern matched exactly that of a *ceh-13::lacZ* reporter gene and of the identically regulated *ceh-13::gfp* reporter gene in strain FR317 (Fig. 1E). Furthermore, confocal microscopical analysis of 50-cell-stage embryos containing the *ceh-13::gfp* marker gene revealed low levels of *ceh-13* expression in the Da and Dp cells (Fig. 1F). A summary of the first *ceh-13* activation in the AB, D and E lineages is shown in Fig. 2.

In situ hybridization experiments revealed that *ceh-13* mRNA was not present at early embryonic stages. It appeared first in the Ep cell of 26-cell-stage embryos and soon afterwards in all ABxxxp cells, only shortly before CEH-13 (Fig. 3 and data not shown). This suggested that *ceh-13* is an early

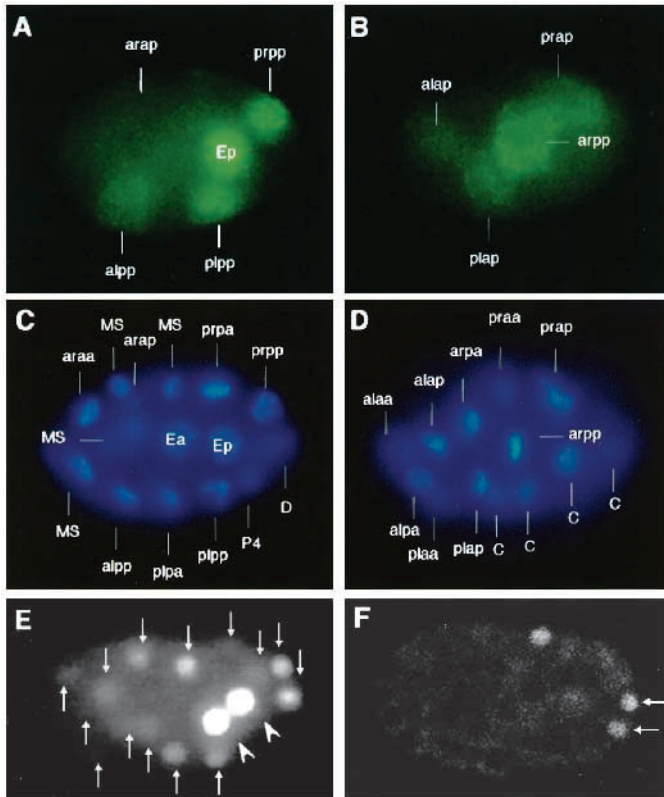
zygotic gene, whose expression pattern is determined at the level of transcription. Therefore, and since the anti-CEH-13 and the CEH-13::LACZ or CEH-13::GFP patterns overlapped exactly, we conclude that the strain containing the integrated *ceh-13::gfp* reporter gene (FR317) can be used to analyse the establishment of the CEH-13 expression pattern during early embryonic development in more detail.

### Correct *ceh-13* expression in the Ep cell depends on its intestinal fate but not on the position of the cell in the embryo

The fact that early *ceh-13* expression occurs in the AB, D and E lineage, but not in the MS and C lineages (see Fig. 2), suggested that the onset of *ceh-13* expression may depend on specific lineage and cell fate cues. To address this hypothesis, we have tested whether the Ep cell fate is necessary and sufficient for *ceh-13* expression. First, we analysed the *ceh-13::gfp* expression pattern in a *pop-1(zu189)* mutant background. *pop-1(zu189)* is a maternal-effect lethal mutation and in embryos isolated from *pop-1* homozygous mothers, the anterior daughter of EMS, MS, adopts the fate of its posterior sister cell E (Lin et al., 1995). The genomic *ceh-13::gfp* insertion (swIs1) was crossed into the *pop-1 lin-17/hT1(I;V)I; him-5/hT1(I;V)V* strain. Embryos from *pop-1 lin-17 I; swIs1[rol-6(su1006) ceh-13::gfp]II* homozygotes were lineaged until we could detect fluorescence, i.e., until the Epl and Epr cells were generated (we have looked at Epl and Epr, since they usually show stronger fluorescence than the Ep cell). We then looked for ectopic *ceh-13* expression in the MS descendants. Out of 18 lineaged embryos, 4 showed bright and 8 showed weak signals in the MSpa and MSpp cells (Fig. 4A). No signal was observed in any of the MSa daughters. Furthermore, MSa and MSp daughters in *lin-17 I; swIs1[rol-6(su1006) ceh-13::gfp]II* control embryos ( $n=6$ ) did not show fluorescence at all (Fig. 4B). Given these results, we conclude that the E fate is sufficient for ectopic expression of *ceh-13* in the MSp cell.

