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The Role of Maternal VegT in Establishing the Primary Germ Layers in *Xenopus* Embryos

Jian Zhang,* Douglas W. Houston,*
Mary Lou King,* Christopher Payne,†
Christopher Wylie,†‡ and Janet Heasman†§‖
*University of Miami School of Medicine
Department of Cell Biology and Anatomy
Miami, Florida 33136
†Institute of Human Genetics
‡Department of Paediatrics
§Department of Cell Biology and Neuroanatomy
University of Minnesota School of Medicine
Minneapolis, Minnesota 55455

Summary

VegT is a T-box transcription factor whose mRNA is synthesized during oogenesis and localized in the vegetal hemisphere of the egg and early embryo. We show that maternally expressed VegT controls the pattern of primary germ layer specification in *Xenopus* embryos. Reduction of the maternal store completely alters the fates of different regions of the blastula so that animal cell fate is changed from epidermis and nervous system to epidermis only, equatorial cell fate is changed from mesoderm to ectoderm, and vegetal cell fate is changed from endoderm to mesoderm and ectoderm. Vegetal cells lose their capacity both to form endoderm and to release mesoderm-inducing signals. These results show that a single maternally expressed gene controls the patterning of the *Xenopus* blastula.

Introduction

In early vertebrate embryos, the first visible sign of cell diversity is gastrulation, in which the cells of the blastula become rearranged to form the three primary germ layers. In *Xenopus*, this occurs with a distinct pattern in which the three domains of the blastula (the animal cap, equatorial zone, and vegetal mass) become rearranged to form the ectoderm, mesoderm, and endoderm, respectively. It is thought that specification of the germ layers involves two overlapping mechanisms: maternally expressed gene products, stored in the egg, establish initial differences between cell groups, which are then amplified by intercellular signaling. However, no maternal molecules have so far been identified that have been shown to be essential for primary germ layer specification.

Single cell transplantation studies show that at the early blastula stage, cells are still pluripotent with respect to germ layer, and that pluripotency is lost by the beginning of gastrulation (Heasman et al., 1984; Snape et al., 1987; Wylie et al., 1987), placing the timing of germ layer specification mechanisms between these stages. It is generally accepted that the mesoderm forms in the equatorial region as a result of signals from the vegetal mass (Nieuwkoop, 1969; Smith, 1989). Until recently, it was thought that this signaling event started at the 64-cell stage and was due to maternal signaling molecules translated from stored maternal mRNAs (Heasman 1997, for review). However, more recent heterochronic recombination experiments have suggested that vegetal masses do not release mesoderm-inducing signals until after zygotic transcription at the midblastula transition (MBT), suggesting that mesoderm-inducing genes may be zygotic and regulated by maternal transcription factors (Wylie et al., 1996). The timing and mechanism of endoderm formation is also uncertain. Isolated vegetal masses express several endoderm markers (*Xsox17*α, *IFABP*, *Xlhox8* [Wright et al., 1989; Henry et al., 1996; Hudson et al., 1997]) in the absence of animal or equatorial components, suggesting that formation of endoderm in the vegetal mass is autonomous. However, the respective contributions of the maternal and zygotic genomes are not known.

In this study, we have used a depletion analysis to study the function of a localized maternal transcription factor, VegT (also known as Antipodean [Stennard et al., 1996], Xombi [Lustig et al., 1996], Brat [Horb and Thomsen, 1997], and XTbx6r [Smith, 1997]), in early development. VegT is a member of the T-box family of transcription factors, the founder-member of which, the mouse *T* gene (*Brachyury*), is necessary for the development of the notochord and somites posterior to somite seven (Smith, 1997; Papaioannou and Silver, 1998, for review). Related proteins have been found in many animal groups and are known to play roles in cell lineage specification. Maternal VegT mRNA is localized in *Xenopus* to the vegetal hemisphere of the full-grown oocyte and is inherited by cells that develop from this region of the egg (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Zygotic transcription of VegT begins before gastrulation and is restricted to equatorial, presumptive mesodermal cells, particularly on the dorsal side. Overexpression and ectopic expression studies show that VegT mRNA can induce both mesoderm and endoderm, while a dominant negative form inhibits mesoderm formation and severely disrupts normal development (Horb and Thomsen, 1997).

Here, we have studied specifically the role of maternal VegT. We show that maternal VegT is essential for endoderm germ layer formation and for establishing the vegetal signal first described by Nieuwkoop (1969). As VegT is a transcription factor, this work indicates that the mesoderm-inducing signals produced by the vegetal mass are zygotic. Furthermore, loss of maternal VegT function causes dramatic changes in the fates of the other two domains of the blastula. The animal cap does not form nervous tissue, but forms a ventral epidermal cyst. The equatorial zone does not form mesoderm; it forms only ectodermal derivatives. The vegetal mass undergoes convergence extension movements and expresses mesodermal and ectodermal markers. Models of germ layer formation are discussed in light of these results.

‖To whom correspondence should be addressed.

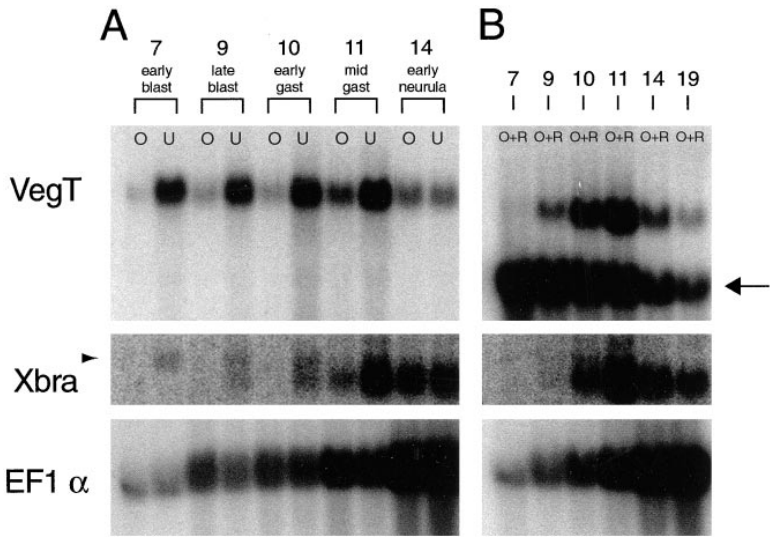

Figure 1. Injection of an Oligo Antisense to VegT mRNA Depletes the Endogenous Maternal VegT mRNA, but Does Not Prevent the Transcription of Zygotic VegT

(A) Northern blot, probed for VegT RNA from embryos derived from one batch of oocytes frozen at the stages indicated. O, 4 ng VT9M oligo injected; U, uninjected. The blot was stripped and reprobed for Xbra and EF1α. The arrowhead points to residual VegT signal that was incompletely stripped before adding Xbra probe.

