TGF-β signals and a prepattern in *Xenopus laevis* endodermal development


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SUMMARY

We have analyzed two gene products expressed in the early endoderm of *Xenopus laevis*: Xlhbox-8, a pancreas-specific transcription factor and intestinal fatty acid binding protein (IFABP), a marker of small intestinal epithelium. Expression of the pancreas marker relies on cell signaling mediated by both the TGF-β and FGF classes of secreted peptide growth factors, whereas, expression of the more posterior small intestinal marker does not. Endodermal explants devoid of mesoderm express both markers in a regionalized manner. Cortical rotation is required for the expression of the more anterior marker, Xlhbox-8, but not for the small intestinal marker, IFABP. These findings suggest that endodermal patterning is dependent, in part, on the same events and signals known to play important roles in mesodermal development. Furthermore, inhibition of TGF-β signaling in the endoderm leads to ectopic expression of both mesodermal and ectodermal markers, suggesting that TGF-β signaling may play a general role in the segregation of the three embryonic germ layers.

Key words: endoderm, TGF-β, cortical rotation, *Xenopus*, endoderm, Xlhbox-8, IFABP

INTRODUCTION

Early in vertebrate embryogenesis, the developing embryo contains three germ layers. The ectoderm, or outer layer, gives rise to the epidermis and nervous system. The mesoderm, which during gastrulation is positioned between the ectoderm and endoderm, differentiates into a variety of tissues including notochord, muscle, kidney, gut mesenchyme and blood. The endoderm, or inner germ layer, forms the lining of both the gastrointestinal and respiratory tracts. Recent studies have focused on how ectodermal cells are directed towards either an epidermal or neural fate (Hemmadi-Brivanlou and Melton, 1994; Lamb et al., 1992; reviewed in Saxen, 1989 and Ruiz i Altaba, 1993). Additionally, the induction and patterning of the mesoderm have been intensely investigated (reviewed in Kessler and Melton, 1994; Klein and Melton, 1994; Slack, 1993). Though the role of the prospective endoderm in the induction of mesoderm has been studied in depth, the generation and patterning of the endoderm itself has received less attention, especially at the molecular level. Because organs along both the gastrointestinal and respiratory tracts contain an endodermally derived epithelium, understanding how this germ layer is generated and patterned is an important issue for understanding organogenesis.

In the *Drosophila* midgut (reviewed in Skaer, 1993 and Bienz, 1994) and most vertebrate systems, two key events in the development of the gut are the creation of a tube, lined by endoderm and surrounded by mesoderm, and the patterning of this tube such that individual organs emerge in a proper anteroposterior and dorsoventral pattern. In the vertebrate gut, the greatest amount of tissue and histological diversity exists along the anteroposterior axis and, for this reason, we have initially analyzed the expression of two endodermal markers whose expression domains differ along this axis. Intestinal fatty acid binding protein (IFABP) is a low molecular weight cysteine rich protein that is abundantly expressed in small intestinal epithelium. It is thought that IFABP is involved in the cellular uptake of fatty acids, as well as their transport within the cell to particular organelles (reviewed in Sweetser et al., 1987). In the rat small intestinal epithelium, mRNA for IFABP and another fatty acid binding protein (LFABP or liver-FABP) constitute 2-3% of the entire pool of translatable RNA (Bass et al., 1985). During anuran metamorphosis, *Xenopus* IFABP expression decreases as the tadpole intestinal epithelium is shed. As the more complex adult-like epithelium forms after metamorphic climax, IFABP expression is restored (Shi and Par Hayes, 1994). Xlhbox-8 is a homeodomain-containing transcription factor that is expressed in a thin band of endoderm that will eventually line both the dorsal and ventral pancreas (Wright et al., 1988). The expression of both markers can be detected at the mid to late tailbud period (stages 25-28) by RT-PCR. Interestingly, both markers are expressed in the gut endoderm prior to the conversion of the yolky endodermal cells to a primary columnar epithelium (Shi and Par Hayes, 1994; Wright et al., 1988). In other words, the expression of both markers can be detected before overt signs of intestinal or pancreatic endodermal differentiation (Nieuwkoop and Faber, 1967).