To determine whether gut determination is necessary for *ceh-13* expression in Ep, we have incubated FR307 4-cell-stage embryos (*swEx307[rol-6(su1006) + ceh-13::GFP]; rol-6(n1276e187)II*) with trypan blue. This polysulfated hydrocarbon dye binds strongly to the cell membranes and inhibits the interaction between P<sub>2</sub> and EMS and thus prevents gut induction, resulting in two EMS daughters that both have MS-like characteristics (Bossinger and Schierenberg, 1996). The treated embryos ( $n=12$ ) show several signs of normal cell differentiation, e.g. muscle twitching and programmed cell death, indicating that trypan blue in the applied concentration is not generally toxic (Bossinger and Schierenberg, 1996). However, using two different markers (birefringency and binding of the gut-specific antibody ICB4; Laufer et al., 1980; Okamoto and Thomson, 1985), no signs of gut differentiation could be found (12/12). Furthermore, in trypan-blue-treated FR307 embryos, the 4-EMS descendants behaved like MS cells and did not show any CEH-13::GFP fluorescence (data not shown), suggesting that gut induction is necessary for normal CEH-13 expression in Ep.

To further investigate this question, we have analyzed the distribution of CEH-13::GFP in the *skn-1(zu67)* background. Mutations in the maternal-effect gene *skn-1* prevent EMS from producing both pharyngeal and intestinal cells (Bowerman et al., 1992). Instead, its daughters E and MS both make body



**Fig. 1.** Expression of *ceh-13* in *C. elegans* early embryos. (A–D) 28-cell-stage embryo (this embryo has 16 AB-derived cells, we call it a 16-AB stage). (A, C) Ventral and (B, D) dorsal views. (A, B) Immunofluorescent staining of Ep and ABxxxp cells with anti-CEH-13 antibodies. The AB<sub>arap</sub> cell stains very weakly. (C, D) The same embryo stained with the DNA-binding dye DAPI. The names of the blastomeres are indicated (see Materials and Methods for blastomere identification); lower cases are used for AB-derivatives. (E) 32-AB-stage embryo expressing the *ceh-13::gfp* transgene. All fluorescent nuclei are visible. CEH-13::GFP is present in Epl and Epr (arrowheads) and in 14 out of 16 daughters of ABxxxp (arrows). The image has been generated by adding fluorescent pictures of two different focal planes. (F) Confocal microscope section of an about 50-cell-stage embryo showing CEH-13::GFP in Da and Dp (arrows). All embryos are oriented with the anterior end to the left. Embryos measure approximately 50  $\mu$ m along their anteroposterior axis.

wall muscles and hypodermal cells similar to the C blastomere, the anterior daughter of P<sub>2</sub> (Bowermann et al., 1992). We have lineaged embryos from *skn-1(IV); swIs1[rol-6(su1006)+ceh-13::gfp] II* (FR525) homozygous mothers until the 2 daughters of Ep were born and scored for the presence of CEH-13::GFP in these cells. Since the *zu67* allele shows incomplete penetrance (Bowerman et al., 1992, 1993), we also scored gut fluorescence in our lineaged embryos after a few hours of further development. The Ep daughters of *skn-1* embryos with differentiated gut ( $n=10$ ) always showed wild-type levels of CEH-13 (equal levels of GFP fluorescence in Ep daughters and AB descendants, Fig. 4D). Out of 21 lineaged embryos that did not develop gut granules, however, 15 showed a significantly reduced CEH-13::GFP signal in the two daughter cells of Ep (Fig. 4C, E, F). This demonstrates that mutations in *skn-1* can interfere with the normal expression of *ceh-13*, but only in Ep cells that do not differentiate gut granules. Surprisingly, 6 out

of 21 lineaged *skn-1* animals that do not form gut granules showed normal *ceh-13* expression in the Ep daughter cells. Again, this may be caused by incomplete penetrance of the *skn-1* allele *zu67*.

