Developmental Biology 236, 330–343 (2001)
doi:10.1006/dbio.2001.0347, available online at http://www.idealibrary.com on IDEAL®

Endoderm Specification and Differentiation in *Xenopus* Embryos

Marko E. Horb[1] **and Jonathan M. W. Slack**

Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom

It is known from work with amniote embryos that regional specification of the gut requires cell–cell signalling between the mesoderm and the endoderm. In recent years, much of the interest in *Xenopus* endoderm development has focused on events that occur before gastrulation and this work has led to a different model whereby regional specification of the endoderm is autonomous. In this paper, we examine the specification and differentiation of the endoderm in *Xenopus* using neurula and tail-bud-stage embryos and we show that the current hypothesis of stable autonomous regional specification is not correct. When the endoderm is isolated alone from neurula and tail bud stages, it remains fully viable but will not express markers of regional specification or differentiation. If mesoderm is present, regional markers are expressed. If recombinations are made between mesoderm and endoderm, then the endodermal markers expressed have the regional character of the mesoderm. Previous results with vegetal explants had shown that endodermal differentiation occurs cell-autonomously, in the absence of mesoderm. We have repeated these experiments and have found that the explants do in fact show some expression of mesoderm markers associated with lateral plate derivatives. We believe that the formation of mesoderm cells by the vegetal explants accounts for the apparent autonomous development of the endoderm. Since the fate map of the *Xenopus* gut shows that the mesoderm and endoderm of each level do not come together until tail bud stages, we conclude that stable regional specification of the endoderm must occur quite late, and as a result of inductive signals from the mesoderm. © 2001 Academic Press

Key Words: **endoderm; *Xenopus*; Xlhbox8; Pdx-1; IFABP; Xcad2; Edd; region-specific induction; mesoderm; gut.**

INTRODUCTION

The endoderm in *Xenopus* is derived from cells located in the vegetal hemisphere of the early embryo (Dale and Slack, 1987). Although it is commonly assumed that the large yolky cells are extraembryonic (e.g., Beddington and Robertson, 1999), recent results from our lab show that all parts of the endoderm present at the neurula stage contribute to the organs of the gastrointestinal and respiratory tracts, including the pancreas, liver, gall bladder, stomach, intestine, and lungs (Chalmers and Slack, 2000). The *Xenopus* tadpole gut is composed of an outer smooth muscle layer, derived from the mesoderm, and an inner epithelial layer, derived from the endoderm (Chalmers and Slack, 1998).

Development of the gut occurs in three stages: formation, regional specification, and differentiation (Fig. 1). **Formation** is the establishment of the endodermal germ layer. The cells must first decide whether to become endoderm, mesoderm, or ectoderm. In *Xenopus*, vegetal cells become committed to an endodermal fate cell-autonomously as a result of the localized maternal determinant *VegT* (Clements *et al.,* 1999; Dale, 1999; Xanthos *et al.,* 2001; Yasuo and Lemaire 1999; Zhang *et al.,* 1998) and this process is completed by the time of gastrulation (Wylie, 1987). Formation is assessed by the expression of pan-endodermal markers such as *Endodermin.* **Regional specification** within the endoderm is the commitment of each tissue region, which is manifested on culture in a neutral medium but may still be reversible (Slack, 1991). Here, the cells are being told where they are in regard to their position along the anterior–posterior, dorsal–ventral, and right–left axes. Regional specification is manifested by the expression of transcription factors such as *Xlhbox8* and *Xcad2.* Results from both chick and mouse have shown that regional specification of the endoderm is controlled by the adjacent mesoderm. In contrast, experiments in *Xenopus* have suggested that regional specification of the endoderm occurs

[1] To whom correspondence should be addressed. E-mail: m.horb@bath.ac.uk.

0012-1606/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

cell-autonomously (see below). **Differentiation** is the synthesis of functional proteins and mRNAs that are specific to a particular cell type or organ, be it pancreas, liver, stomach, or intestine. Examples of markers of endodermal differentiation include *IFABP*, *LFABP*, and *insulin*. In this study, these markers are used to infer the previous occurrence of regional specification.

It is known from numerous experiments on the chick embryo that the gut mesoderm can respecify the regional character of the endoderm (e.g., Yasugi, 1993; Roberts *et al.,* 1998; Rawdon, 2001). However, much of the work done on endoderm formation in *Xenopus* has suggested that regional specification occurs early in development, prior to gastrulation, and in the absence of mesoderm. These studies have relied upon vegetal explants isolated from the late blastula embryo. It was shown that both *Xlhbox8* and *IFABP* are expressed in particular regions within vegetal explants (Gamer and Wright, 1995; Henry *et al.,* 1996) and that the anterior endoderm becomes specified by the early blastula stage (Zorn *et al.,* 1999). In all of these cases, no expression of mesoderm markers was detected in the vegetal explants. As a result of these studies, it has become commonly assumed that regonal specification of the endoderm occurs very early in development and that the subsequent differentiation of organs from the endodermal germ layer occurs in an autonomous fashion. While these results appear conclusive, they contradict some earlier studies by Okada (1960), using not *Xenopus* but embryos of the urodele amphibian *Cynops (=Triturus) pyrrhogaster*. These showed that the endoderm will not differentiate in the absence of mesoderm and that the fate of the endoderm depends on the regional character of the adjacent mesoderm. Although Okada's study was carried out without the benefit of molecular markers, the results do resemble those obtained more recently with amniote embryos. We are then faced with the question: does the specification of the endoderm in *Xenopus* differ markedly from that in both amniotes and urodeles, or are the results showing autonomous development incorrect?

To investigate this, we have examined the specification and differentiation of the endoderm in *Xenopus* embryos by using various explants of mesoderm and endoderm from blastula, gastrula, neurula, and tail-bud-stage embryos. Our results confirm that endoderm formation is autonomous. However, we find that regional specification occurs only when there is concurrent formation of mesoderm. This was not detected in previous studies with vegetal explants because of the use of inappropriate mesodermal markers. We have performed isolation experiments of pieces of endoderm with and without mesoderm at later stages and these indicate that regional specification of the endoderm does not occur until tail bud stages, after the mesoderm has attained its final position in the embryo. We show, using heterotopic recombinations, that at this stage the mesoderm can still impart a regional character to the endoderm. Our final conclusion is that the endoderm possesses no stable regional specification until the tail bud stage and that it then acquires it through local inductive signals from the mesoderm.

Our experiments cannot exclude an early labile regional specification that depends on continuous interaction with the mesoderm or other neighbouring tissues. However, our earlier fate-mapping studies have shown that relative displacement between the endoderm and the mesoderm occurs subsequent to the midneurula stage (Chalmers and Slack, 2000). This means that any early transient labile specifications would be reprogrammed in the intact embryo through later interactions.

MATERIALS AND METHODS

Embryological Dissections

Stage 15 whole endoderm plus mesoderm (WEM) explants were made by first removing the cement gland and head region of the embryo. Then, the neural plate was split and the posterior portion of the embryo removed, cutting just below the blastopore. Last, the lateral portions were cut away. This left the archenteron floor endoderm on the dorsal side and the mes-ectoderm on the lateral and ventral sides. For anterior endoderm plus mesoderm (AEM) and posterior endoderm plus mesoderm (PEM) explants, the WEM explants were cut in half. A similar procedure was used for the stage 25 dissections. First, the head was removed. Second, the tail was removed. Last, the spinal cord and notochord were removed. Again, the dorsal surface of the explant consisted of the floor of the archenteron. The explants were bisected to generate AEM and PEM explants. For WE explants, the ectodermal and mesodermal layers were manually removed from the WEM explants. All explants were dissected in the presence of trypsin (100 μg/ml), to facilitate removal of the mesoderm, and remained in this solution for 30 min. Afterwards, the trypsin was neutralized by placing the explants in soybean trypsin inhibitor (10 μg/ml) for 30 min. All of the explants were cultured *in vitro* in NAM/2 medium (Beck and Slack, 1999a). Embryos were staged according to Nieuwkoop and Faber (1967). Explants of anterior and posterior mesoderm were dissected by removing the head and spinal cord from stage 25 embryos. Subsequently, the explants were bisected and the endoderm was removed with a tungsten needle. Anterior and posterior halves were kept separate and cultured in NAM/2 until stage 42.

Recombinations of mesoderm and endoderm were performed at stage 25. For anterior endoderm–posterior mesoderm recombinants (AEPM), AEM explants were dissected, and the endoderm was removed and placed aside. Next, PEM explants were dissected; the endoderm was removed and discarded, while the mesectoderm was kept. Last, the anterior endoderm was then placed within the posterior mesectoderm and allowed to heal for 30 min. The opposite was performed for posterior endoderm–anterior mesoderm recombinants (PEAM). Recombinations were cultured until stage 42.

For animal cap experiments (see Fig. 7F), 500 pg of *mixer* mRNA was injected at the 1 to 2 cell stage. Animal caps were dissected at stage 8–9 and grown to stage 35–40. Five to ten animal caps were harvested for RNA isolation.