In this report, an explant system is used to study the mechanisms involved in endodermal development. Endodermal
explants autonomously express both Xlhbox-8 and IFABP. Microinjection of dominant negative receptors into prospective endoderm suggests that the expression of the pancreas marker Xlhbox-8 depends on cell signaling mediated by TGF-β and FGF factors, whereas expression of IFABP does not. Explants dissected from UV-irradiated embryos show a difference in the expression of Xlhbox-8 and IFABP which suggests that, like mesodermal patterning, early endodermal development is regionally differentiated by the cortical rotation.

Previous studies showed that both an endoderm-specific antigen (4G6) and a pancreas-specific transcription factor (Xlhbox-8) could be turned on in animal caps (prospective ectoderm) by activin (Jones et al., 1993; Gamer and Wright, 1995). We show here that processed Vg1, a TGF-β with an expression pattern during normal development that is consistent with it having a role in endodermal development, also induces endodermal markers in animal caps.

Through the use of various blocking reagents, including dominant negative receptors, we show that prospective endoderm can express specific mesodermal and ectodermal markers. This alteration in the fate of the prospective endoderm suggests that TGF-β signaling may play a general role in the determination of the embryonic germ layers.

MATERIALS AND METHODS

Embryological methods

Embryos were obtained from females injected with 600-700 units of human chorionic gonadotropin (Sigma) the night preceding an experiment, fertilized in vitro with homogenized testis and cultured in 0.1 M MMR on agarose-coated dishes. Explants, cut with eye-brow hair knives, were cultured at 16-23°C in 0.5× MMR with staging based on the table of normal development (Nieuwkoop and Faber, 1967). To explant dorsovegetal or ventrovegetal poles, 4-cell embryos were selected with clear pigmentation differences along the dorsal/ventral axis, placed in 0.1× MMR-1% ficoll and marked ventrovegetally with a dried agarose chip saturated with a 1% solution of Nile Blue sulfate.

RNA extraction and RT-PCR

RNA isolation, reverse transcription and PCR amplification were performed as described previously (Wilson and Melton, 1994). The cycle number in parenthesis and the sequence of all primers used in the PCR step are listed below (5’-3’):

- **Cardiac actin:** upstream-GCTGACAGAATGCGAAG; downstream-TCGGATCTGGAGATGTTG. (20) (Stutz and Sphor, 1986)
- **EF-1α:** upstream-CAGATTTGCTGTCGGATATGC; downstream-ACTGCCCTTGTAGCTCCTTAG. (20) (Krieg et al., 1989)
- **Epidermal cytokeratin:** upstream-CACCAAGACAGATGTAC; downstream-CAACCTTCCCAATACCA. (20) (Jones et al., 1989)
- **Globin:** upstream-GCTTCAACACTGAGAGTGT; downstream-CAGGCTTGTGGACCTGGC. (20) (Banville and Williams, 1985)
- **IFABP:** upstream-CTGGTCCTCTACAGGAC; downstream-GTATGGCCAAATGTC. (25) (Shi and Par Hayes, 1994)
- **Insulin:** upstream-ATGGCTCTATGGATGAC; downstream-AGAGAATCAGTCTGGC. (25) (Shuldiner et al., 1989)
- **Xlhbox-8:** upstream-CACAGTTCCAAATG; downstream-GGAATCAAGCGTACAG. (25) (Kintner and Melton, 1987)
- **Xbra:** upstream-GGATCCGTATACCTGCTG; downstream-ATGATCTGAGCAAGCA. (25) (Smith et al., 1991)
- **Xtwist:** upstream-AGAAACTGGAGCTGGATC; downstream-GGCTTCAAGGCGACT. (25) (Hopwood et al., 1989)

UV irradiation

Dejellied embryos were irradiated from the vegetal pole with short wave UV light for 75 seconds at 30 minutes postfertilization as described previously (Ku and Melton, 1993). Tailbud (stg. 25-30) embryos were assigned a value on the dorsoanterior index, DAI (Kao and Ellinson, 1988).