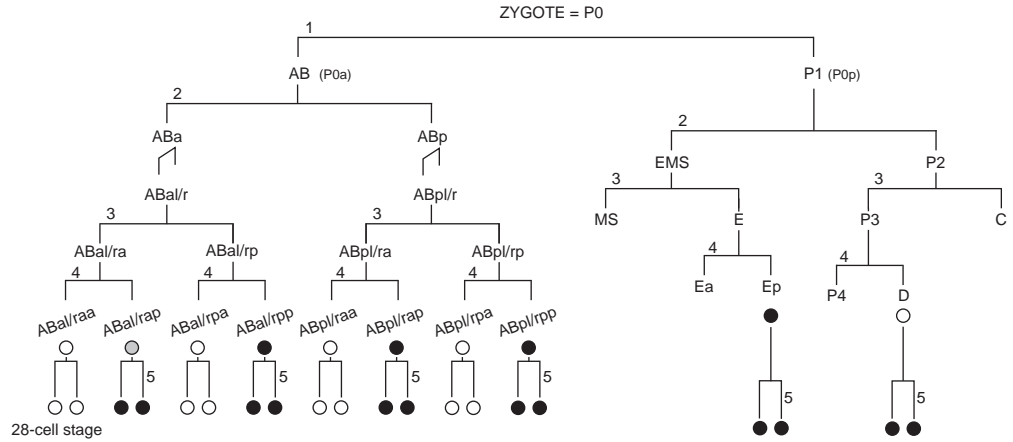
Weak *ceh-13* in *skn-1* animals without gut differentiation contrasts the situation in trypan-blue-treated embryos where, in absence of gut induction, no *ceh-13* expression occurs. This difference may be explained by the fact that *ceh-13* regulation is only partially SKN-1 dependent. SKN-1 is required for specifying the identity of the EMS blastomere and both of its descendants E and MS, but MS does not express *ceh-13*. Thus, SKN-1 is necessary but not sufficient to specify the E fate and additional factors must act to establish the gut founder cell. It has been suggested that P<sub>2</sub> is the source of a polarizing signal that orients the EMS cleavage axis and causes EMS to divide asymmetrically and to produce MS and E fates (Goldstein, 1992, 1993). If this induction is inhibited by treating 4-cell-stage embryos with trypan blue, no gut is formed and no *ceh-13* expression is observed in the Ep cell, indicating that P<sub>2</sub>-EMS interaction may be required for *ceh-13* activation. In *skn-1* embryos, this interaction may still occur and provide the cue for the activation of low levels of *ceh-13* expression even in the absence of differentiated gut. Altogether, our data with *pop-1* and *skn-1* mutants and the results with trypan-blue-treated embryos demonstrate that normal *ceh-13* expression in Ep correlates with the intestinal cell fate, but does not depend on the position of the cell in the embryo.

#### Asymmetric *ceh-13* activation does not depend on cell-cell contacts

An important finding is that *ceh-13* activation in the E and soon afterwards in the AB lineages always occurs in the posterior daughters of anteroposterior cell division (cf. Fig. 2). In the D lineage, weak expression was observed in the Da and Dp cells, and it is tempting to speculate that onset of *ceh-13* expression below the detection level may already occur in the D cell, the posterior daughter of the P<sub>3</sub> cell. From our data, thus, an apparent rule emerges; the potential to produce CEH-13 is present in the posterior daughters of the 4th anteroposterior division in the E and possibly the D lineage and of the 5th division in the AB lineage (which corresponds also to the 4th anteroposterior cleavage, since the 3rd cleavage in the AB lineage occurs along the left-right axis) and is distributed to the daughters of these cells.