RT-PCR

RT-PCR was performed as previously described with slight modifications (Wilson and Hemmati-Brivanlou, 1995; Wilson and

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

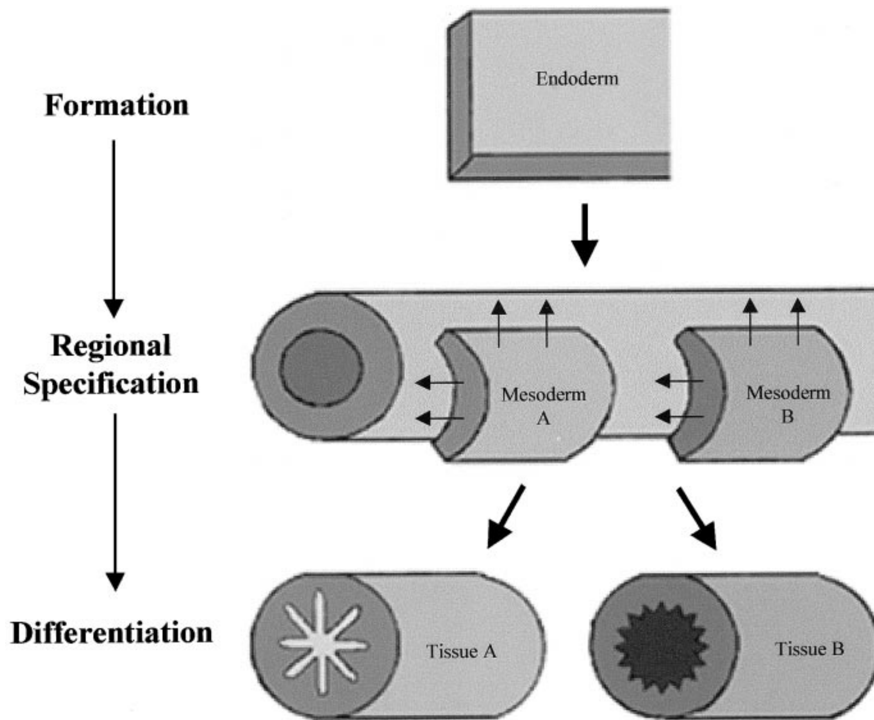

FIG. 1. Model for the development of the digestive tract occurs in three stages. (1) Formation. Establishment of endodermal cell fate occurs early in embryogenesis and is autonomous. This observation comes from recent work in *Xenopus* showing that localized transcription factors will activate the transcription of Edd and that loss of maternal VegT leads to a complete loss of endoderm. (2) Regional specification. Once cell fate is established, the mesoderm then acts to pattern the endoderm. Results from mouse, chick, frog, and fly embryos have shown that specification of the endoderm depends on the character of the overlying mesoderm. Since different mesoderm can respecify the endoderm, patterning in the endoderm is labile until the third stage. (3) Differentiation. By the last stage, cell fate has become determined irreversibly. This is characterized by the synthesis of mRNAs specific for a particular function. (Adapted from Horb, 2000.)

Melton, 1994). Groups of five explants were pooled for each measurement. After the cDNA synthesis, the volume was raised to 50 μl, and 1 μl of 0.5 M EDTA was added. The samples were phenol/chloroform-extracted and ethanol-precipitated. The cDNA was then resuspended in a total volume of 50 μl or greater depending of the relative levels of *EF1a*. RT-PCR was repeated at least three times using different samples in the presence of ^{32}P, except for the analysis described in Fig. 7F. For the animal cap experiment, the PCR was nonradioactive and three extra cycles were added for each primer. The primers for PCR and cycle numbers are as follows:

Xlhbox8 (28 cycles) upstream: 5′-TGCCAACTTCATCCCAGC-CC-3′ (818–837); downstream: 5′-GGCAGATGAAGAGGGCTC-3′ (998–1015).

Insulin (28 cycles) upstream: 5′-ATGGCTCTATGGATGCAG-TG-3′ (4–23); downstream: 5′-AGAGAACATGTGCTGTGGCA-3′ (277–295) (Henry *et al.,* 1996).

IFABP (24 cycles) upstream: 5′-CTGGTTCCTACAGGAC-3′ (286–301); downstream: 5′-GTATGCCCAATGTGCC-3′ (468–483) (Henry *et al.,* 1996).

LFABP (24 cycles) upstream: 5′-ACCGAGATTGAACAGAAT-GG-3′ (31–50); downstream: 5′-CCTCCATGTTTACCACGG-AC-3′ (156–174) (Henry and Melton, 1998).

Xhex (24 cycles) upstream: 5′-TGTGGAAAGAGGAATCG-ACA-3′ (75–94); downstream: 5′-GTCCCATTAGATGCGCT-GTT-3′ (204–224).

Xcad2 (25 cycles) upstream: 5′-CCACCAACGGTAAGACAA-3′ (542–559); downstream: 5′-GGAGATACCAAGTTGCTG-3′ (855–871).

Edd (21 cycles) upstream: 5′-TATTCTGACTCCTGAAGG-TG-3′ (1445–1465); downstream: 5′-GAGAACTGCCCATGTGC-CTC-3′ (1564–1583) (Sasai *et al.,* 1996).

EF1α (21 cycles) upstream: 5′-CAGATTGGTGCTGGATAT-GC-3′ (1096–1115); downstream: 5′-ACTGCCTTGATGACTCCT-AG-3′ (1345–1364) (Henry *et al.,* 1996).

XFD-13 (FoxF1) (25 cycles) upstream: 5′-AACCCTCTGTCC-TCCAGCCT-3′ (1181–1200); downstream: 5′-GGTTAGTGGAATG-ACTAACTT C-3′ (1491–1512).

XNkx-2.5 (25 cycles) upstream: 5′-AGAGATGGGAAGCCT-TGC-3′ (663–680); downstream: 5′-TCTACCAAGCTCGGATCG-3′ (931–948).

XTbx5 (27 cycles) upstream: 5′-GCCTGCATGTATGCTAGT-TC-3′ (1343–1362); downstream: 5′-GCCTGATGAGAAGACTGA-TG-3′ (1583–1602).

xFOG (27 cycles) upstream: 5′-TATGCCCAGAAGTTACAG-GAA-3′; downstream: 5′-CACCTCCTTTTTGTGCCAGTG-3′ (Deconinck *et al.,* 2000).

Xtwist (25 cycles) upstream: 5′-AGAAACTGGAGCTGGATC-3′

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

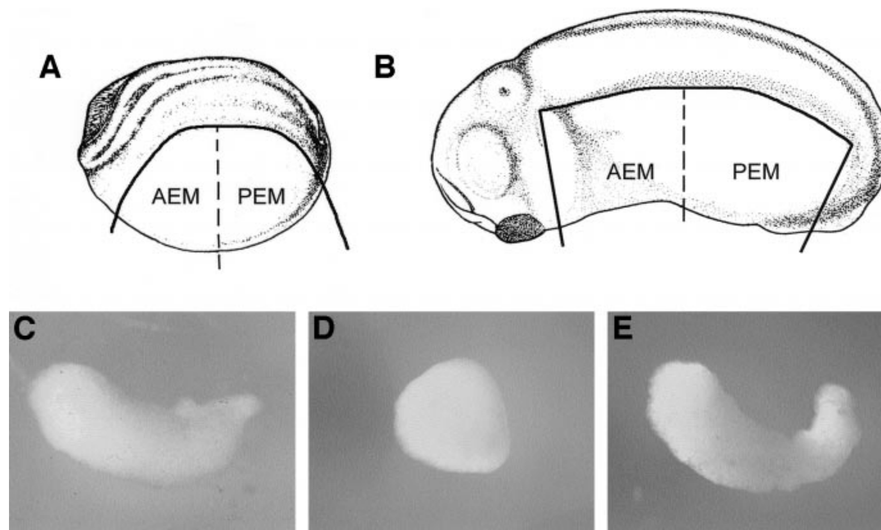

FIG. 2. Schematic diagram illustrating how the explants were made. (A) Neurula stage 15 embryo. The bold line indicates where the initial dissection was made to remove the neural plate, cement gland region, and blastopore. This isolated fragment is called whole endoderm plus mesoderm (WEM). The dashed line indicates where the WEM was bisected to generate the anterior (AEM) and posterior (PEM) explants. (B) Tail bud stage 25 embryo. As before, the bold line indicates where the head, spinal cord, and tail regions were dissected away. (C) Stage 15 whole endoderm (WE) explant. The WE explants elongate much more than the other explants. Notice, too, that there are no recognizable organ structures. (D) Stage 15 anterior endoderm (AE) explant. Unlike the WE explants, the AE explants remain as round balls and do not elongate. (E) Stage 15 posterior endoderm (PE) explant. These explants elongate much more than the AE explants.

(730–747); downstream: 5′-GGCTTCAAAGGCACGACT-3′ (1014–1031) (Henry *et al.,* 1996).

α-T_3 globin (21 cycles, 62°C) upstream: 5′-GCCTACAACCTG-AGAGTGG-3′ (328–346); downstream: 5′-CAGGCTGGTGAGC-TGCCC-3′ (511–529) (Henry *et al.,* 1996).

Cardiac actin (21 cycles, 62°C) upstream: 5′-GCTGACAGAA-TGCAGAAG-3′ (987–1004); downstream: 5′-TTGCTTGGAGG-AGTGTGT-3′ (1194–1212) (Henry *et al.,* 1996).