In vitro transcription and microinjection of synthetic mRNA

RNA was transcribed in vitro as described previously (Krieg and Melton, 1984). Embryos were injected in a solution of 0.5× MMR-3% ficoll. For UV rescue experiments, a single vegetal blastomere of an 8-cell embryo was injected with 10 nl of the appropriate mRNA. All other injections were performed at the 1- to 2-cell stage with 10-20 nl of RNA dissolved in water. For experiments involving vegetal pole injections, embryos were turned on their sides and injected into the bottom-most region of the vegetal pole.

Histological analysis

In situ hybridization was performed on paraffin-embedded 15 μm thick sections. After dewaxing in xylene and rehydration, sections were fixed for 20 minutes at room temperature in 4% paraformaldehyde-PBS-3% sucrose. After 2× 5 minute PBS washes, the sections were gently digested with 3 μg/ml proteinase K at 37°C for 30 minutes. The digestion was terminated by soaking the slides in 0.2% glycine-PBS for 15 seconds at room temperature. After 2× 5 minute PBS rinses, the slides were taken through the following steps: 0.2 N HCl, 15 minutes at room temperature; 2× 5 minutes PBS wash; 0.1 M triethanolamine pH 8.0 with acetic anhydride added to 0.25% for 10 minutes at room temperature; 1× PBS wash and finally a single wash in DEPC-treated water for 5 minutes. Prehybridization was performed at 60°C overnight, in a sealed humidified chamber, in buffer containing the following: 50% formamide, 5× SSC, 1 mg/ml torula RNA, 100 μg/ml heparin, 1× Denhardt’s, 0.1% Tween 20, 0.1% CHAPS, 10 mM EDTA. Hybridization was performed in prehybridization buffer plus the appropriate digoxigenin-labeled probe at 3 μg/ml final concentration at 60°C for 10 hours. To remove unhybridized probe, the slides were treated in the following manner: slides were covered in 1:1 prehybridization buffer-2× SSPE-0.3% CHAPS, 60°C, 10 minutes; 1× prehybridization buffer-2× SSPE-0.3% CHAPS, 37°C, 10 minutes; 2× 30 minute washes in 2× SSPE-0.3% CHAPS at room temperature; digestion with RNase A at 20 μg/ml in 4× SSPE at 37°C for 30 minutes; a single wash in 2× SSPE-0.3% CHAPS at room temperature for 1 hour; a single wash in 2× SSPE-0.3% CHAPS-50% formamide at 50°C for 1 hour; 2× 10 minute washes at room temperature in 2× SSPE-0.3% CHAPS and finally 3× 10 minute washes in TBS at room temperature. Detection of probe:mRNA hybrids was accomplished by first washing slides for 15 minutes in TBST, then blocking in TBST-20% lamb serum for 30 minutes at room temperature. A sheep anti-digoxigenin-alkaline phosphatase-coupled antibody (Boehringer Mannheim) was then added to slides at a 1:2000 dilution in TBST-10% lamb serum for 1 hour at room temperature. After 3× 5 minute washes in TBST at room temperature, slides were incubated in alkaline phosphatase buffer for 10
minutes which consisted of the following: 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween20, 5 mM levamisole (Sigma). Slides were than covered with BM purple-alkaline phosphatase substrate (Boehringer Mannheim) in a humidified chamber. After sufficient color development, slides were fixed in 1× MEMFA for 45 minutes at room temperature, washed in PBS-0.1% Tween20 at room temperature and finally mounted in 100 mM Tris pH 7.5, 1% n-propyl-gallate (Sigma), 80% glycerol.

Immunohistochemistry was performed on paraffin-embedded 15 μm thick sections. After dewaxing and rehydration, slides were transferred to 1× PBS for 5 minutes followed by incubation in blocking buffer for 1 hour, which consisted of 5% BSA, 1% goat serum and 1× PBS. Primary antibody (Gamer and Wright, 1995) was diluted 1:200 in 1× PBS, 1:10 blocking buffer and applied directly to slides overnight at room temperature. After 3×5 minutes PBS washes, a donkey anti-rabbit HRP-coupled secondary antibody (Amersham) was diluted 1:200 in 1× PBS, 1:10 blocking buffer and applied to slides for 2 hours at room temperature. After 3×5 minutes PBS washes, HRP activity was visualized with DAB.