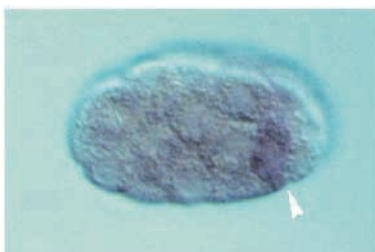
This asymmetric *ceh-13* activation pattern may be the result of asymmetric segregation of regulatory factors or of cell-cell interactions, or both. Because *pop-1* mutants express *ceh-13* ectopically in their MS<sub>p</sub> cells (Fig. 4A), it is unlikely that asymmetric activation depends on specific cell-cell interactions of Ep with its neighboring cells. To confirm this assumption, we have removed the late P<sub>2</sub>-blastomere after the division of the AB cells from 2 to 4 through a laser-induced hole in the eggshell ( $n=6$ , Fig. 5). In these embryos, gut induction had already occurred and *ceh-13::gfp* is correctly expressed in Ep. This result demonstrates that after gut induction cell-cell contacts between E and C, Cp and P<sub>3</sub>, as well as between Ep and D, Cp and P<sub>4</sub>, are not required for correct *ceh-13::gfp* activation in Ep. Furthermore, removal of P<sub>2</sub> had no influence on the *ceh-13* expression pattern in the ABxxxp cells of the 32-AB cell stage ( $14 \pm 2$  fluorescent nuclei,  $n=6$ ; data not shown). These results suggest that *ceh-13* activation in Ep and in the

**Fig. 2.** Summary of the initial *ceh-13* expression in the AB, E and D lineages of *C. elegans*. Numbers indicate the number of anteroposterior divisions from the beginning of development. 'a' stands for anterior, 'p' for posterior, 'l' for left and 'r' for right. The 3rd division in the AB lineage is a left-right division, whose daughters are indicated as ABx/l/r. Filled circles, cells expressing *ceh-13*; open circles, cells not expressing *ceh-13*.



ABxxxp cells does not depend on interactions with any of the P<sub>2</sub> descendants.

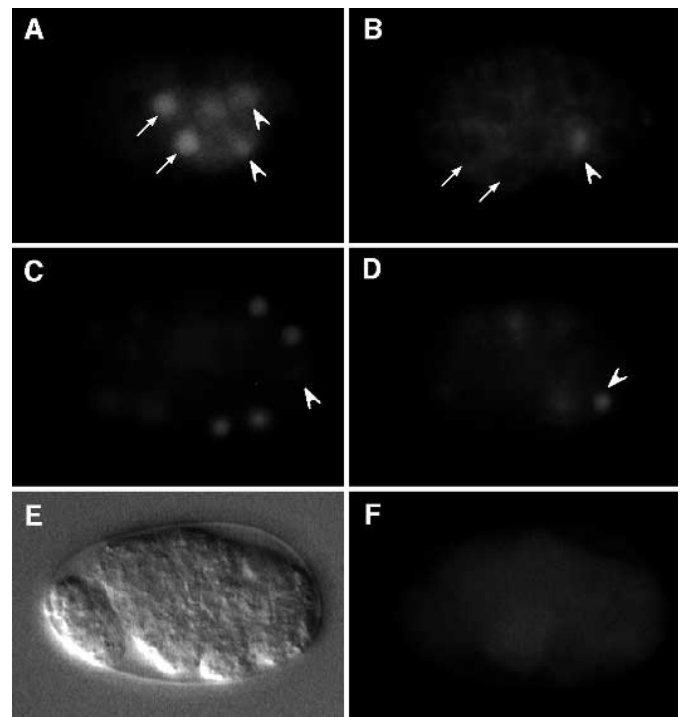
In order to investigate a possible role of postdivision cell-cell interactions in *ceh-13* activation, *ceh-13::gfp*-carrying embryos were incubated with cytochalasin D. Cytochalasin D disrupts the *C. elegans* actin cytoskeleton within 1 minute of exposure time and blocks cell divisions upon continuous incubation without stopping nuclear divisions (Hill and Strome, 1988). The embryos were treated with cytochalasin D at different timepoints in development, followed under the microscope until the number of their nuclei reached the equivalent of 28- or 46-cell-stage embryos and assayed for *ceh-13::gfp* expression. Some embryos were further incubated for later scoring of the intestinal-specific rhabditin granules by fluorescence (Cowan and McIntosh, 1985; Laufer et al., 1980; Goldstein, 1995a,b). No GFP expression could be detected in arrested zygotes ( $n=8$ ). 2-cell-stage embryos also showed no GFP expression, even if gut granules could later be scored ( $n=10$ , Fig. 6A,B). 4-cell-stage-blocked embryos, however, could be classified into three categories: non-fluorescing embryos ( $n=4$ ), embryos with *ceh-13::gfp* expression in all ABp nuclei only ( $n=6$ , Fig. 6D), and embryos with expression in all ABp and EMS nuclei ( $n=6$ , Fig. 6F; in these embryos, EMS expression was always correlated with gut granule formation). From these results, we conclude that, after the 4-cell stage, neither normal cell-cell contacts nor an intact actin cytoskeleton are required for the onset of *ceh-13* expression in the ABp and EMS lineages, which therefore differ from the ABa and P<sub>2</sub> lineages. Alternatively, however, it is also possible that normal cell-cell interactions are required, but that inductions occur between non-normal cells in the blocked embryos.