In Situ *Hybridization*

Whole-mount *in situ* hybridization was performed according to Harland (1991), but without the RNase treatment. Antisense *Xlhbox8* RNA was made by cutting *Xlhbox8-CS2* with *Eco*RI and transcribing with T7; antisense *IFABP* RNA was made by cutting with *Xho*I and transcribing with T7; antisense *Xcad2* RNA was made by cutting with *Not*I and transcribing with T7; antisense *Edd* RNA was made by cutting pBS-#55 with *Eco*RI and transcribing with T7; antisense XFD-13 was made by cutting with *Eco*RI and transcribing with T7. *In situ* hybridizations were repeated several times by using samples from separate experiments.

RESULTS

Our lab has recently completed a fate map of the *Xenopus* gut at midneurula stage 15 (Chalmers and Slack, 2000). We therefore decided to examine what role mesoderm–endoderm interactions may have in gut formation and patterning by examining the regional specification of the endoderm at this stage. We also examined explants taken at stage 25, by which time we expect the relative movement of endodermal and mesodermal layers to be completed. We dissected four different types of explants (Fig. 2): WEM, AEM, PEM, and WE.

The WEM explants were produced by removing the neural plate, blastopore, and cement gland regions as shown in Fig. 2A. This left the endoderm and the lateral and ventral mes-ectoderm intact. AEM and PEM explants were then produced by cutting the WEM explants in half (Fig. 2A). As a result of the dissection procedure, the dorsal part of the explant, which is the archenteron floor, becomes exposed to the medium and the explants thus develop with the endoderm on the dorsal side and the mes-ectoderm on the ventral side. These explants were cultured until stage 42, when organogenesis has begun, and the expression of several markers of anterior and posterior endoderm was examined by whole-mount *in situ* hybridization. Based on our recent fate map, we would predict that the AEM explants contain pharynx, oesophagus, liver, pancreas, stomach, and small intestine, while PEM explants contain some small intestine and all of the large intestine (Chalmers and Slack, 2000). In agreement with this, AEM explants frequently contained beating hearts and pharyngeal arches. PEM explants normally contained bifurcated elongated structures (see Fig. 3). Molecular characterisation of the types of mesoderm produced by these explants is presented in the next section and the associated Fig. 5.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

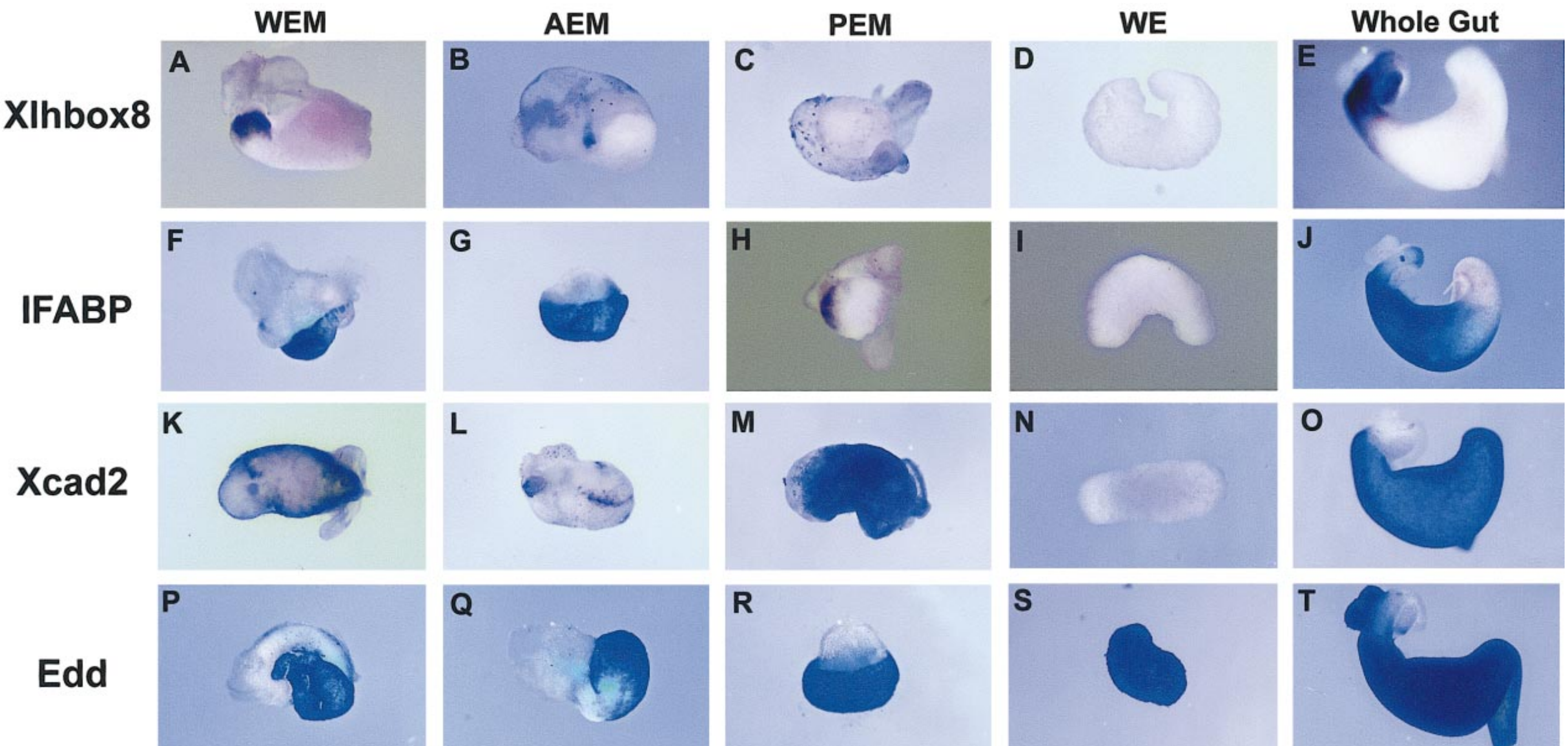

FIG. 3. Expression pattern analysis of endodermal markers in stage 15 explants by whole-mount in situ hybridization. (A–E) Xlhbox8 expression. Notice that expression of Xlhbox8 is only seen in WEM and AEM explants, but not in PEM or WE explants. In isolated whole guts at stage 42, Xlhbox8 is expressed in the pancreas and the stomach. (F–J) IFABP expression. IFABP is expressed in WEM, AEM, and PEM explants, but not in WE explants. Also, IFABP is expressed in both AEM and PEM explants, as predicted by the fate map. In isolated whole guts, IFABP is expressed from the base of the stomach through the middle of the intestine. No IFABP expression is detected in the posterior intestine. (K–O) Xcad2 expression. Xcad2 is also expressed in WEM, AEM, and PEM explants. In contrast to IFABP, however, Xcad2 is expressed more highly in PEM explants than in AEM explants. In isolated whole guts, Xcad2 is expressed from the beginning of the small intestine through to the proctodeum. (P–T) Edd expression. Edd is expressed in all four explants. It is not expressed throughout the AEM explants, but is expressed throughout the PEM explants. In contrast to the other markers, Edd is expressed throughout the WE explants. In isolated whole guts at this stage, expression has become downregulated in the pancreas or stomach.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

TABLE 1
Endodermal Marker Expression in Explants (*in situ* results)

Stage	Marker	WEM	AEM	PEM	WE
15	*Xlhbox8*	11/14	9/14	0/14	0/14
15	*IFABP*	17/20	13/15	10/17	0/18
15	*Xcad2*	13/15	5/13	13/14	0/15
25	*Xlhbox8*	12/14	10/13	0/13	0/22
25	*IFABP*	16/17	16/16	7/15	0/20
25	*Xcad2*	15/15	12/16	8/11	0/15

WE explants were produced by removing the mesodermal and ectodermal layers manually. Suprisingly, these explants elongate much more than any of the mesoderm-containing explants, but do not contain any recognisable morphological structures (Fig. 2C). The extensive cell movements made by the endodermal explants suggest that the elongation of the whole embryo is not caused by the mesoderm as we had thought, but rather is driven by the endoderm (Larkin and Danilchik, 1999; Drawbridge and Steinberg, 2000). In fact, explants of mesoderm alone do not elongate, but rather form large vesicles (data not shown). AE and PE explants were produced by cutting the WE explants in half. Interestingly, PE explants (Fig. 2E) elongate much more than AE explants (Fig. 2D), suggesting that it is the posterior endoderm that is responsible for the elongation of the WE explants.

Regional Specification of the Endoderm Requires the Mesoderm

To determine what type of endoderm is present in these explants, we examined the expression of three anterior–posterior markers of endoderm specification or differentiation by whole-mount *in situ* hybridization: *Xlhbox8*, *IFABP*, and *Xcad2*. Explants were made at stages 15 and 25 and the results were similar for both stages. Typical specimens explanted at stage 15 are shown in Fig. 3, and the complete results for both stages are collected in Table 1.