**RESULTS**

**Autonomous expression of endodermal markers in explants**

Vegetal pole explants were used to analyze the autonomous development of the endoderm (Dale and Slack, 1987; Holtfreter, 1938a,b). Explants were dissected from blastula embryos (stage 8-9) and cultured in isolation until the late tailbud period (stage 30-35). Both Xlhbox-8 and IFABP are expressed in vegetal pole explants (Fig. 1A). We also detect expression of the pancreatic endocrine product insulin (Shuldiner et al., 1989) in explanted vegetal poles (Fig. 1A). This is consistent with Xlhbox-8 being a homologue of the mouse transcription factor IPF-1, which is capable of transactivating the murine insulin promoter and is co-expressed in insulin-producing β-cells in mice (Ohlsson et al., 1993). The expression of endodermal markers is regionalized in the sense that the dorsal end of the vegetal pole expresses Xlhbox-8 and both the dorsal and ventral vegetal pole express IFABP (Fig. 1B). The restriction of Xlhbox-8 expression to dorsal vegetal tissue is in agreement with fate mapping data showing that the dorsal-vegetal region of the early embryo gives rise to the anterior gut endoderm (Dale and Slack, 1987; Moody and Kline, 1990; Nakamura and Kishiyama, 1971).

The vegetal poles used in this study are not contaminated with prospective mesoderm as we do not detect expression of globin, Xtwist or cardiac actin which are ventral, ventrolateral and dorsolateral mesodermal markers, respectively (Fig. 1A). It is noteworthy that the vegetal poles do not contain the ventral gut mesoderm that gives rise to the mesenchyme surrounding the gut tube in normal development as Xtwist marks the mesoderm from which the gut mesenchyme delaminates (Hopwood et al., 1989).

To further analyze endodermal marker expression, histological analysis was performed on vegetal pole explants. As evident in Fig. 2C-E, both IFABP and Xlhbox-8 are expressed in a limited number of cells in vegetal pole explants. Moreover, Xlhbox-8 expression occurs over a smaller region of the vegetal pole which is consistent with the finding that Xlhbox-
Fig. 2. Expression patterns of Xlhbox-8 and IFABP both in whole embryos and vegetal pole explants. (A) Sagittal section through a stage 38 embryo that was subsequently immunostained with an anti-Xlhbox-8 antibody (Gamer and Wright, 1995). A thin band of endodermal nuclei stretching from the dorsal pancreas, through the duodenum and down to the ventral pancreas is indicated by the bracketed area. (B) Sagittal section through a stage 38 embryo that was in situ hybridized to an anti-sense probe that detects IFABP mRNA. (C) Section of a stage 38 vegetal pole explant that was probed with an anti-sense IFABP probe. (D) The adjacent section to that in C except that a control sense IFABP probe was used in the in situ. (E) Section of a stage 38 explant that was subsequently immunostained with an anti-Xlhbox-8 antibody. Positively stained nuclei are contained within the bracketed area which represents the dorsally derived side of the explant. Abbreviations: n, notochord; p, pancreas; ph, pharynx; si, small intestine.

Expression patterns of Xlhbox-8 and IFABP

Fig. 2.

8 expression is confined to the dorsal side of the vegetal pole whereas IFABP expression is more dispersed. By counting the number of positively stained nuclei in the case of Xlhbox-8 or positively stained cells for IFABP, it appears that fewer cells express both markers in explants as compared to whole embryos (Fig. 2A-E). The temporal component of each markers expression is identical when explants are compared to whole embryos (data not shown).

Inhibition of cell signaling blocks Xlhbox-8 expression and leads to the ectopic expression of non-endodermal markers

Mesodermal development can be altered by dominant negative receptors that inhibit FGF, BMP-2/4, activin and Vg1 signaling pathways (Amaya et al., 1991; Graff et al., 1994; Maeno et al., 1994; Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994, Kessler and Melton, 1995). To examine the potential role of these signaling molecules in the expression of Xlhbox-8 and IFABP, in vitro transcribed mRNA for a particular dominant negative receptor was injected into the vegetal pole of a fertilized egg. At the blastula stage, vegetal pole explants were dissected and then cultured until the late tailbud period (Fig. 3A). While both the activin and FGF dominant negative receptors completely blocked Xlhbox-8 expression, a dominant negative BMP-2/4 receptor had no effect on the expression of the pancreas marker (Fig. 3B). In contrast, IFABP expression was unaffected by the FGF, activin and BMP-2/4 dominant negative receptors (Fig. 3B).