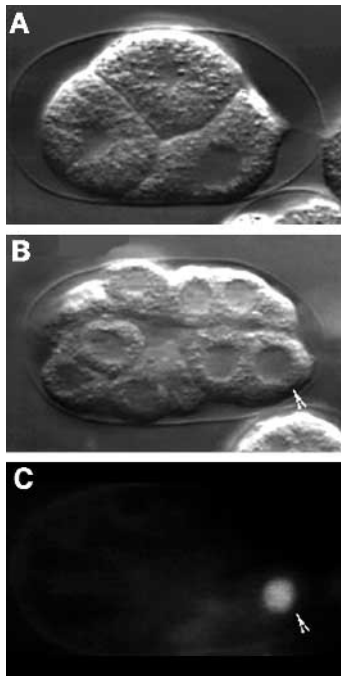
**Fig. 3.** Localization of *ceh-13* mRNA in a 26-cell-stage *C. elegans* embryo by whole-mount in situ hybridization. Nomarski picture showing staining in Ep (arrowhead).

### Contact between AB and P1 is not required for *ceh-13* expression

It has been proposed that a polarising induction of P<sub>1</sub> participates in specifying anteroposterior differences within the AB lineage (Hutter and Schnabel, 1994). To test whether an AB-P<sub>1</sub> interaction is necessary for *ceh-13* activation, we isolated AB and P<sub>1</sub> blastomeres and cultured them separately. When the



**Fig. 4.** *ceh-13::GFP* expression in *pop-1* and *skn-1* mutant backgrounds. (A) 50-cell-stage *lin-17 pop-1* embryo showing expression of the *ceh-13::gfp* reporter in the daughter cells of Ep (arrowheads), and in the ectopic intestinal cells MSpa and MSpp (arrows). (B) *Lin-17* control embryos show normal CEH-13::GFP expression in the Ep cells (arrowhead), but no expression in MSP daughters (arrow). (C) *Skn-1* embryo showing weaker expression than normal in the Ep cell (arrowhead). (D) *Skn-1* escaper (with gut fluorescence) showing normal expression in the Ep cell (arrowhead). (E) Nomarski picture of C after a few hours of development. (F) Same embryo as in E, UV excited. No gut autofluorescence was detected.



**Fig. 5.** CEH-13::GFP expression in an embryo in which P<sub>2</sub> has been removed. (A) Laser removal of P<sub>2</sub> was performed at the time when the two anterior AB cells started to divide. (B) The same embryo at the equivalent of the 28-cell stage. Ep is indicated with an arrowhead. (C) Same embryo as in B: the CEH-13::GFP fusion protein was expressed in Ep (arrowhead).