Xlhbox8 is an anterior endoderm marker being expressed in the duodenum and the pancreas (Fig. 3E; Wright *et al.,* 1989; Gamer and Wright, 1995; Chalmers and Slack, 1998). *Intestinal fatty acid binding protein* (*IFABP*) has generally been considered to be a posterior endoderm marker (Henry *et al.,* 1996). It is expressed more posteriorly than *Xlhbox8* with strongest expression in the small intestine, but weaker expression is also present in the duodenum (Fig. 3J; Shi, 1994). Our fate-map data show that it would be expected to be expressed in both anterior and posterior halves (Chalmers and Slack, 2000). *Xcad2* is expressed in both the small and the large intestine (Fig. 3O) and is predominantly a posterior endoderm marker; however, it is not exclusively posterior because the expression domain does overlap *Xlhbox8* and *IFABP* to a degree (Chalmers *et al.,* 2000).

In the explants, *Xlhbox8* is expressed in WEM and AEM explants, but not in PEM or WE explants (Figs. 3A–3D). *IFABP*, on the other hand, is expressed in both AEM and PEM explants, with stronger expression in AEM explants (Figs. 3F–3H). This is in agreement with our fate map, which shows that the small intestine is derived from the middle of the endoderm at stage 15 (Chalmers and Slack, 2000). As with *Xlhbox8*, we were unable to detect any expression of *IFABP* in the WE explants (Fig. 3I). *Xcad2* is expressed in both AEM and PEM, but more strongly in PEM explants (Figs. 3K–3M). Again, no expression of *Xcad2* was detected in WE explants (Fig. 3N). The lack of expression of any of these markers of endoderm specification or differentiation in explants of whole endoderm alone suggests that regional specification of the endoderm may not occur cell-autonomously as previously proposed, but may, in fact, require the presence of mesoderm. The stage 25 explants behave very similarly to the stage 15 explants, thus demonstrating that, even as late as stage 25, the endoderm has not been regionally specified.

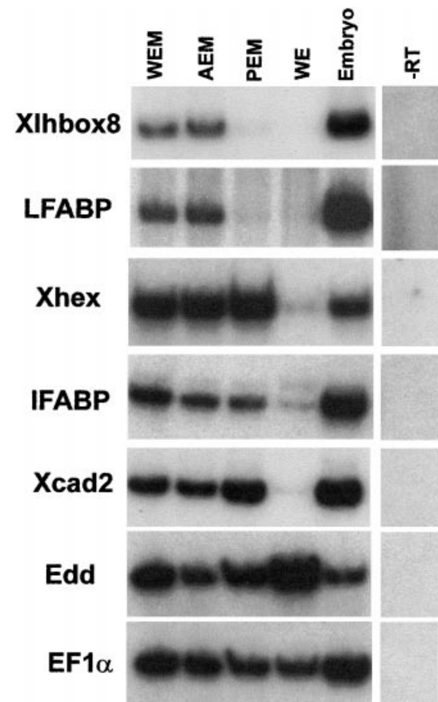

FIG. 4. RT-PCR analysis of stage 15 explants cultured until stage 42. cDNA was generated from five explants and used to analyze the expression of several endodermal differentiation markers by PCR. None of the markers tested, including *Xlhbox8*, *LFABP*, *Xhex*, *IFABP*, and *Xcad2*, are expressed in the isolated whole endoderm. *Xlhbox8* and *LFABP* are only expressed in the AEM explants, but not, as expected, in the PEM explants. *Xhex*, *IFABP*, and *Xcad2* are expressed in both AEM and PEM explants. In contrast to the other markers, *Edd* expression is increased in the WE explants compared to the others. This agrees with our assertion that the WE explants have not undergone regional specification, as *Edd* is normally lost from the pancreas and stomach.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

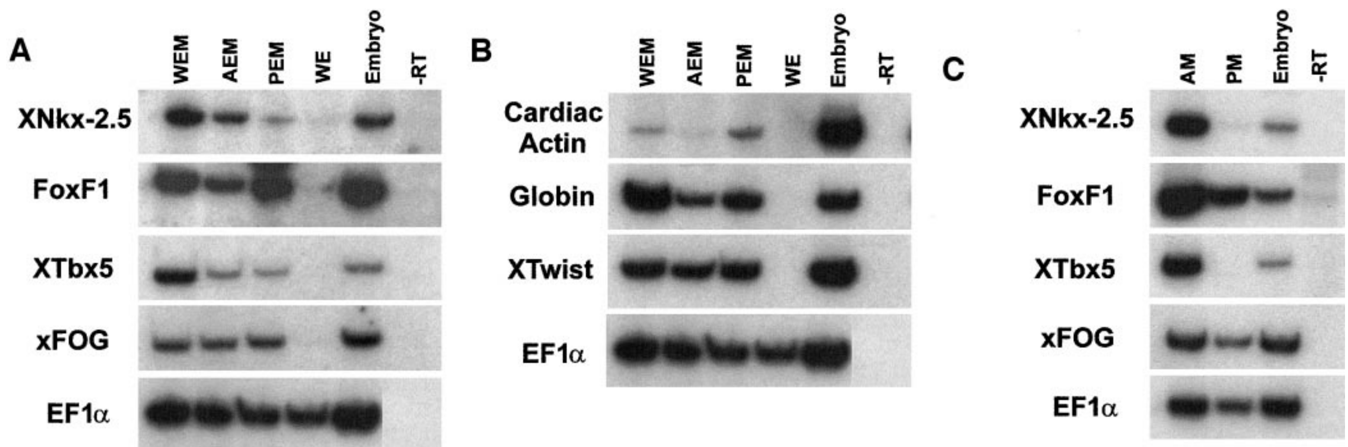

FIG. 5. Mesodermal marker expression in neurula stage 15 explants. (A) *XNkx-2.5*, *FoxF1*, *XTbx5*, and *xFOG* are not detected in the whole endoderm explants. All of these markers are, however, expressed in the explants with mesoderm to various degrees. Notice that *XNkx-2.5* is expressed most strongly in the AEM explants. This is in agreement with its endogenous expression pattern in the mesoderm surrounding the duodenum. (B) Neither *cardiac actin*, α-T_3 *globin*, nor *Xtwist* is expressed in the whole endoderm explants. Again, expression of each of these markers is seen in the explants with the mesoderm to varying degrees. (C) To confirm that the markers used to detect the presence of mesoderm are indeed expressed in the mesoderm, we examined their expression in explants of isolated anterior or posterior mesoderm from stage-25 embryos.

There are several other possible explanations for the discrepancy between our results and those of others. First, it is possible that the WE explants are dying and this may be the reason they do not express any endodermal markers. To determine whether the WE explants express any endodermal markers at all, we examined the expression of *Endodermin* (*Edd*). Early in development *Edd* is expressed throughout the whole endoderm (Sasai *et al.,* 1996), but by stage 42 the expression in the stomach and pancreas decreases, while expression in the liver and intestine remains (Fig. 3T; Chalmers and Slack, 1998). *Edd* was expressed in all of our explants, including the WE explants (Fig. 3P–3S). This confirmed that the WE explants were viable and were committed to form endoderm. Unlike the regionally specific markers, *Edd* is expressed throughout the explant, suggesting that its activation really is autonomous and does not require the presence of mesoderm. *Edd* is commonly considered a pan-endodermal marker. Although this is true during the earliest developmental stages, by stage 40 its expression normally becomes downregulated in the anterior endoderm, excluding the liver, and by stage 48 *Edd* expression is only detected within the liver (Chalmers and Slack, 1998). The change in *Edd* expression correlates well with the timing of endodermal differentiation, in that when the endoderm begins to differentiate, *Edd* is shut off. The failure to shut it off in the WE explants is consistent with the view that the endoderm is not regionally specified.

Second, it is possible that the whole endoderm requires a covering of some sort for proper differentiation to occur. To test this, whole endoderm explants were wrapped within an animal cap and cultured until stage 42. No expression of endodermal differentiation markers was observed in these explants (data not shown).

Third, the discrepancy between our results and others may be due to the use of different techniques. Since previous groups mainly used RT-PCR to examine the expression of endodermal markers, the use of whole-mount *in situ* hybridization here may not be sensitive enough. To address this, we looked at the expression of several markers of endodermal specification and differentiation by RT-PCR. The explants were dissected at stage 15 and cultured until stage 42. In agreement with our *in situ* results, the PCR results confirm that regional specification of the endoderm requires the mesoderm. *Xlhbox8* expression is detected in WEM and AEM explants, but not in PEM or WE explants (Fig. 4). Both *IFABP* and *Xcad2* are expressed in WEM, AEM, and PEM explants, but neither is expressed in WE explants. Identical results were obtained with stage 25 explants (data not shown). In agreement with our *in situ* results, *Xcad2* did show higher levels of expression in PEM explants than in AEM explants. This was more apparent in the stage 25 explants. We also looked at the expression of two liver markers, *LFABP* (Henry and Melton, 1998) and *Xhex* (Newman *et al.,* 1997). Interestingly, while *LFABP* is only present in AEM explants, *Xhex* is expressed in both AEM and PEM explants (Fig. 4). As with the other markers, neither *LFABP* nor *Xhex* is expressed in the WE explants. The lack of any endodermal specification or differentiation markers in the neurula and tail bud WE explants by both PCR and whole-mount *in situ* hybridization confirms that regional specification of the endoderm does not occur autonomously. Finally, certain anterior endodermal genes