The effects of the dominant negative activin receptor when expressed in vegetal pole explants are not limited to blocking Xlhbox-8 expression. It can also ectopically induce expression of markers characteristic of germ layers other than endoderm. In particular, expression of cardiac actin, N-CAM and epidermal keratin, markers of dorsolateral mesoderm, neural tissue and epidermis, respectively, is seen following injection of a truncated activin receptor (Fig. 3B,C). While a dominant negative FGF receptor blocks Xlhbox-8 expression, it does not induce expression of cardiac actin (Fig. 3B). High levels of a truncated BMP receptor can induce cardiac actin expression. However, as the amount of injected mRNA is decreased (4000 pg to 400 pg), the truncated BMP receptor, unlike the truncated activin receptor, fails to block endodermal markers or induce markers of other germ layers (data not shown).

The dominant negative activin receptor blocks signaling by both activin and Vg1 in animal caps (Schulte-Merker et al., 1994; Kessler and Melton, 1995); thus, experiments utilizing this reagent cannot allow us to determine which of these signals may be involved in Xlhbox-8 expression. To help resolve this issue, an activin antagonist, follistatin (Asashima et al., 1991b; Kessler and Melton 1995; Kogawa et al., 1991; Nakamura et al., 1989; Schulte-Merker et al., 1994) was injected into the vegetal pole of a 1-cell embryo. Follistatin had no effect on either IFABP or Xlhbox-8 expression (Fig. 3D). The follistatin mRNA used in this experiment inhibited activin in an animal cap assay and induced expression of the neural marker N-CAM in vegetal pole explants (Fig. 3C,E). From this, we conclude that activin is unlikely to play a role in the expression of Xlhbox-8 and that Vg1 or possibly another TGF-β-related factor is necessary for Xlhbox-8 expression.

IFABP expression, in contrast to Xlhbox-8, does not depend on FGF or TGF-β signaling. Furthermore, expression of a dominant negative form of RAS (Cai et al., 1990; Feig and Cooper, 1988) abolishes Xlhbox-8 expression and has no effect on IFABP expression (Fig. 3D). Thus, it appears that IFABP is expressed by cells that are determined via a RAS-independent signaling mechanism excluding signaling by BMP-2, activin and Vg1, all of which are inhibited by a dominant negative form of RAS in induction assays (data not shown).

Vg1 can induce endodermal markers in prospective ectoderm

Vg1 is a maternal mRNA localized to the vegetal cortex of oocytes and eggs (Weeks and Melton, 1987). Processed Vg1 can induce mesoderm in animal caps (Dale et al., 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995) and both its temporal and spatial expression patterns are appropriate for a signaling role in the development of the endoderm. To test the ability of Vg1 to induce endodermal marker expression, mRNA encoding a processable form of Vg1 (B-Vg1) was injected into the animal pole of a 1-cell embryo, from
which, animal caps were dissected and cultured in isolation (Fig. 4A). B-Vg1 induced Xlhbox-8 and IFABP at levels that are known to be sufficient for induction of the dorsolateral mesodermal marker cardiac actin (Fig. 4B; Thomsen and Melton, 1993). Activin can also induce expression of both markers in animal caps (Fig. 4C, data not shown). As neither the truncated BMP receptor nor the truncated activin receptor block expression of the intestinal marker in explanted vegetal poles, we do not believe that BMP-2, activin or Vg1 plays a role in the expression of this marker in the vegetal pole. Though it is certainly possible that IFABP expression, in the endoderm, might rely on multiple active signaling pathways one or more of which is not blocked by the dominant negative constructs used in this study.