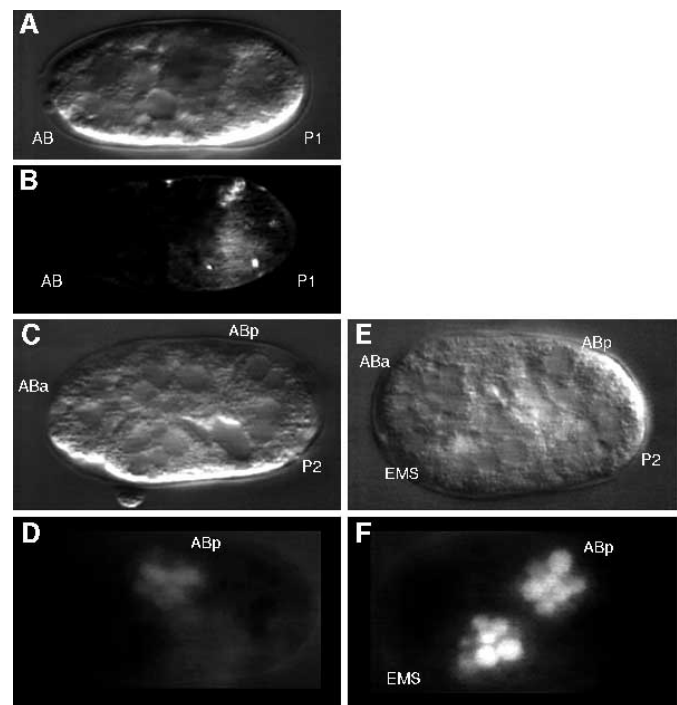
isolates reached the equivalent of the 26-cell stage (16 ABa like cells, 10 P<sub>1</sub>-derived cells), each isolate was examined for CEH-13 expression (see Materials and Methods). Five of six AB isolates contained CEH-13 in up to 8 of the total 16 cells (Fig. 7A). All of eight P<sub>1</sub> isolates were positive for CEH-13 in the Ep cell as demonstrated by cell lineaging analysis (Fig. 7B). Even cell isolations carried out very early in the 2-cell stage (5 minutes after the start of cytokinesis in P<sub>0</sub> for AB isolates, and 7 minutes for P<sub>1</sub> isolates) were positive for CEH-13. These results were confirmed by removing either the AB or the P<sub>1</sub> cell from 2-cell embryos through a laser-introduced hole in the eggshell (results not shown). Altogether, our data suggest that contact between P<sub>1</sub> and AB is not likely to be required for *ceh-13* activation.

## DISCUSSION

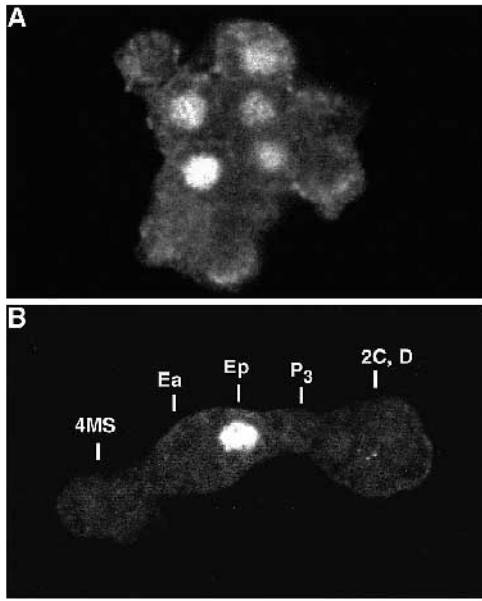
### *ceh-13* activation depends on cell fate and on anteroposterior cell polarity

The homolog of the *Drosophila* *HOM-C* gene *labial*, *ceh-13*, is the first gene of the *C. elegans* *Hox* gene cluster to be activated during development (Wittmann et al., unpublished data). The early expression of *ceh-13* begins at the 26-cell stage, shortly before the onset of gastrulation in the intestinal precursor cell Ep, and soon afterwards in all ABxxxp cells. One division round later, the *ceh-13::gfp* reporter gene becomes activated in the Da and Dp cells, however, no *ceh-13* expression is seen in the MS and C lineages at this stage.