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

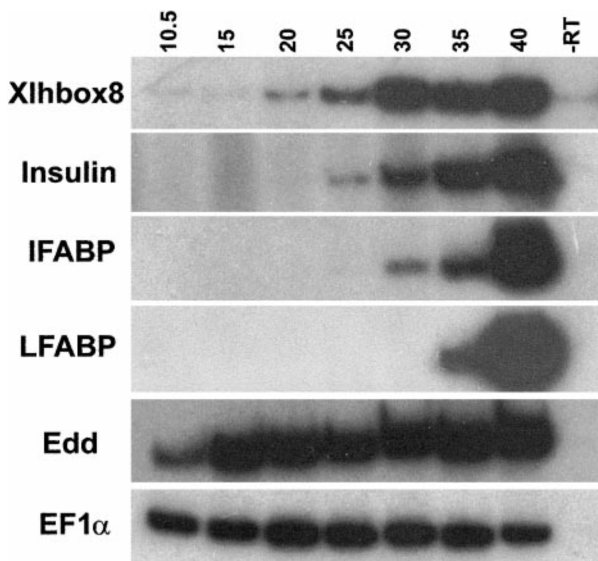

FIG. 6. Temporal expression of endodermal differentiation markers in whole embryos. Whole embryos were collected at various stages from 10.5 to 40. In agreement with previous results, a low level of *Xlhbox8* expression is detectable at stages 20 and 25, but there is a substantial increase in expression beginning at stage 30. Other markers are first expressed between stages 25 and 35.

such as *xhex* are known to be activated at gastrula stages (Jones *et al.,* 1999; Newman *et al.,* 1997; Zorn *et al.,* 1999) and it is possible that these remain on in the explants. *Xhex* was examined by RT-PCR in stage 15 explants, but no expression was detected (data not shown).

To make our results secure, we needed to characterise the mesoderm present in the WEM, AEM, and PEM explants and to confirm that there was really no mesoderm in the WE explants. To this end, we examined the expression of various mesoderm markers, namely, *XFD-13*, *XNKx-2.5*, *XTbx5*, and *xFOG*. *XFD-13* has recently been cloned and shown to be expressed throughout the gut mesoderm at stage 35 (Köster *et al.,* 1999). According to the new nomenclature for winged helix/forkhead transcription factors, *XFD-13* has been renamed *FoxF1* and will be named as such throughout the paper (Kaestner *et al.,* 2000; http://www.biology.pomona.edu/foxbyspp.html). In mouse and chick, *Nkx2.5* has been reported to be expressed in the mesoderm in a small ring around the duodenum (Lints *et al.,* 1993). In *Xenopus*, Nkx-2.5 is initially expressed in both mesodermal and endodermal tissues during early tail bud stages (Evans *et al.,* 1995). By tadpole stages, however, *XNkx-2.5* is found to be expressed in the heart and in the mesoderm surrounding the duodenum (Patterson *et al.,* 2000; Smith *et al.,* 2000). The third marker, *XTbx5*, has been shown to be essential for heart development (Horb and Thomsen, 1999). Initially, *XTbx5* is expressed in the lateral mesoderm during tail bud stages, but becomes restricted to the heart and sinus venosus later in development. The last marker, *xFOG*, was recently cloned and shown to repress the development of red blood cells (Deconinck *et al.,* 2000). At tail bud stages, *xFOG* is expressed in the ventral mesoderm in a pattern similar to *α-globin*.

The WE explants isolated from neurula and tail bud stages do not express any of the mesoderm markers examined but expression is detected in the explants with mesoderm (Fig. 5A). We also examined the expression of mesodermal markers not associated with the gut mesoderm: *cardiac actin*, *α-T_3 globin*, and *Xtwist*. These were also not expressed in the WE explants, but were expressed in the explants with mesoderm (Fig. 5B). To further confirm the mesodermal localization of the mesodermal markers, we examined their expression in isolated anterior and posterior mesoderm explants taken from stage 25. As shown in Fig. 5C, all of these markers are present within at least one of the mesodermal explants.

To try to determine when the endoderm does become specified, we attempted to isolate endoderm free of mesoderm at progressively later stages than stage 25. But in the late 20 stages, we found it impossible to remove the mesoderm cleanly, even after trypsin treatment. So, instead, we examined the temporal expression of *Xlhbox8*, *insulin*, *LFABP*, and *IFABP* by RT-PCR in order to find the initial stage of differentiation, which would represent the latest possible time of specification. The results from this time course are presented in Fig. 6 and they show that the initial stages of endoderm differentiation occur between 25 and 30, with substantial levels of *insulin* and *Xlhbox8* present by stage 30. The low level of Xlhbox8 present at stages 20 and 25 is in agreement with previous results, which showed a basal level of expression present from stage 12 that increases substantially after stage 25 (Gamer and Wright, 1995). This initial endoderm differentiation most likely represents the development of the dorsal pancreas since it coincides with *insulin* expression, which has recently been shown to only be expressed in the dorsal pancreas during early tadpole stages (Kelly and Melton, 2000). Later endoderm differentiation takes place between stages 30 and 35 with the appearance of the liver and intestinal markers, *LFABP* and *IFABP*. These results confirm that regional specification of the endoderm need not occur until stage 25. The very low levels of *Xlhbox8* seen before this stage may not be sufficient for biological activity, and are evidently not stable as they are not maintained in stage 15 or 25 WE explants isolated from mesoderm and cultured to later stages.

Vegetal Explants Contain Mesoderm

We were initially suprised to find that the WE explants do not express any markers of regional endodermal specification, since earlier results by others with vegetal explants had shown that not only endoderm formation but also regional specification is autonomous. One possibility to explain this discrepancy is that the explants in earlier studies actually did contain mesoderm but that the appro-

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

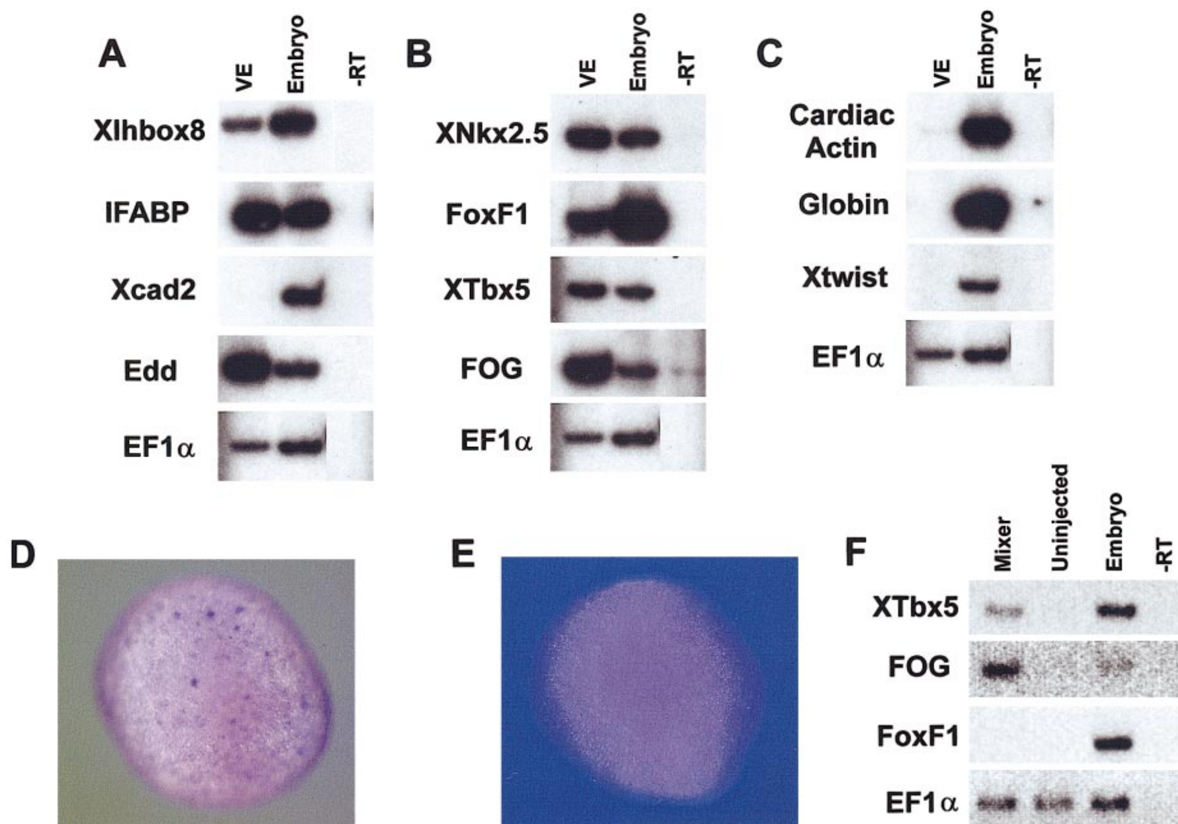

FIG. 7. RT-PCR analysis of vegetal explants. The explants were isolated at late blastula stage 9 and grown *in vitro* until stage 42. (A) Endodermal marker expression. *Xlhbox8* and *IFABP*, but not *Xcad2*, are expressed. This suggests that posterior endoderm does not become specified in these vegetal explants. (B) Mesodermal marker expression. All four of the mesodermal markers tested here show expression in the vegetal explants: *XNkx-2.5*, *FoxF1*, *XTbx5*, and *xFOG*. (C) Expression of previously used mesodermal markers *cardiac actin*, *globin*, and *Xtwist* is absent in the vegetal explants. This confirms that our explants were dissected properly. (D, E) *In situ* hybridization for *FoxF1* (D) and nonexpressed control gene (E) showing scattered positive cells expressing *FoxF1* in the vegetal explant. (F) Mesodermal marker expression in *mixer*-injected animal caps. *Mixer*-injected animal caps express both *XTbx5* and *xFOG*, but not *FoxF1*. Uninjected animal caps do not express any of these three markers.

priate mesoderm markers were not used to detect it. Indeed, only *Xbra*, *cardiac actin*, *type II collagen*, *Xtwist*, and *α-T_3 globin* were previously used as markers for the presence or absence of mesoderm in vegetal explants (Gamer and Wright, 1995; Henry *et al.,* 1996; Henry and Melton, 1998; Lemaire *et al.,* 1998). Although all are early markers of mesoderm differentiation, none of these has been shown to be expressed in the gut mesoderm. For example, by tadpole stages, *Xbra* is expressed exclusively in the tail bud and notochord (Gont *et al.,* 1993; Smith *et al.,* 1991). Having assembled the panel of gut mesoderm markers described above, we decided to reexamine the issue of mesoderm production by vegetal explants by RT-PCR.