(B) Northern blot of sibling embryos to those in Figure 1A that were injected with oligo VT9M at the same time as those in Figure 1A and, 24 hr later, were also injected with synthetic VegT mRNA (300 pg) before fertilization (O + R). The injected mRNA is indicated by the arrow. The appearance of sibling gastrulae treated in the same fashion from this experiment is shown in Figure 5A.

Results

Zygotic VegT mRNA Is Expressed in Embryos Depleted of Maternal VegT mRNA

Maternal VegT mRNA is localized to the vegetal hemisphere of the full-grown oocyte. To study the function of the stored mRNA, we caused its degradation by injecting antisense oligos into the vegetal poles of defolliculated oocytes. Of 12 oligos selected that were complementary to the target mRNA, only 1 (VT9M) depleted the mRNA substantially, and this oligo was used throughout these experiments. Control, uninjected oocytes and oligo-injected oocytes were fertilized by the host-transfer technique and allowed to develop. The levels of VegT mRNA were compared in the control and oligo-injected batches at the early blastula (stage 7), late blastula (stage 9), early (stage 10) and midgastrula (stage 11), and neurula (stage 14) stages (Figure 1A). Embryos derived from oligo-injected oocytes have 5%–10% of the control level of maternal mRNA throughout the blastula and early gastrula stages. In contrast, RT-PCR shows that oocytes depleted of VegT have no reduction in the maternal levels of the related T-box gene Xbra (data not shown). During the gastrula stages, zygotic VegT mRNA begins to be expressed by both control and oligo-injected embryos, so that by the neurula stage the amount of VegT mRNA is restored to control levels in oligo-injected embryos.

The Phenotype of VegT-Depleted Embryos

Depletion of VegT mRNA causes a highly reproducible and dose-dependent phenotype. Figure 2 shows the characteristic appearance of VegT-depleted embryos at the tailbud stage. There are four characteristic external features of the high-dose phenotype (embryos labeled "H" in Figure 2): the embryos have a darkly pigmented ventral surface, the dorsal surface is unpigmented, the body axis is shortened, and there is a lack of head structures. Although most external features of the head are missing, the anterior/posterior axis can be judged by the presence of the cement gland at one end (arrow). Less severe phenotypes, caused by the injection of less oligo (embryos labeled "L" in Figure 2), have enlarged heads, shortened body axes, and reduced and abnormal gut endoderm.

How Does the VegT Phenotype Arise?

We examined the development of VegT-depleted embryos, concentrating on those with the most extreme phenotype. As VegT is a maternal transcription factor and both the full-grown oocyte and embryo are transcriptionally inactive until the MBT, we anticipated that maternal VegT would function after the onset of transcription. As expected, VegT-depleted embryos develop normally throughout the cleavage and blastula stages, with the same rate of cleavage and the same dorsal/ventral differences in pigmentation pattern as the controls.

Major developmental changes occur in VegT-depleted embryos compared to controls at the time of gastrulation. Figures 3A–3C show a developmental series of embryos taken from one experiment comparing the appearance of control, uninjected (U) and VegT-depleted (V) embryos. The first obvious difference is that VegT-depleted embryos have altered behavior at gastrulation. While uninjected sibling controls form the characteristic blastoporal pigment ring, no blastopore appears in VegT-depleted embryos. They remain without blastopores throughout the sibling gastrulation period (typically 6 hr). Then, as controls reach the end of gastrulation, indentations appear at the vegetal poles of VegT-depleted embryos. These structures form pits that lack the typical blastoporal pigment lines of control gastrulae (arrow in Figure 3A). Also, there is no spreading of animal cells over the surface of the embryo, leaving all or most of the marginal zone on the surface. These effects on gastrulation are highly reproducible (occuring in all cases; over 200 examined) and are specifically due to the depletion of maternal VegT, because the normal timing and appearance of the blastopore and pigmentation pattern is rescued by the reintroduction of synthetic VegT mRNA into oocytes that have been depleted of their maternal VegT (Figures 1A and 5A).

Although there is no visible gastrulation in the equatorial zone, cells of the vegetal region do move inside the embryo, leaving all or most of the equatorial zone on

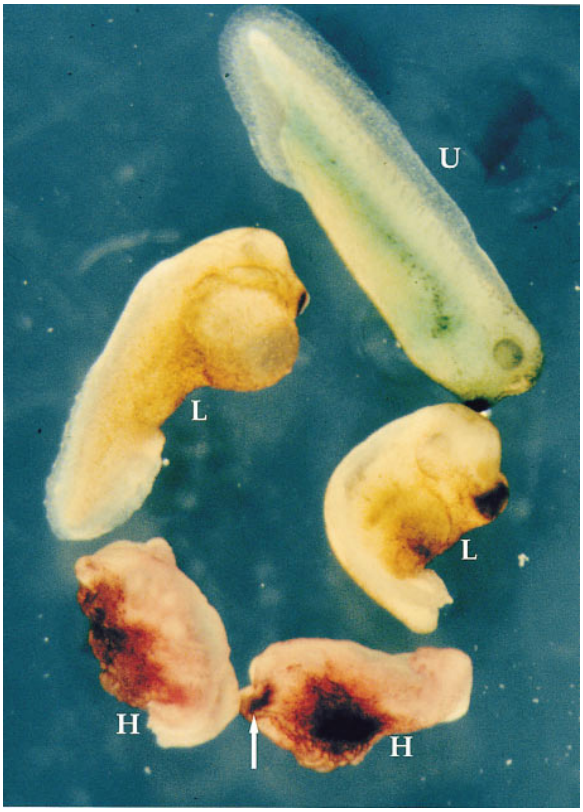

Figure 2. The Phenotype of Maternal VegT-Depleted Embryos

The appearance of embryos depleted of VegT mRNA as oocytes at the late tailbud stage (stage 34). Oocytes were uninjected (U), injected with 4 ng of oligo (L), or with 5 ng of oligo (H). The arrow points to the cement gland.

the outside. This was shown in two ways. First, vital dye marks placed on the vegetal pole of both control and experimental embryos at the late blastula stage leave the surface by the neurula stage (Figures 3D and 3E). Second, lineage label injected into vegetal cells at the 16–32-cell stage is located in internal tissues at the late gastrula stage (data not shown).