In *Drosophila*, position-specific signals located along the anteroposterior axis establish the pattern of *HOM-C* gene expression (Lawrence and Morata, 1994). In the nematode *C. elegans*, it is not yet clear how the pattern of *HOM-C/Hox* gene expression is regulated. Our data suggest that cell fate determinants rather than positional information play a fundamental role in *ceh-13* activation. Indeed, *ceh-13* is ectopically expressed in the MS lineage of *pop-1* embryos, indicating that the activation of this gene in the intestinal precursor cell Ep is independent on the position of this cell in the embryo. Furthermore, cytochalasin D and P<sub>2</sub>-removal experiments suggest that *ceh-13* activation does not depend on the interaction of Ep with its neighbouring cells. Rather, the Ep cell fate itself is necessary and sufficient for correct activation of *ceh-13*. This is also supported by strongly reduced levels of CEH-13::GFP in *skn-1* embryos, which fail to produce intestinal cells (Bowermann et al., 1992), and by the complete absence of *ceh-13* expression in embryos where gut induction was inhibited by trypan blue treatment (Bossinger and Schierenberg, 1996). Furthermore, incubation of 4-cell-stage embryos with cytochalasin D blocks the onset of *ceh-13* expression in the ABa cell, but not in the ABp cell, suggesting that the regulation of *ceh-13* expression is modulated by cell-specific cues. Altogether, our results indicate that the onset of *ceh-13* expression during early embryonic development is cell fate dependent.



**Fig. 6.** CEH-13::GFP expression in cytochalasin-D-blocked embryos. (A) Nomarski picture of an embryo blocked at the 2-cell stage and incubated in cytochalasin D until the embryo reached the equivalent of the 28-cell stage. (B) Fluorescent picture of the same embryo as in A showing rhabditin granules in P<sub>1</sub> but no *ceh-13::gfp* expression. (C) Nomarski picture of a 4-cell-stage-blocked embryo. (D) Fluorescent picture of the embryo in C with CEH-13::GFP expression in the ABp cell. (E) Nomarski picture of another 4-cell-stage-blocked embryo. (F) Same embryo as in E. CEH-13::GFP fluorescence was detected in the ABa cell. No expression was present in the ABp and EMS cells.



**Fig. 7.** Confocal microscope sections of the results of 2-cell-stage blastomere isolations, processed for anti-CEH-13 fluorescence at the equivalent of the 26-cell stage. (A) 8 of the 16 progeny of an AB isolate showed nuclear CEH-13; the identity of these 8 cells was not determined. 5 of the positive nuclei are shown in this section. (B) One cell of the 10 progeny of P<sub>1</sub> showed nuclear CEH-13. Each cell was identified by following the cell lineage of the isolate and also by DAPI-staining of the fixed isolate. The stained cell is Ep.

Our findings are consistent with recent data of another member of the *C. elegans Hox* gene cluster, *mab-5*. Cowing and Kenyon (1996) demonstrated that the expression of *mab-5* in mesodermal and ectodermal tissues during embryonic and postembryonic development is highly correlated with cell fate and does not depend on cell position. These findings imply that mechanisms other than cell-extrinsic A-P positional signals play an important role in the activation and regulation of the *C. elegans Hox* genes.