Vegetal explants were dissected at stage 9 and grown to stage 42. As shown previously by others, we find that both *Xlhbox8* and *IFABP* are expressed in vegetal explants (Fig. 7A). In contrast, the posterior endoderm marker, *Xcad2*, is not expressed. This suggests that posterior endoderm is not produced by vegetal explants, while anterior-type endoderm is. We next examined the expression of *XNkx-2.5*, *FoxF1*, *XTbx5*, and *xFOG* in these vegetal explants. We were surprised to find that all four of these mesoderm markers are expressed in vegetal explants (Fig. 7B). It may be argued that our vegetal explants were not dissected properly and that we may have inadvertently included mesodermal cells. To confirm that our vegetal explants were dissected properly, we examined the expression of the other mesoderm markers that have been used previously to show the absence of mesoderm in the vegetal explants: *cardiac actin*, *α-T_3 globin*, and *Xtwist*. As shown in Fig. 7C, none of these markers is expressed in our vegetal explants, thus confirming that our dissections were accurate. An *in situ* study of *FoxF1* expression in these vegetal explants suggests that a small number of cells in some explants are mesodermal in character (Fig. 7D). *In situs* for *XTbx5* also showed some positive cells, but none were found for *XNkx-2.5* (data not

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

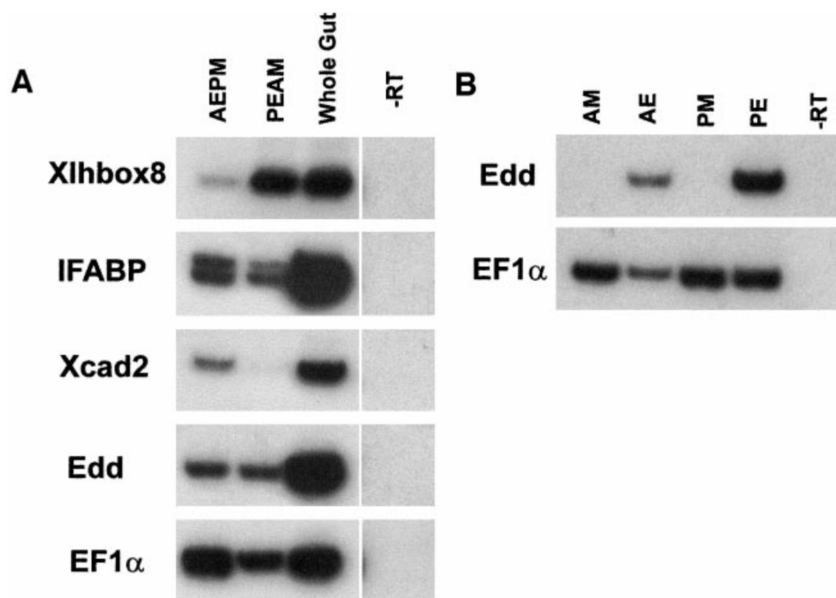

FIG. 8. RT-PCR expression of endodermal markers in mesoderm–endoderm recombinants. (A) *Xlhbox8* is only expressed in the PEAM recombinants, and barely in the AEPM recombinants. Conversely, *Xcad2* is only expressed in the AEPM recombinants. *IFABP* is, as expected, expressed in both recombinants. The regional specificity is characteristic of the mesoderm and not the endoderm. (B) To confirm that our mesodermal explants were themselves free from contaminating endoderm, we examined the expression of *Edd* in anterior endoderm (AE), anterior mesoderm (AM), posterior endoderm (PE), and posterior mesoderm (PM) explants. No expression of *Edd* is seen in the mesodermal explants, while abundant expression of *Edd* is seen in the endodermal explants.

shown). It may be significant that previously published pictures of endodermal marker expression in vegetal explants show somewhat spotty expression at the edge of the explants (e.g., Henry *et al.,* 1996). We also examined whether it was possible to isolate vegetal explants from later stages that were devoid of mesoderm. To this end, we dissected vegetal explants from stage 10.5 embryos, cutting below the blastopore, and cultured them to stage 42. As with the stage 9 explants, stage 10.5 vegetal explants also contained mesoderm (data not shown).

Since the initial studies with vegetal explant, numerous endoderm-specific cDNAs have been cloned, and several of these have been shown to activate endoderm differentiation markers when overexpressed in animal caps. These include *Mixer* (Henry and Melton, 1998), *XSox17* (Hudson *et al.,* 1997), *Gata5* (Weber *et al.,* 2000), and *xBic-C* (Wessely and De Robertis, 2000). As with the vegetal explants, none of the mesoderm markers examined were expressed in the animal caps, including *Xbra*, *Xtwist*, muscle actin, and globin. We wanted to determine whether any of these endoderm-specific transcription factors were activating endodermal differentiation autonomously or whether lateral plate mesoderm was being produced much like the vegetal explants. We chose to use *mixer* as a representative of this group to test whether these factors do indeed induce ectopic mesoderm. We examined *mixer*-injected animal caps for the presence of mesoderm and found that both *XTbx5* and *xFOG* were expressed, while *FoxF1* was not expressed (Fig. 7F). Although not tested we believe that, much like the vegetal explants and the *mixer*-injected animal caps, the other endoderm-specific cDNAs do not activate endoderm differentiation markers in animal caps autonomously, but rather do so through the activation of mesoderm markers.

These results thus demonstrate that, in contrast to previous reports, vegetal explants and *mixer*-injected animal caps do contain or produce mesoderm. This difference is due to the use of different mesoderm markers; whereas previous reports only looked at axial mesoderm markers, we have looked at lateral, ventral, and gut mesoderm markers. These results confirm our analysis with the neurula and tail-bud-stage explants and suggest that the expression of regional endoderm markers in the vegetal explants is probably due to the presence of mesodermal cells and the subsequent inductive interactions between the endoderm and mesoderm arising within the explants. These results presented above demonstrate that our own endodermal explants taken from later stages do not contain any mesoderm, and are thus better suited than the vegetal explants to determine whether endodermal differentiation occurs cell-autonomously.

Mesoderm–Endoderm Recombinations

From the results presented so far, it remains possible that the mesoderm is only playing a permissive role in endoder-

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

mal specification and differentiation. To investigate whether the mesoderm can, in fact, instruct the endoderm, we recombined isolated endoderm with isolated mesoderm. Anterior endoderm was isolated from stage 25 tail bud embryos and recombined with posterior mesoderm (AEPM), while posterior endoderm was recombined with anterior mesoderm (PEAM). We examined the expression of *Xlhbox8*, *IFABP*, *Xcad2*, and *Edd* by RT-PCR and found that the regional expression of these endodermal markers depends on the nature of the adjacent mesoderm (Fig. 8A). *Xlhbox8* is expressed in PEAM recombinants, but barely in AEPM recombinants. *Xcad2*, on the other hand, is expressed in AEPM but not in PEAM recombinants. *IFABP* did not show much regional specificity in these recombinations, but we would not expect this based on the fate map. To confirm that there was no endodermal contamination in our mesodermal explants, we examined the expression of *Edd* in isolated anterior or posterior mesoderm. As shown in Fig. 8B, no *Edd* marker expression is present in our mesodermal explants. In contrast, high levels of *Edd* are seen in the anterior and posterior endodermal explants. Therefore, the regional expression of both *Xlhbox8* and *Xcad2* in the endoderm in our recombinants must be due to signals released from the anterior and posterior mesoderm, respectively. These results demonstrate that the mesoderm can indeed play an instructive role in endodermal specification and differentiation.