At the neurula stage, the noninvaginated marginal zone can be seen as a nonpigmented area on the outside of the embryo. The body axis of the embryo forms in this nonpigmented region. Specifically, enlarged folds appear in the nonpigmented half of VegT-depleted embryos when sibling control embryos are at the midneurula stage (Figure 3B). These folds strongly resemble neural folds, and this was confirmed by in situ hybridization with the neural marker *NCAM* (Figure 3F). At the tailbud stage, VegT-depleted embryos with external appearance similar to the high-dose phenotype shown in Figure 2 have both notochords and neural tubes visible in histological sections. These are derived from vegetal cells because lineage label placed in vegetal blastomeres at the 16-cell stage is predominantly found in the notochord and neural tube in VegT-depleted embryos (Figure 3J), compared to the endoderm and somites of control embryos (data not shown).

The body of the embryo at the tailbud stage continues to lack pigmented cells, while the animal cap forms a cyst on the ventral surface. The fact that the ventral cyst is the nonobliterated blastocoel was confirmed in two ways. First, when lineage label is injected into the A tier blastomeres of the 32-cell stage embryo, (the cells whose progeny form the roof of the blastocoel), the labeled progeny are consistently located in the ventral pigmented cysts at the tailbud stage and are not found elsewhere (Figure 3H). Second, India ink injected into the blastocoel of blastula stage is located in this ventral area at the tailbud stage (data not shown).

Maternal VegT Is Required for the Correct Expression of Endodermal and Mesodermal Markers

The morphological appearance of VegT-depleted embryos suggested abnormal germ layer specification. To test this, we asked whether germ layer–specific markers were expressed normally. In these experiments, we used embryos with the extreme phenotype, similar to those shown in Figure 2.

First, we studied whether maternal VegT depletion affected the expression of the endoderm-specific markers *endodermin*, *Xsox17*α, *Xlhox8*, *insulin*, and *IFABP* in tailbud stage embryos by RT-PCR (stage 33, Figure 4A, and stage 38, Figure 4B). In all cases (3 experiments), the endodermal markers were reduced or absent. These effects were rescued by the injection of VegT mRNA into the vegetal hemisphere of VegT-depleted embryos at the 8-cell stage (lane 4, Figure 4A; lane 3, Figure 4B).

Since gastrulation movements were clearly abnormal in VegT-depleted embryos, we next asked whether mesodermal markers were expressed normally. Figure 5B shows that mesodermal markers are expressed in VegT-depleted embryos but that early mesodermal markers are delayed in their expression relative to control levels, corresponding with the gastrulation abnormality. Sibling embryos to those shown in Figure 5A were assayed by RT-PCR at the late blastula (stage 9), midgastrula (stage 11), and early neurula (stage 13) stages for the dorsal mesodermal markers *chordin* and *XMyoD*, the general mesodermal marker *Xbra*, and endomesodermal markers *Xwnt 8* and *Mix1* (Figure 5B). *Xbra* was also studied by Northern analysis (Figure 1B). While control embryos expressed mesodermal markers at the late blastula stage, the VegT-depleted embryos showed no expression of *Mix1* and *Xwnt 8* and reduced expression of *Xbra* (10%–20% control levels) and *chordin* (50% control levels) at this time. Expression levels remained low at the midgastrula stage but rose to control levels by the early neurula stage, except for *Mix1*, which was switched off in both experimental and control embryos at the early neurula stage.

In summary, these experiments indicated that maternal VegT is essential for the differentiation of endodermal tissues and that in its absence, mesodermal differentiation is delayed.

In the Absence of Endoderm Differentiation, the Site of Mesoderm Formation Shifts to Cells Derived from the Vegetal Mass

Since early mesodermal markers are expressed in VegT-depleted embryos and yet these embryos do not undergo the gastrulation movements characteristic of mesodermal cells, we next asked whether mesodermal

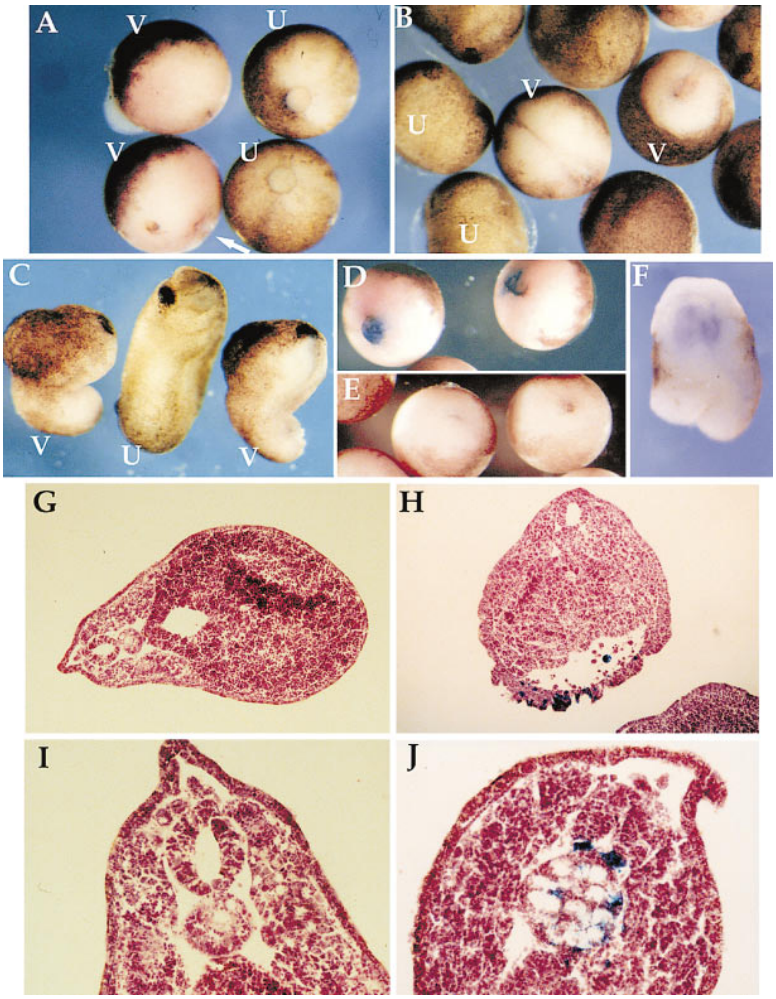

Figure 3. The Development of VegT-Depleted Embryos

(A) Gastrulae derived from oocytes depleted of maternal VegT are unable to form normal blastopores, compared to sibling uninjected control embryos. Arrow points to the ectopic ingression site that appears at the late gastrula stage in VegT-depleted embryos.

(B) Neurulae from the same batch of embryos shown in (A). The neural folds of VegT-depleted embryos are nonpigmented.

(C) Tailbud embryos from the same batch of embryos as shown in (A). V, VegT-depleted using 5 ng oligo; U, uninjected control.