Cortical rotation is required for the pattern of the endoderm

Dorsal axis formation in amphibians requires a reorganization of cytoplasm during the first cell cycle. UV irradiation blocks cortical rotation resulting in a ventral posteriorized embryo with radial symmetry (Cooke and Smith, 1987; reviewed in Gerhart et al., 1989). Mesoderm in UV-irradiated embryos is ventrolateral in character and thought to result from the absence of a specialized endodermal signaling center that in normal embryos induces dorsal mesoderm in the overlying marginal zone (Boterenbrood and Nieuwkoop, 1973; Nieuwkoop, 1973). Since a pancreas marker is expressed by endodermal cells derived from the dorsal side of the vegetal pole, we tested whether the expression of this marker is affected in explants dissected from UV-ventralized embryos. Fig. 5A shows that Xlhbox-8 expression is inhibited in explants derived from severely ventralized embryos (D.A.I. = 0), as well as in the whole UV embryo. In addition, IFABP expression in explants dissected from UV irradiated embryos is comparable to that seen in control explants.

Processed Vg1 can rescue UV-ventralized embryos resulting in normal dorsal axis formation and a phenotypically normal embryo (Thomsen and Melton, 1993). Vegetal pole explants cut from UV-irradiated embryos that were subsequently rescued by Vg1 mRNA injection at the 8-cell stage, express levels of Xlhbox-8 similar to those seen in control explants. In the whole UV embryo, Xlhbox-8 expression is restored to normal levels by Vg1 injection. IFABP expression in ventralized embryos is severely diminished; however, rescue of the UV phenotype with processed Vg1 restores IFABP expression to normal levels (Fig. 5A).

Vegetal pole explants dissected from wild-type embryos that were injected with B-Vg1 at the 1-cell stage do not express cardiac actin and a modest but reproducible increase in Xlhbox-8 expression is observed (Fig. 5B). In the same experiment, no effect is seen

Fig. 3. Xlhbox-8 expression relies on TGF-β and FGF signals. (A) At the 1-cell stage, synthetic mRNA encoding inhibitory proteins were injected into the vegetal pole. At stage 9, vegetal pole explants were dissected and cultured to the mid-tailbud period (stage 33-36). RNA levels were assayed by RT-PCR. (B) Both a truncated activin (tAR) and truncated FGF receptor (XFD) block expression of Xlhbox-8 but a truncated BMP receptor has no effect. IFABP expression is unaffected by all three truncated receptors. 4-5 nanograms of mRNA for each receptor was used in this experiment and the result does not differ from experiments using 400-500 picograms of mRNA except that the truncated BMP receptor does not efficiently induce cardiac actin expression at 400 picograms (data not shown). (C) Induction of the ectodermal markers NCAM and epidermal cytokeratin in vegetal poles expressing a truncated activin receptor. The activin-specific antagonist follistatin (XFS) neutralizes vegetal poles but does not induce expression of epidermal cytokeratin. 2 nanograms and 500 picograms of mRNA were used in the truncated activin receptor and follistatin injections, respectively. (D) A dominant negative form of RAS (2 nanograms) blocks expression of Xlhbox-8 but has no effect on IFABP expression. The activin-specific antagonist follistatin has no effect on the expression of either IFABP or Xlhbox-8. Neither follistatin nor the dominant negative RAS induce expression of the mesodermal marker cardiac actin. (E) The follistatin mRNA used in parts C and D of this figure blocks induction in animal caps by injected activin mRNA as judged by expression of the pan-mesodermal marker Brachyury (Xbra). 1 nanogram of mRNA encoding follistatin was co-injected with 2 picograms of mRNA encoding activin βB. WE lanes represent RT-PCR products where mRNA from an unmanipulated embryo was used for cDNA synthesis. The WE-RT lanes represent assays in which reverse transcriptase was left out of the cDNA synthesis step with whole embryo RNA as a template. This serves as a control for DNA contamination. Elongation factor 1 alpha (EF-1α) is included as a control to insure that similar amounts of RNA are used in each lane.
on IFABP expression. The B-Vg1 mRNA used in this experiment is active as it induces both Xlhbox-8 and IFABP in explanted animal caps (Fig. 5B).