DISCUSSION

Endoderm Formation Is Autonomous, but Regional Specification Is Not

In the 1950s, Okada used the amphibian *Cynops* (=*Triturus) pyrrhogaster* to demonstrate that the endoderm would differentiate only in the presence of mesoderm and that the regional character of endodermal differentiation was determined by the mesoderm (reviewed in Okada, 1960). These initial studies were based on morphological characteristics using histology to determine the presence or absence of mesoderm and the type of endoderm present. In 1995, however, Gamer and Wright showed that regional markers within the endoderm are expressed in the absence of mesoderm in another amphibian, *Xenopus laevis*. In that study, they examined the expression of *Xlhbox8* in vegetal explants and were able to show that its expression was turned on independent of mesoderm. Since that time, all of the papers published on endoderm development in *Xenopus* have apparently confirmed that the endoderm not only is formed autonomously, but also acquires regional specification autonomously (Clements *et al.,* 1999; Henry *et al.,* 1996; Henry and Melton, 1998; Lemaire *et al.,* 1998; Yasuo and Lemaire, 1999; Zhang *et al.,* 1998; Zorn *et al.,* 1999). We tried to extend these results using neurula and tail bud embryos to examine the specification of the endoderm but our results show that the endoderm cannot become regionally differentiated in the absence of mesoderm. Our results now bring *Xenopus* into line both with the urodele studied by Okada, and with mouse and chick, showing that regional specification and consequent differentiation of the endoderm depends on signals released from the mesoderm (Okada, 1960; Rawdon, 2001; Roberts *et al.,* 1998; Wells and Melton, 1999, 2000; Yasugi, 1993).

We have concluded that the mesoderm really is essential to the regional specification of the endoderm and that the vegetal explants used in previous studies did in fact produce some gut-type mesoderm. At neurula and tail bud stages, the endoderm and mesoderm form distinct layers and are easily separated. In contrast, during blastula stages, from which vegetal explants are made, the mesodermal and endodermal cells overlap and are not easily separable. Also, it may be possible for a few vegetal cells to switch from a labile endodermal to a mesodermal commitment as a consequence of the isolation procedure. In the previous studies, the markers used to establish whether any mesoderm was present in the vegetal explants have never been shown to be expressed in the gut mesoderm. *Xbra* is expressed only in the tail bud at early tadpole stages (Gont *et al.,* 1993; Smith *et al.,* 1991); *muscle specific actin* is localized to the somitic and heart mesoderm; *type II collagen* is localized to the notochord (Amaya *et al.,* 1993); *Xtwist* is expressed in the neural crest (Hopwood *et al.,* 1989; Schuh *et al.,* 1993); and although α-T_3 *globin* is expressed in the ventral mesoderm at tail bud and early tadpole stages, it is unlikely to be expressed in the mesoderm surrounding the gut at late tadpole stages. In contrast, we have used two mesoderm markers, *FoxF1* and *XNkx-2.5*, that are expressed in the gut mesoderm. *FoxF1* was recently cloned by Köster *et al.* (1999) and they showed that it is expressed throughout the mesoderm surrounding the gut. *XNkx-2.5* is also expressed in the gut mesoderm, localized to the region surrounding the duodenum. We have also shown that these vegetal explants express other mesoderm markers normally found in parts of the lateral plate: *XTbx5* and *xFOG*. This expression is found only in a few cells and may represent a regulative response to isolation.

More recently, several endoderm-specific transcription factors have been shown to function in a manner similar to the vegetal explants. When the mRNA is overexpressed in animal caps, endodermal differentiation markers are turned on, while mesodermal markers are not. These results seem to strengthen the argument that the endoderm differentiates autonomously. Our results with *mixer*-injected animal caps, however, show that mesoderm is present (Fig. 7F). This result therefore strengthens our argument that the mesoderm is necessary for the endoderm to differentiate. We believe that the other endoderm-specific transcription factors will also be found to induce some mesoderm markers in animal caps. Although *mixer*-injected animal caps do not express all of the mesoderm markers tested, we do not find this suprising since *mixer* is normally expressed in only a subset of the vegetal cells. It will be of interest to see whether the

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

presence of specific mesoderm markers can be correlated with the induction of specific endoderm markers.

Status of Anterior–Posterior Endodermal Markers

In this study, we have examined the specification of the endoderm by using both anterior and posterior explants of endoderm and mesoderm. Both *in situ* hybridisation and RT-PCR were used to determine the localisation of four endoderm markers, *Xlhbox8*, *IFABP*, *Xcad2*, and *Edd*. Previous studies into endoderm formation in *Xenopus* have usually just relied upon *Xlhbox8* and *IFABP* to mark anterior and posterior endoderm, respectively. The results in this paper have a bearing on how these markers are regarded. While our results confirm that *Xlhbox8* is an anterior marker, the fate map clearly shows that the normal expression domain of *IFABP* is derived from both anterior and posterior halves of the neurula endoderm (Chalmers and Slack, 2000). Our *in situ* hybridisation and RT-PCR data show that *IFABP* is expressed in both anterior and posterior explants, as predicted from the fate map. So *IFABP* is not really a posterior endoderm marker, but should be considered more of an intermediate marker. *Xcad2* is a better candidate for a posterior endoderm marker, although even this is not exclusively expressed by tissues derived from the posterior half of the neurula.

Endoderm Specification Occurs Late in Xenopus

It is commonly assumed that endodermal regional specification occurs during gastrulation in the *Xenopus* embryo, but our results contradict this view. If specification of the endoderm occurred early in development, then appropriate regional markers should thereafter be expressed by endoderm explants. However, even as late as stage 25, explants of the whole endoderm will not express any regional differentiation markers. These WE explants are indeed alive and of endodermal character as shown by the expression of *Edd* and by the fact that they elongate. Recently, it has been shown that ventral explants from neurula embryos will elongate as much as seen in whole embryos and it has been proposed that cell rearrangements within the mesoderm and the ectoderm are responsible for the lengthening of the embryo (Larkin and Danilchik, 1999). Our results with the WE explants, however, suggest that it may be the endoderm, rather than the mesoderm or the ectoderm, that is responsible for the anterior–posterior lengthening of the axis that occurs during neurula and tail bud stages.

Our recent fate map of the *Xenopus* gut showed that the presumptive epithelial and smooth muscle cells of gut organs are not aligned with one another at stage 15, but move into correspondence at a later stage. This observation, together with the data presented here showing that endoderm is not specified even by stage 25, suggests that regional specification in normal development occurs only after the endoderm and mesoderm have reached their final relative positions. Several results agree with this position. First, we have shown in transgenic *Xenopus* embryos that the *elastase* promoter becomes activated about stage 31 (Beck and Slack, 1999b). Second, XlHbox8 protein is first visible at stage 33 (Wright *et al.,* 1989). Third, tadpoles exposed to retinoic acid for a brief period during stages 25–35 will develop abnormalities in the gut, while earlier or later treatments have little effect (Zeynali and Dixon, 1998). Fourth, in *Xenopus*, grafts of endoderm into new locations prior to stage 28 will develop according to their new location, while grafts after stage 28 will develop according to their fate (Zeynali *et al.,* 2000). Last, we have shown that high levels of expression of *Xlhbox8*, *insulin*, *LFABP*, and *IFABP* mRNA do not begin until stages 30–35 (Fig. 6). In conclusion, these results would place the time of regional specification of the endoderm between stages 28 and 32.

Our results cannot exclude a labile expression of endodermal markers at earlier stages within intact embryos. For example, there is a low level of *Xlhbox8* expressed before stage 25. Since this is not sustained in WE explants, it must require continuous signalling from nonendodermal tissues for its maintenance. Because in normal development there is relative movement of endoderm and mesoderm until early tail bud stage, any such labile expression would not be expected to contribute to the ultimate regional specification of the endoderm acquired either in intact embryos or in tissue recombinations.

These results support the conception that the endoderm develops in three stages: formation, regional specification, and differentiation (Fig. 1; Horb, 2000). First, a general endodermal cell state is established cell-autonomously by VegT. Second, signals released from the mesoderm specify several different states along the anteroposterior axis of the endoderm. This most likely occurs between stages 25 and 30 for the dorsal pancreas and between stages 30 and 35 for the rest of the liver and intestine. Finally, cell differentiation occurs from stage 30 onwards. This is accompanied by a radial intercalation of endodermal cells driving the elongation and later the coiling of the gut. This sequence of events is similar to that described in higher vertebrates.

ACKNOWLEDGMENTS

We thank the members of the Slack lab for their encouragement in developing this paper. Special thanks go to David Tosh for his helpful insights and long discussions. M.E.H. also thanks Lori Dawn Horb for her help in drawing Fig. 1. We also thank Manfred Köster for the *XFD-13* cDNA clone, Doug Melton for the *Mixer* cDNA clone, Christopher Wright for the *Xlhbox8* cDNA clone, and Eddy De Robertis for the *Edd* cDNA clone.

REFERENCES

Amaya, E., Stein, P. A., Musci, T. J., and Kirschner, M. W. (1993). FGF signaling in the early specification of mesoderm in *Xenopus*. *Development* **118**, 477–487.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

Beck, C. W., and Slack, J. M. W. (1999a). A developmental pathway controlling outgrowth of the *Xenopus* tail bud. *Development* **126,** 1611–1620.

Beck, C. W., and Slack, J. M. W. (1999b). Gut specific expression using mammalian promoters in transgenic *Xenopus laevis. Mech. Dev.* **88,** 221–227.

Beddington, R. S. P., and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96,** 195–209.