(D) (stage 12) and (E) (stage 16), VegT-depleted embryos. Dye marks were placed on the vegetal poles of VegT-depleted embryos at the late blastula stage and photographed here at sibling late gastrula stage (D) and midneurula stage (E). The dye marks pass inside the embryos during this time.

(F) Whole-mount in situ hybridization to show the location of the neural marker NCAM within the unpigmented cells in a VegT-depleted tailbud stage embryo.

(G–J) Histological sections of control (G and I) and VegT-depleted embryos (H and J). A comparison between (G) and (H) shows the abnormal ventral vesicle in (H). In (H), one animal cell (A1) of the embryo was injected with β-galactosidase mRNA at the 32-cell stage, and descendant blue cells lie in the wall of the ventral vesicle. (I) and (J) show a higher power comparison of the dorsal axial structures of a control (I) and VegT-depleted (J) embryo. In (J), a single vegetal cell was injected with β-galactosidase mRNA at the 16-cell stage, and blue progeny were located in the neural tube and notochord.

gene expression occurs in the correct region in VegT-depleted embryos. To do this, we dissected individual midblastulae into their three prospective germ layer–forming regions, as shown in Figure 6A, and cultured them overnight. At the time of dissection, the VegT-depleted embryos had the same appearance as the control embryos.

It has previously been shown that isolated equatorial regions (derived from B and C tier cells) of wild-type blastulae undergo changes in shape and elongation as a result of convergence extension movements (Keller and Danilchik, 1988). Figure 6B (top row) shows this behavior in dissected equatorial zones from control embryos cultured until sibling embryos reached early

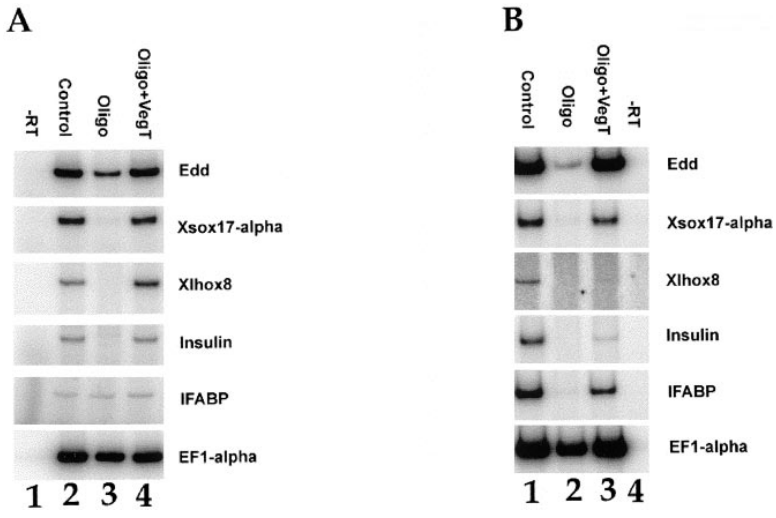

Figure 4. Zygotic Endodermal Differentiation Markers Are Not Expressed in Maternal VegT-Depleted Embryos and Are Rescued by the Injection of VegT mRNA

(A) and (B) show RT-PCR analysis of the levels of expression of the endodermal markers *endodermin* (edd), *Xsox17*α, *Xlhbox8*, *insulin*, and *IFABP*. *EF1*α was assayed as a loading control. (A) Pairs of control (uninjected), VegT-depleted (oligo), and Oligo + VegT RNA (oligo + VegT) injected embryos frozen at stage 33, and (B), at stage 38 for analysis.

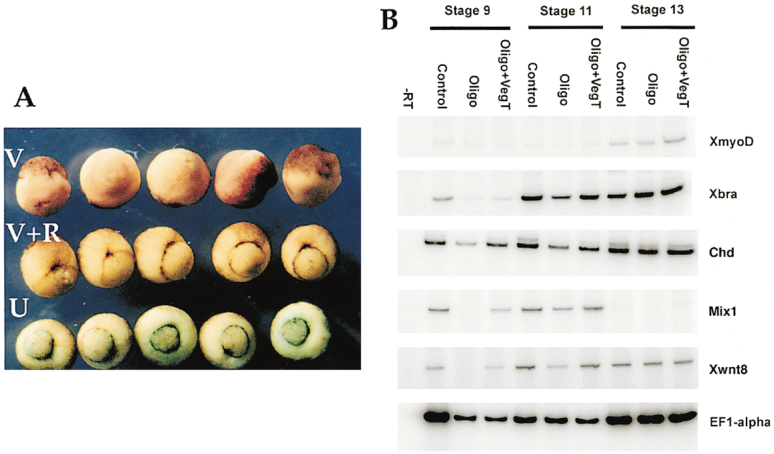

Figure 5. The Expression of Mesodermal Markers Is Delayed in VegT-Depleted Embryos, and This Is Rescued by the Injection of VegT mRNA

(A) The appearance of oligo-injected (V), oligo + VegT mRNA–injected (V + R), and uninjected sibling control embryos (U) at the midgastrula stage.

(B) Sibling embryos of these were frozen and assayed for mesodermal markers by RT-PCR at the late blastula (stage 9), midgastrula (stage 11), and early neurula stage (stage 13). Relative levels of expression were determined normalized to EF1α.

tailbud stage. In contrast, VegT-depleted equatorial explants fail to undergo convergence extension movements in a dose-dependent manner (Figure 6B) (four experiments, 45/45 cases in total). Surprisingly, we find that vegetal masses lacking maternal VegT elongate in culture, unlike control vegetal explants that form typical round balls of tissue (Figures 6C and 6D).

These observations suggested that the vegetal masses had acquired mesodermal characteristics, and this was confirmed biochemically. Figure 6E shows RT-PCR analysis of tissue-specific markers in equatorial and vegetal explants assayed at the neurula stage. The equatorial explants of VegT-depleted blastulae lack the expression of the dorsal and general mesodermal markers, zygotic *VegT*, *Xwnt 8*, *gsc*, *XMyoD*, and *Xbra*. In contrast, VegT-depleted vegetal masses express all the mesodermal markers except *MyoD* (Figure 6, lane 5), the reverse of the control pattern.

In a second experiment, high- (5 ng) and low-dose (3 ng) equatorial and vegetal explants were cultured until siblings reached early tailbud stage (Figure 6F) and assayed for neural and mesodermal markers. The same pattern was observed: vegetal masses of VegT-depleted embryos form mesoderm. Two further observations were made at these later stages. Neural and epidermal markers (*NCAM*, *nrp-1*, epidermal keratin *XK81*) are expressed by equatorial explants of control embryos and not by vegetal masses, but in VegT-depleted embryos, they are expressed both by equatorial and vegetal explants. Second, increasing doses of oligo do not cause further reduction in the amounts of expression of notochord markers (*chordin* and *Xbra*) or neural markers (*nrp-1* and *NCAM*), but they do cause a reduction in the expression of the somite marker *MyoD* in VegT-depleted vegetal masses.