In addition to cell fate determinants, polarity cues are also important in regulating early *ceh-13* expression, an interpretation supported by the observation that the first activation of *ceh-13* in the E, AB and probably also D lineages always occurs in the posterior daughters of the 4th embryonic anteroposterior division (corresponding to the 5th division in the AB lineage). This suggests that these cells contain information regarding their position along the last division axis and that this information is passed along to the *ceh-13* regulatory pathway. The polarity of the E cell, e.g., does not depend on its position in the embryo, since in *pop-1* mutants the two daughters of the MS cell, which ectopically adopts the fate of its posterior sister cell E (Lin et al., 1995), show the same difference (i.e., only the daughters of the MSp cell express *ceh-13*). In summary, our data demonstrate that the cells first expressing *ceh-13* in early embryos are always the posterior daughters of anteroposterior cell division, regardless of where they are born, and suggest the existence of an anteroposterior cellular polarity system that is involved in the activation of *ceh-13*. This cellular polarity system may act cell-autonomously and be present in all daughters of the 4th anteroposterior divisions of the embryo, irrespective of whether these cells express *ceh-13* or not. Alter-

natively, however, there could be a global, cell-extrinsic polarity system acting in the embryo.

The existence of an animal-wide system directing anteroposterior cellular polarity has already been proposed (Way et al., 1994; Priess, 1994). Such a system may also apply in later developmental stages and for other genes. For example, a MEC-3-expressing cell is always the anterior daughter of a mechanoreceptor-generating asymmetric division (reviewed in Way et al., 1994) and MAB-5::LACZ first appears in the posterior daughters of the ABpxpp cells (Cowing and Kenyon, 1992). VAB-7, another homeodomain-containing protein is first present in the posterior daughters of Cxx cells (Ahringer, 1996). This suggests that similar polarity cues acting in *ceh-13* activation may be involved in the regulation of these genes. The control of *HOM-C/hox* gene expression by cellular rather than global embryonic polarity cues does not seem to be restricted to *C. elegans*. In *Drosophila*, higher levels of LAB have been observed in the delaminating procephalic neuroblasts compared to the protein levels observed in the ectodermal sisters of these cells during the development of the nervous system (Diederich et al., 1989). The cell division that generates these neuroblasts, is asymmetric and controlled by the INSCUTEABLE protein (Kraut et al., 1996), suggesting that *labial* expression is also correlated with cellular polarity. Therefore, the mechanisms integrating cellular polarity and homeobox gene expression, particularly *labial*-like gene activity, may have been conserved during evolution.

#### Molecular mechanisms of *ceh-13* regulation

The fact that *ceh-13* activation is dependent on cell fate and cell polarity strongly suggests that the regulatory information, or at least a part of it, is segregated asymmetrically to the *ceh-13*-expressing cells. During the first few embryonic divisions, differentially localized maternal gene products account for the various developmental potentials of the first few embryonic cells (Priess, 1994). Our data show that at least two of these proteins, SKN-1 and POP-1, influence *ceh-13* expression in Ep and may function as cell-specific or cell-lineage-specific modulators of *ceh-13* activation. It remains to be determined whether these two regulatory proteins directly bind to the promoter region of *ceh-13*.

The activation of *ceh-13* expression may depend on other activator and inhibitor molecules that are differently distributed during early embryonic development. 1- and 2-cell embryos blocked by cytochalasin D did not express GFP::CEH-13, suggesting that early embryos may contain one or several inhibitors of *ceh-13* expression. After the 4-cell stage, the cytochalasin D sensitivity is released in ABp and EMS, but not in ABa and P2. The finding that 4-cell-stage-blocked embryos can be classified into non-fluorescing embryos, embryos with *ceh-13::gfp* expression in all ABp nuclei only, and embryos with expression in all ABp and EMS nuclei, suggest that these inhibitors could be inactivated in the ABp and EMS lineages as the result of inducing events. In the ABa lineage, the inhibitor of *ceh-13* expression may then be segregated away from the *ceh-13*-expressing ABaxp cells. Alternatively, the ABa descendants may require a positional signal or a cell-cell interaction. In the ABp and E lineages, *ceh-13* activation may depend on an activator molecule that is distributed to the *ceh-13*-expressing Ep and ABpxp cells. The correct distribution of both, the inhibitor in the ABa

lineage and the activator in the ABp and E lineages, may depend on the same polarity system that is active during the 4th embryonic anteroposterior cell division. Further analysis will be needed to reveal the mechanisms of early *ceh-13* activation and to understand the role of asymmetric cell divisions in *ceh-13* expression and in embryonic patterning in general.

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