Chalmers, A. D., and Slack, J. M. W. (1998). Development of the gut in *Xenopus laevis. Dev. Dyn.* **212,** 509–21.

Chalmers, A. D., and Slack, J. M. W. (2000). The Xenopus tadpole gut: Fate maps and morphogenetic movements. *Development* **127,** 381–392.

Chalmers, A. D., Beck, C. W., and Slack, J. M. W. (2000). Regional expression in the epithelia of the *Xenopus* tadpole gut. *Mech. Dev.* **96,** 125–128.

Clements, D., Friday, R. V., and Woodland, H. R. (1999). Mode of action of VegT in mesoderm and endoderm formation. *Development* **126,** 4903–4911.

Dale, L., and Slack, J. M. W. (1987). Fate map for the 32-cell stage of *Xenopus laevis. Development* **99,** 527–551.

Dale, L. (1999). Vertebrate development: Multiple phases to endoderm formation. *Curr. Biol.* **9,** R812–R815.

Deconinck, A. E., Mead, P. E., Tevosian, S. G., Crispino, J. D., Katz, S. G., Zon, L. I., and Orkin, S. H. (2000). FOG acts as a repressor of red blood cell development in Xenopus. *Development* **127,** 2031–2040.

Drawbridge, J., and Steinberg, M. S. (2000). Elongation of Axolotl tailbud embryos requires GPI-linked proteins and organizer-induced, active, ventral trunk endoderm cell rearrangements. *Dev. Biol.* **223,** 27–37.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J., and Papalopulu, N. (1995). tinman, a Drosophila homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: XNkx-2.3, a second vertebrate homologue of tinman. *Development* **121,** 3889–3899.

Gamer, L. W., and Wright, C. V. (1995). Autonomous endodermal determination in Xenopus: Regulation of expression of the pancreatic gene XlHbox 8. *Dev. Biol.* **171,** 240–251.

Gont, L. K., Steinbeisser, H., Blumberg, B., and De Robertis, E. M. (1993). Tail formation as a continuation of gastrulation: The multiple cell populations of the *Xenopus* tailbud derive from the late blastopore lip. *Development* **119,** 991–1004.

Harland, R. M. (1991). *In situ* hybridization: An improved whole mount method for *Xenopus* embryos. *Methods Cell Biol.* **36,** 675–685.

Henry, G. L., Brivanlou, I. H., Kessler, D. S., Hemmati-Brivanlou, A., and Melton, D. A. (1996). TGF-beta signals and a pattern in *Xenopus laevis* endodermal development. *Development* **122,** 1007–1015.

Henry, G. L., and Melton, D. A. (1998). Mixer, a homeobox gene required for endoderm development. *Science* **281,** 91–96.

Hopwood, N. D., Pluck, A., and Gurdon, J. B. (1989). A Xenopus mRNA related to Drosophila twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59,** 893–903.

Horb, M., and Thomsen, G. H. (1999). Tbx5 is essential for heart development. *Development* **126,** 1739–1751.

Horb, M. E. (2000). Patterning the endoderm: The importance of neighbours. *BioEssays* **22,** 599–602.

Hudson, C., Clements, D., Friday, R. V., Stott, D., and Woodland, H. R. (1997). Xsox17α and -β mediate endoderm formation in Xenopus. *Cell* **91,** 397–405.

Jones, E. A., Abel, M. H., and Woodland, H. R. (1993). The possible role of mesodermal growth factors in the formation of endoderm in *Xenopus laevis. Roux's Arch. Dev. Biol.* **202**, 233–239.

Jones, C. M., Broadbent, J., Thomas, P. Q., Smith, J. C., and Beddington, R. S. (1999). An anterior signalling centre in Xenopus revealed by the homeobox gene XHex. *Curr. Biol.* **9,** 946–954.

Kaestner, K. H., Knöchel, W., and Martinez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev.* **14,** 142–146.

Kelly, O. G., and Melton, D. A. (2000). Development of the pancreas in *Xenopus laevis. Dev. Dyn.* **218,** 615–627.

Köster, M., Dillinger, K., and Knöchel, W. (1999). Genomic structure and embryonic expression of the Xenopus winged helix factors XFD-13/13′. *Mech. Dev.* **88,** 89–93.

Larkin, K., and Danilchik, M. V. (1999). Ventral cell rearrangements contribute to anterior-posterior axis lengthening between neurula and tailbud stages in *Xenopus laevis. Dev. Biol.* **216,** 550–560.

Lemaire, P., Darras, S., Caillol, D., and Kodjabachian, L. (1998). A role for the vegetally expressed Xenopus gene Mix.1 in endoderm formation and in the restriction of mesoderm to the marginal zone. *Development* **125,** 2371–2380.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993). Nkx-2.5: A novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants [published erratum appears in *Development* **119,** 969]. *Development* **119,** 419–431.

Matsushita, S. (1999). Fate mapping study of the endoderm in the posterior part of the 1.5-day-old chick embryo. *Dev. Growth Differ.* **41,** 313–319.

Newman, C. S., Chia, F., and Krieg, P. A. (1997). The XHex homeobox gene is expressed during development of the vascular endothelium: Overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* **66,** 83–93.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis* (Daudin)." Reprinted Garland (1994).

Okada, T. S. (1960). Epithelio-mesenchymal relationships in the regional differentiation of the digestive tract in the amphibian embryo. *Roux's Arch. Ent. Mech.* **152,** 1–21.

Patterson, K. D., Drysdale, T. A., and Krieg, P. A. (2000). Embryonic origins of spleen asymmetry. *Development* **127,** 167–175.

Rawdon, B. B. (2001). Early development of the gut: New light on an old hypothesis. *Cell Biol. Int.* **25,** 9–15.

Roberts, D. J., Smith, D. M., Goff, D. J., and Tabin, C. J. (1998). Epithelial-mesenchymal signaling during the regionalization of the chick gut. *Development* **125,** 2791–2801.

Sasai, Y., Lu, B., Piccolo, S., and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J.* **15,** 4547–4555.

Schuh, T. J., Hall, B. L., Kraft, J. K., Privalsky, M. L., and Kimelman, D. (1993). v-erbA and citral reduce the teratogenic effects of all-trans retinoic acid and retinol, respectively, in Xenopus embryogenesis. *Development* **119,** 785–798.

Shi, Y. B., and Hayes, W. P. (1994). Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev. Biol.* **161,** 48–58.

Slack, J. M. W. (1991). "From Egg to Embryo," 2nd Ed., pp. 31–33. Cambridge Univ. Press, Cambridge, U.K.

Smith, D. M., Grasty, R. C., Theodosiou,, N. A., Tabin, C. J., and Nascone-Yoder, N. M. (2000). Evolutionary relationships be-

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

tween the amphibian, avian, and mammalian stomachs. *Evol. Dev.* **2,** 348–359.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D., and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. *Cell* **67,** 79–87.

Weber, H., Symes, C. E., Walmsley, M. E., Rodaway, A. R. F., and Patient, R. K. (2000). A role for GATA5 in *Xenopus* endoderm specification. *Development* **127**, 4345–4360.

Wells, J. M., and Melton, D. A. (1999). Vertebrate endoderm development. *Annu. Rev. Cell Dev. Biol.* **15,** 393–410.

Wells, J. M., and Melton, D. A. (2000). Early mouse endoderm is patterned by soluble growth factors from adjacent germ layers. *Development* **127,** 1563–1572.

Wessely, O., and De Robertis, E. M. (2000). The *Xenopus* homologue of *Bicaudal-C* is a localized maternal mRNA that can induce endoderm formation. *Development* **127,** 2053–2062.

Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by BMP-4. *Nature* **376,** 331–333.

Wilson, P. A., and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell–cell communication. *Curr. Biol.* **4,** 676–686.

Wright, C. V., Schnegelsberg, P., and De Robertis, E. M. (1989). XlHbox 8: A novel Xenopus homeo protein restricted to a narrow band of endoderm. *Development* **105,** 787–794.

Wylie, C. C., Snape, A., Heasman, J., and Smith, J. C. (1987). Vegetal pole cells and commitment to form endoderm in *Xenopus laevis. Dev. Biol.* **119,** 496–502.

Xanthos, J. B., Kofron, M., Wylie, C., and Heasman, J. (2001). Maternal VegT. is the initiator of a molecular network specifying endoderm in *Xenopus laevis. Development* **128,** 167–180.

Yasugi, S. (1993). Role of epithelial-mesenchymal interactions in differentiation of epithelium of vertebrate digestive organs. *Dev. Growth Differ.* **35,** 1–9.

Yasuo, H., and Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in Xenopus embryos. *Curr. Biol.* **9,** 8609–8879.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. *Cell* **94,** 515–524.

Zeynali, B., and Dixon, K. E. (1998). Effects of retinoic acid on the endoderm in Xenopus embryos. *Dev. Genes Evol.* **208,** 318–326.

Zeynali, B., Kalionis, B., and Dixon, K. E. (2000). Determination of anterior endoderm in Xenopus embryos. *Dev. Dyn.* **218,** 531–536.

Zorn, A. M., Butler, K., and Gurdon, J. B. (1999). Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways. *Dev. Biol.* **209,** 282–297.

Received for publication October 24, 2000
Revised June 4, 2001
Accepted June 4, 2001
Published online July 16, 2001

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.