These results confirm that the depletion of maternal VegT from blastulae causes a change of fate for the vegetal and equatorial region. The capacity to form mesoderm is shifted to the vegetal mass from the equatorial region, and the capacity to form ectoderm is now shared by both regions.

Since high-dose explants had reduced expression of the late mesodermal marker *MyoD*, we examined whether this was the case in whole embryos taken from the same experiment. Figure 6G confirms that there is a dose response of reduction of muscle markers *cardiac actin* and *MyoD* in VegT-depleted embryos at the tailbud stage.

Maternal VegT Expression Is Required for Mesoderm-Inducing Signals to Be Released by Vegetal Cells

As well as differentiating into endoderm, a second characteristic of the vegetal mass of the early embryo is that it produces inducing signals. These signals were first assayed by Nieuwkoop, who showed that vegetal masses dissected from blastula stage embryos induced animal caps placed on those vegetal masses to form mesoderm (Nieuwkoop, 1969). Next, we tested whether this property of vegetal cells is retained by VegT-depleted blastulae.

We dissected midblastulae into animal caps, vegetal masses, and equatorial zones, as in Figure 6A, and cocultured vegetal masses of VegT-depleted and control embryos with control and experimental animal caps in the four possible combinations. When control animal caps were cultured on control vegetal masses until sibling embryos reached the midneurula stage, the vegetal cells induced the animal caps to contract and elongate (Figure 7A). In contrast, control and VegT-depleted animal caps placed on VegT-depleted vegetal explants were completely unable to round up and elongate (Figures 7C and 7D). Instead, the animal cells spread out as thin sheets of cells continuous with the vegetal tissue. We know from the previous experiment that vegetal masses express epidermal keratin, so, as both have epidermal characteristics, this presumably allows animal cap–derived epidermis and vegetally derived epidermis to be continuous with each other. However, when animal caps from VegT-depleted embryos were cultured with control vegetal masses, they behaved in a similar fashion to controls, rounding up and elongating on the surfaces of the vegetal masses (Figure 7B).

To perform gene expression analysis on the animal caps, we repeated the Nieuwkoop assay, this time keeping the animal and vegetal parts of the recombinants together for only 2 hr before dissecting them apart. This allowed us to study gene expression in the animal caps separately from the vegetal masses, since we know that mesoderm markers are expressed by the vegetal masses. We confirmed both morphologically (Figure 7E) and biochemically (Figure 7F) that VegT-depleted vegetal masses

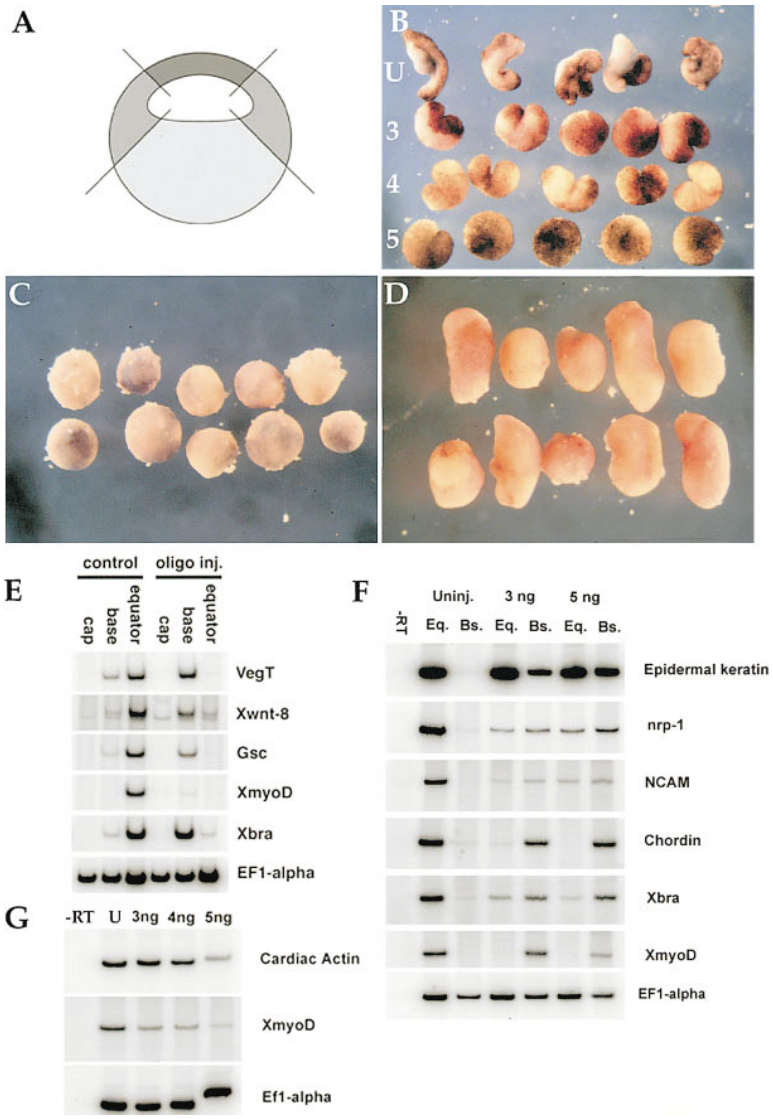

Figure 6. In VegT-Depleted Embryos, Mesoderm Forms in the Vegetal Mass and Not in the Equatorial Zone

(A) shows the dissection carried out on control, uninjected and VegT-depleted midblastulae to explant the three regions, the animal caps, equatorial zones, and vegetal masses.

(B) shows the appearance of the equatorial explants from control (U), 3, 4, and 5 ng oligo-injected blastulae after culture to sibling early tailbud stage.

(C and D) Vegetal masses derived from control (C) and 3 ng oligo-injected (D) blastulae after culture to sibling early tailbud stage. These are from the same experiment as shown in Figures 6B and 6F.

(E) RT-PCR analysis of mesodermal markers from animal (cap), equatorial (equator), and vegetal (base) explants from uninjected and VegT-depleted embryos cultured to the mid-neurula stage (stage 16). The markers *VegT*, *Xwnt 8*, *goosecoid* (*gsc*), and *Xbra* are expressed strongly in the equatorial regions of controls and in the vegetal masses of VegT-depleted embryos. *EF1*α is used as a loading control.