**DISCUSSION**

Our data suggest that the pattern of the gut endoderm is dependent on events and molecules known to be important in the induction and patterning of the mesoderm. Inhibition of cortical rotation leads to the loss of Xlhbox-8 expression in vegetal pole explants, whereas, IFABP expression is unaffected. Similarly, both a truncated activin and FGF receptor block expression of Xlhbox-8 in explants with no effect on IFABP expression. This suggests that TGF-β and FGF signaling play a role in the patterning of the anteroposterior axis of the gut endoderm with a specific signaling function in the establishment of the anterior endoderm. Though speculative, the activity of the aforementioned signaling pathways would depend on the cortical rotation. In this scheme, the cortical rotation would not only lead to dorsal axis formation but also to the anteroposterior pattern of the endoderm.

Xlhbox-8 expression requires cortical rotation in vegetal pole explants whereas IFABP expression does not. Additionally, the pancreas marker is expressed in cells that are derived from the dorsal vegetal region of blastula unlike IFABP which is expressed in cells derived from both the dorsal and ventral vegetal regions. These results agree with previous histological studies on the early endoderm. In the studies of Ogi, Nieuwkoop and their colleagues, it was shown that blastula stage endoderm when juxtaposed to prospective ectoderm can induce mesoderm in the ectoderm-derived portion of the recombinant (Nieuwkoop, 1973; Ogi, 1967, 1969). The type of induced mesoderm depended on the regional derivation of the endodermal cells used in the recombinant (Boterenbrood and Nieuwkoop, 1973); that is, dorsal vegetal endoderm induced dorsal mesoderm when placed in contact with prospective ectoderm whereas ventral vegetal endoderm induced ventral types of mesoderm in the same assay. Thus, it has been shown previously that the blastula stage endoderm possesses an asymmetry. In this report, we demonstrate that an endodermal asymmetry exists in the autonomous expression of two genes that are expressed just prior to organogenesis.

An unexpected finding of the UV experiments is that IFABP expression is greatly reduced in ventralized embryos. Explants from UV embryos express IFABP and it is possible that the excess of ventrolateral mesoderm in the UV embryo acts negatively on IFABP expression. Consistent with this, rescue of UV-irradiated embryos with Vg1 restores IFABP expression in whole embryos. IFABP expression is induced in animal caps by the dorsal mesoderm inducers Vg1 and activin. However, dominant negative receptors that block signaling by Vg1, activin, BMP-2,4 and FGF have no effect on the expression of IFABP. These results can be accounted for by one of three models. First, it is possible that IFABP expression requires a cell-cell signaling event that is mediated by Vg1 and a second unknown factor. The signal transduction pathway stimulated by this putative factor would have to be insensitive to the

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**Fig. 4. Induction of endodermal markers by activin and Vg1 protein.** (A) At the 1-cell stage synthetic mRNA encoding a TGF-β ligand was injected into the animal pole. Animal caps were dissected from blastula stage embryos (stage 9) and cultured in isolation until the mid-tailbud period (Stage 33-36). Gene expression was monitored by RT-PCR. (B) To express the mature forms of Vg1 and BMP-2, mRNA encoding chimeric forms of each gene were used in our injections. In the case of Vg1, a chimeric mRNA was used where the pro region of BMP-2 was fused to the mature region of Vg1 (Thomsen and Melton 1993). For BMP-2, the pro region of activin B was fused to the mature region of BMP-2 (A-BMP-2). The B-Vg1 chimera induces expression of cardiac actin, IFABP and Xlhbox-8 in animal caps consistent with previously published results that demonstrated the ability of B-Vg1 to induce axial mesoderm (Thomsen and Melton 1993). The A-BMP-2 chimera induces expression of globin, a ventral mesodermal marker, in animal caps. Neither IFABP nor Xlhbox-8 expression is efficiently induced in animal caps by BMP-2. The amount of RNA injected is indicated in picograms. (C) Activin can induce cardiac actin, IFABP (data not shown) and Xlhbox-8 in animal caps. The amount of injected RNA is indicated in femtograms. WE lanes represent RT-PCR products where mRNA from an unmanipulated embryo was used for cDNA synthesis. The WE-RT lanes represent assays in which reverse transcriptase was left out of the cDNA synthesis step with whole embryo RNA as a template. This serves as a control for DNA contamination. Elongation factor 1 alpha (EF-1α) is included as a control to insure that similar amounts of RNA are used in each lane.
inhibitory effects of the three truncated receptors used in this study and the dominant negative form of RAS. Second, it is also possible that IFABP expression depends on the same signals as those shown herein to be important for Xlhbox-8 expression and that no effect is seen on the expression of this marker in vegetal poles because the various inhibitory constructs used in this study are not translated to a sufficient level after injection to have an effect. Third, the induction seen in animal caps by Vg1 and activin might not necessarily reflect the true abilities of these signaling factors as the mesoderm and endoderm derive from a portion of the embryo that is distinct from the animal pole.