(F) Oocytes were uninjected or injected with 3 or 5 ng oligo, and equatorial zones (Eq.) and vegetal masses (Bs.) were dissected at the midblastula stage. The explants were cultured until the early tailbud stage and then analyzed by RT-PCR for ectodermal and mesodermal markers.

(G) Whole embryos from the same experiment as Figures 6B–6D and 6F analyzed by RT-PCR for the expression of muscle markers at the early tailbud stage.

are unable to induce dorsal or ventral mesoderm in wild-type or VegT-depleted animal caps. We also confirmed that animal caps derived from VegT-depleted embryos are able to respond to mesoderm-inducing signals from wild-type embryos by expressing mesodermal markers. Thus, the failure of the animal caps of intact VegT-depleted embryos to undergo epiboly and form nervous tissue must be secondary to the loss of normal endodermal and mesodermal specification, and not a direct effect of VegT depletion on the animal cap cells.

Discussion

While several maternal transcription factors have been studied in *Xenopus*, including *XTcf3* (Molenaar et al., 1996), *xnf7* (El-Hodiri et al., 1998), *Xrel* (Bearer, 1994), *ets 1* (Stiegler et al., 1993), and *XLPOU60* (Whitfield et al., 1993), none have definitively been assigned specific roles in establishing the three primary tissue types. The results described here show that there are at least three roles for the maternal transcription factor VegT in tissue formation.

First, the coculture experiments above indicate that maternal VegT is required for vegetal cells of the blastula to produce the endogenous vegetal signal(s) that cause caps to form mesoderm. This represents an important departure from the popular view that early vegetal signals cause mesoderm formation. VegT is a transcription factor and will not activate transcription until after MBT. Thus, zygotic inducing factors downstream of VegT, not maternal signaling factors, initiate the endogenous signal. This supports the view that mesoderm induction is a posttranscriptional event in *Xenopus* and that the primary patterning event underlying it is the localization of a maternal transcription factor. This view has been expressed previously, based on heterochronic animal cap/vegetal mass recombination experiments (Wylie et al., 1996). The identification of the endogenous signaling factor(s) will be aided by the identification of maternal VegT as an upstream regulator.

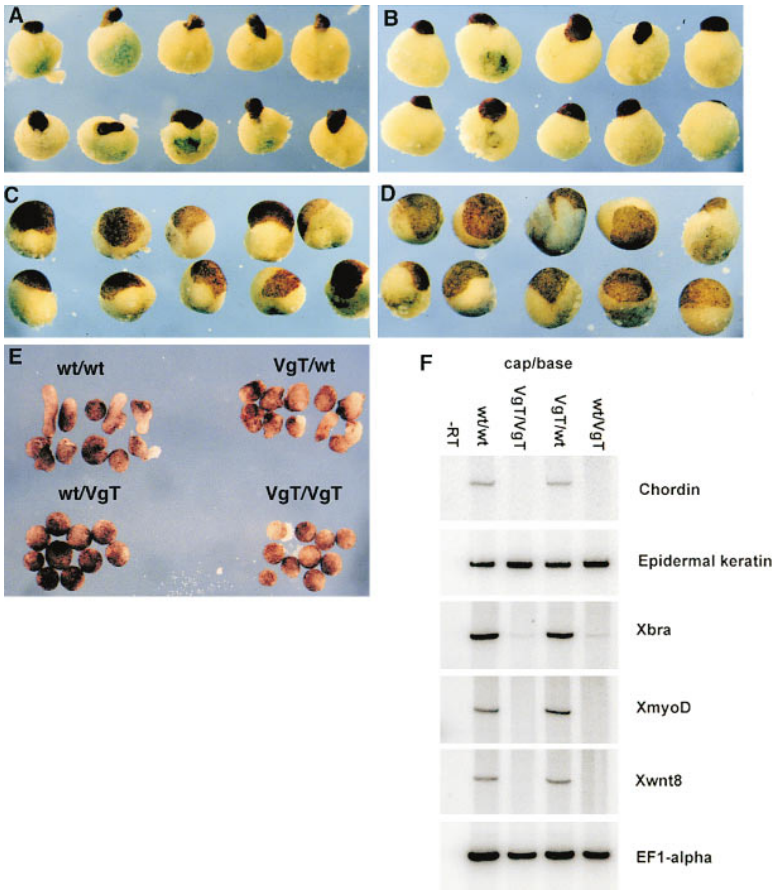

Figure 7. Maternal VegT Expression Is Required for Mesoderm-Inducing Signals to Be Released by Vegetal Cells

(A–D) Nieuwkoop recombinants cultured to the early neurula stage. (A) Uninjected animal cap/ uninjected vegetal mass. (B) VegT-depleted animal cap/uninjected vegetal mass. (C) Uninjected animal cap/VegT-depleted vegetal mass. (D) VegT-depleted animal cap/ VegT-depleted vegetal mass. (E) Animal caps cultured on vegetal masses for 2 hr and then separated and cultured to sibling early tailbud stage. These caps were analyzed by RT-PCR in (F). Upper left are uninjected caps that were cultured with wild-type bases for 2 hr (wt/wt). Upper right are animal caps from VegT-depleted embryos cultured with wild-type bases (VgT/wt). Lower left are wild-type caps cultured with VegT-depleted bases (wt/VgT), and lower right are VgT-depleted caps cultured with VegT-depleted bases (VgT/VgT). (F) RT-PCR analysis on animal caps shown in (E) for mesodermal and epidermal markers.

Second, maternal VegT is required for the differentiation of the embryonic endoderm. Larvae that develop from oocytes depleted of VegT have severely reduced or absent molecular markers of endoderm and lack endodermal organs. This shows that the formation of the endoderm relies entirely on a single regionally localized maternal transcription factor and that VegT is, directly or indirectly, an activator of endoderm. Interestingly, zygotic VegT does not have this role. Zygotic VegT is expressed in maternal VegT-depleted embryos and does not rescue endoderm formation. Also, the pattern of expression of zygotic VegT in the marginal zone at the gastrula stage (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) argues against zygotic VegT playing a role in endoderm formation.

Third, in addition to the absence of endoderm, the depletion of maternally encoded VegT causes a more general shift in the primary germ layer–forming regions of the blastula. The vegetal mass, which normally forms endoderm, now forms mesoderm and ectoderm, and the most vegetal cells (derived from the D tier) move inside the embryo. In culture, the vegetal mass undergoes the convergence extension movements characteristic of mesoderm and expresses mesoderm and ectoderm markers, including zygotic *VegT*, *nrp-1*, and epidermal keratin, *XK81*. The marginal zone does not invaginate during gastrulation but remains outside the embryo and forms ectoderm. In culture, it does not undergo gastrulation movements but expresses markers of epidermis and nervous tissue. A summary of these effects is shown in Figure 8A.

There are several possible explanations for these effects, as suggested in Figure 8B.

In Model 1, maternal VegT is only required in the vegetal mass, causing it to form endoderm and to produce mesoderm-inducing signals. The lack of mesoderm formation in the equatorial zone is secondary to the lack of mesoderm-inducing signals being emitted from the vegetal mass. This model most closely fits with the widely accepted view of mesoderm formation, that the vegetal mass induces adjacent naive animal hemisphere cells. It explains the fact that in VegT-depleted embryos, mesoderm does not form in the equatorial zone, but it does not explain why mesoderm forms in the vegetal area. Indeed, the fact that mesoderm forms ectopically in VegT-depleted embryos when there are no vegetal mesoderm-inducing signals challenges the widely accepted three-signal/four-signal model (Smith, 1989; Sive, 1993) of mesoderm formation by induction.

In Model 2, maternal VegT is required separately in the vegetal mass and equatorial regions. In the vegetal mass, maternal VegT expression in the cells of the vegetal mass causes them to adopt three properties: to make endoderm, to release mesodermal-inducing signals, and to inhibit them from forming mesoderm. In the absence/ depletion of maternal VegT, vegetal cells adopt a mesodermal fate. In the equatorial region, maternal VegT is required for mesoderm formation. In its absence, no

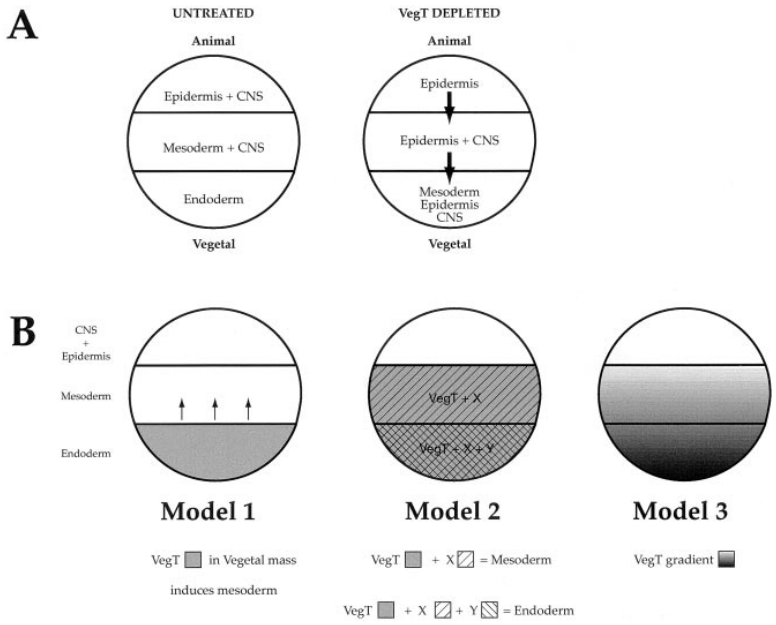

Figure 8. The Role of Maternal VegT in Tissue Specification

(A) The fates of the three regions of the blastula are altered in VegT-depleted embryos. The vegetal mass forms endoderm in wild-type embryos; in VegT-depleted embryos, it forms mesoderm, epidermis, and neural tissue and undergoes convergence extension and ingression movements. The equatorial zone forms mesoderm in controls, but it forms epidermis and CNS in VegT-depleted embryos and does not undergo gastrulation movements. The animal cap forms both neural and epidermal derivatives of ectoderm in wild-type embryos, but forms only epidermal tissue in VegT-depleted embryos.

(B) Models for mesoderm and endoderm tissue formation. In Model 1, maternal VegT is required only in the vegetal mass at MBT for the formation of endoderm. Vegetal mass cells then induce overlying equatorial cells to form mesoderm. In Model 2, maternal VegT is required separately in both the equatorial zone and the vegetal mass at MBT. In the equatorial region, it acts with another factor (X) to activate mesoderm formation. In the vegetal mass, a second factor (Y) is present that, together with VegT and X, activates endodermal differentiation. In Model 3, there is a gradient of maternal VegT activity at MBT. At low concentration, VegT activates mesodermal differentiation pathways, while at high concentration, it activates endodermal differentiation pathways.

mesoderm forms in this region. Instead, it forms epidermis or is induced by the now vegetal mesoderm to form neural tissue. In this model, the different effects of VegT in the two zones, the vegetal mass and equator, are envisioned to be due to an extra maternal transactivating factor (Y) localized in the vegetal region. In the equatorial region, VegT and factor X together activate mesoderm formation. In the vegetal mass, factor Y is also present, and endoderm differentiation is activated while mesoderm is repressed. In the absence of VegT, X is insufficient for mesoderm formation in the equatorial region, but X + Y factors can cause mesoderm formation in the vegetal mass.

The major problem with this interpretation is to understand why wild-type vegetal masses release mesodermal-inducing signals if mesoderm is in fact specified separately by VegT in the equatorial region. One possible explanation is that the vegetal signal is not a mesoderm-inducing signal, but an ectodermal-repressing signal. This would explain why VegT-depleted vegetal masses and equators both form ectodermal derivatives in the absence of VegT. Another possibility is that the role of the vegetal signal may be to form a sharp boundary between vegetal cells, where mesoderm is repressed, and equatorial cells, which are signaled to form mesoderm.

Although the prevalent view of mesoderm formation is that it is induced by vegetal cells, there is some evidence that mesoderm formation occurs autonomously in the equatorial region. Ligation experiments on fertilized eggs showed that components required for the activation of muscle-specific actin genes are localized in the subequatorial region (Gurdon et al., 1985), and disaggregated cell culture studies show that the expression of mesodermal genes *Xwnt 8* and *gsc* in equatorial cells does not require cell–cell interactions (Lemaire and Gurdon, 1994).

In Model 3, there is a gradient of effect of VegT. At high levels in the vegetal mass, VegT activates endoderm and represses mesoderm. At low levels in the marginal zone, it activates mesoderm. In this model, when VegT is depleted, it is depleted completely from the equatorial region and incompletely from the vegetal mass. This would explain why depletion of maternal VegT shifts the pattern of primary germ layer derivatives toward the vegetal pole. A prediction of this model is that higher doses of oligo should cause depletion of mesoderm as well as endoderm, which was seen in these experiments with regard to late muscle markers *MyoD* and *cardiac actin* (Figure 6G).