Recent work suggests that FGF does not act in the initial stages of mesoderm induction, but is instead required to maintain induced mesoderm (Isaacs et al., 1994). We speculate that the FGF pathway plays a similar role in the expression of Xlhbox-8, with FGF signaling required for the maintenance of Xlhbox-8 expression. A TGF-β related signal would then be involved in a primary inductive signal, consistent with the ability of the dominant negative activin receptor to block expression of the pancreas marker.

A dominant negative activin receptor induces expression of ectodermal and mesodermal markers in explanted vegetal poles, consistent with its ability to divert prospective endoderm to a neural fate in the whole embryo (Hemmadi-Brivanlou and Melton, 1994a). A limitation of the truncated activin receptor is that it is capable of blocking signaling by other TGF-βs (Schulte-Merker et al., 1994; Kessler and Melton 1995). Despite this complexity, one interpretation of these experiments is that germ layer specification involves a graded response to TGF-β signaling. The response to high levels of TGF-β signaling would be the induction of endoderm and at lower levels of signaling mesoderm would be induced. In this scheme, neuralization results from a complete lack of TGF-β signaling as suggested in experiments utilizing both the dominant negative activin receptor and the activin antagonist follistatin (Hemmadi-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994). Alternatively, our data are consistent with a model wherein activin acts as a neural inhibitor in the prospective endoderm. As the dominant negative receptors used in this study act to inhibit signal transduction pathways, it is possible that TGF-β signaling might be required for both repression of a non-endodermal fate and activation of an endodermal fate in the vegetal pole. Further investigation will be needed to understand both why the truncated activin receptor can ectopically induce non-endodermal markers in vegetal pole explants and whether or not it is Vg1 or other TGF-βs that mediate signals required for Xlhbox-8 expression. The ability of prospective endoderm to express mesodermal and ectodermal markers is consistent with previous work. It has been shown that the state of determination of the prospective endoderm is labile prior to gastrulation with isolated endodermal cells differentiating as ectodermal or mesodermal tissues when transplanted into the blastocoel of a host embryo (Wylie et al., 1987).

The monoclonal antibody 4G6 specifically recognizes an endodermal antigen that is expressed along the length of the gut tube. Animal caps treated with either activin or FGF express the 4G6 epitope (Jones et al., 1993). Thus, Xlhbox-8 and IFABP are additional endoderm-specific markers that are induced in animal caps by peptide growth factors (Gamer and Wright, 1995).

The regulation of Xlhbox-8 expression has been investigated by others and similar results have been obtained to those reported here (Gamer and Wright, 1995). Specifically, Xlhbox-8 expression was found to rely on both TGF-β signaling and the cortical rotation. Interestingly, dorsalization of embryos with LiCl leads to the loss of Xlhbox-8 expression as well (Gamer and Wright, 1995). Though additional markers for the anterior and posterior endoderm are needed, it is again tempting to speculate that determination of the anteroposterior axis of the gut endoderm relies on similar events and proteins to those involved in mesodermal development. In such a scheme, the entire vegetal hemisphere of the early embryo could be induced or “vegetalized” by a mesendodermal inducer as suggested previously (Takada and Yamada, 1960;


TGF-β signals and a prepattern in Xenopus laevis


Takata, C. and Yamada, T. (1960). Endodermal tissues developed from the isolated newt ectoderm under the influence of guinea pig bone marrow. Embryologia 5, 8-20.


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