Further work is required to distinguish which of these three possibilities is correct. In all of these models, it is necessary to predict that ectodermal derivatives form in the absence of VegT in the equatorial region. This may suggest that ectoderm is the default state, although it is equally possible that in the absence of mesoderm formation in the equator, animal cap signals induce the equatorial cells to form ectoderm.

The work described here emphasizes again the importance of maternal cytoplasmic localization in patterning the early *Xenopus* embryo, and it particularly highlights the fact that the vegetal hemisphere harbors the determinants of endoderm differentiation and of primary germ layer specification in the shape of maternal VegT.

Experimental Procedures

Oocytes and Embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM), as described in Wylie et al. (1996).

Oocytes were injected with oligo using a Medical Systems picoinjector in OCM. Injection of oligo into the vegetal poles of oocytes was more effective at depleting VegT mRNA than injection into the equatorial region, so vegetal injections were used throughout these experiments. Oocytes were cultured a total of 24–48 hr at 18°C before fertilization. In preparation for fertilization, they were stimulated to mature by the addition of 2 μM progesterone to the culture medium and cultured for 10–12 hr. Oocytes were then labeled with vital dyes and introduced into a stimulated female host using the host-transfer technique described previously (Heasman et al., 1991). Three hours after placing in the frog's body cavity, the eggs were stripped and fertilized along with host eggs using a sperm suspension. Embryos were maintained in 0.1 × MMR, and all the colored, experimental embryos were sorted from host embryos. Unfertilized eggs and abnormally cleaving embryos were removed from all batches.

For injections of mRNAs, embryos were transferred to 3% Ficoll in 1 × MMR at the 4-cell stage and orientated according to pigment pattern, so that the dorsal, less-pigmented side could be accurately targeted for injection at the 32-cell stage. VegT mRNA (150–300 pg per embryo) or β-galactosidase mRNA (300 pg per embryo) was injected into blastomeres as described in the text. Embryos were washed thoroughly and returned to 0.1 × MMR during the blastula stage.

Oligos and mRNAs

The oligo used, VT9M, was an 18-mer: C*A*G*CAGCATGTACTT*G*G *C, where * indicates a phosphorothioate bond, and other linkages were phosphorodiester bonds. Oligos were resuspended in sterile, filtered water and injected in doses of 4–5 ng (at 0.5 mg/ml) per oocyte, and cultured immediately at 18°C. RNAs were synthesized by linearizing (*VegT*, *Not* 1; β-*gal*, *Xho*1) the plasmid vectors containing subcloned cDNAs and transcribing the linear template with SP6 polymerase in the presence of cap analog and GTP using the Megascript kit (Ambion). RNAs were ethanol precipitated and resuspended in sterile, distilled water for injection.

Northern Blot Analysis

Embryo RNA was extracted as described (Gurdon et al., 1985). Electrophoresis and Northern blotting were performed as described (Hopwood et al., 1989), using two embryo equivalents per lane. The probes were synthesized by random priming of the excised inserts of *VegT* and *Xbra* (EcoRI). Blots were stripped and rehybridized with elongation factor *EF1*α as a loading control.

Analysis of Gene Expression Using RT-PCR

Total mRNA was prepared from embryos and embryo explants using the Proteinase K method with 10 μg glycogen added as a carrier. RNA was treated with RNase-free DNase 1 (Boehringer Mannheim) prior to cDNA synthesis. RNA (0.5–1 μg) was subjected to reverse transcription (RT) using first strand synthesis kit (Boehringer Mannheim). Semiquantitative RT-PCR was performed according to a previously published method (Wilson and Melton, 1994) with minor modifications. One-tenth of the RT mixture was used as templates for PCR. Each 25 μl reaction contained 100 mM dNTPs, 1.5 mM $MgCl_2$, 1 μCi^{32}-PdCTP, 1 μm of each primer, and 1 U of Taq DNA polymerase (Boehringer Mannheim). Samples were denatured for 3 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min at 55°C, and 30 s at 72°C (for EF1α, 22 cycles were used). For Xlhbox8, the annealing temperature was raised to 59°C, and 28 cycles were used. Five to ten microliters of the PCR mixture was resolved on 5% nondenaturing gels. Quantification was performed using a Molecular Dynamics PhosphorImager. The primers used in this study were as follows: *EF1*α, *goosecoid*, *Xwnt-8*, *Xbra*, *Mix-1* (Wilson and Melton, 1994); *insulin* (Henry et al., 1996); *Xsox17*α (Hudson et al., 1997), *endodermin* (Sasai et al., 1996), *Xenopus* keratin *XK81* (Jonas et al., 1985), and *nrp*1 (Lamb and Harland, 1995).

Other primer pairs were *IFABP* F′:5′-GAAGGTCATAATCCAGC AAG-3′ (Shi and Hayes, 1994), R′:5′-CTGGTGAAAGTTCCAAGAAG-3′; *Xlhbox8* F′:5′-GGCAGTCCTCCAGACATCTC-3′, R′:5′-TCCACTT CATTCTCCGATTC-3′ (Wright et al., 1989); *VegT* F′:5′-CAAGTA AATGTGAGAAACCGTG-3′, R′:5′-CAAATACACACACATTTCC CGA-3′ (Zhang and King, 1996); and *Chordin* F′:5′-TACCGCACC CACTCAAAATAC-3′, R′:5′-TTTCGCAACAGGAGCACAGAC-3′.

Whole-Mount In Situ Analysis

Whole-mount in situ hybridization was performed as described (Harland, 1991), using BM Purple as substrate (Boehringer Mannheim). After satisfactory color development, embryos were fixed in MEMFA for 1 hr at room temperature, washed, and stored in 100% ethanol. Some embryos were also treated in 10% H_2O_2 to bleach out the pigment.

Fixation and Histology

For X-gal staining, embryos were fixed in MEMFA for 2 hr, rinsed in PBS, and stained using X-gal (Hemmati-Brivanlou and Harland, 1989). Embryos were washed in PBS after staining. For sectioning, embryos were dehydrated and embedded in polyethylene distearate wax (Polysciences) and stained using standard procedures for haematoxylin and eosin.

Explant Culture and Nieuwkoop Recombinants

Midblastula stage embryos were devitellined and dissected on agar-coated dishes in 1 × MMR into animal, equatorial, and vegetal segments, as described previously (Heasman et al., 1994). Dissected pieces were cultured in agar depressions in oocyte culture medium until siblings reached the midneurula stage. For Nieuwkoop assays, stage 8 animal cap and vegetal masses were dissected and pushed together in the appropriate combinations using tungsten needles, and cultured until siblings reached the neurula stage, as above. In other experiments, caps and bases were separated after 2 hr of culture and cultured separately until siblings reached early tailbud stages (stage 22).

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