A two-step model for the fate determination of presumptive endodermal blastomeres in *Xenopus* embryos

Hitoyoshi Yasuo and Patrick Lemaire

Background: In *Xenopus*, the endoderm germ layer is derived from the vegetal blastomeres of cleavage-stage embryos. Cell transplantation experiments have revealed that the endodermal fate becomes gradually fixed during the late blastula stages. *Sox17α, Mix.1, Mixer* and *GATA-4* encode vegetal zygotic transcription factors with endoderm-inducing activity. The accumulation of their transcripts during the late blastula stages may cause determination of the endodermal fate. VegT, a T-box transcription factor, the maternal transcripts of which are vegetally localised, is also required for endoderm formation.

Results: We analysed the events leading to the progressive accumulation of the transcripts for *Sox17α, Mix.1, Mixer* and *GATA-4*. Two phases could be distinguished in the endodermal programme. In phase 1, *Sox17α, Mix.1,* and the genes encoding transforming growth factor β-related signalling molecules Xnr1, Xnr2 and Derrière were activated cell-autonomously at around the mid-blastula transition (MBT) by maternal determinants. In phase 2, TGFβ signalling, possibly involving Xnr1, Xnr2 and Derrière, led to the activation of *Mixer* and *GATA-4* in late blastula stages and to the reinforcement of the expression of *Sox17α* and *Mix.1*. Overexpression of *VegT* in animal caps triggered a developmental programme qualitatively similar to that observed in vegetal blastomeres, except that *Xnr1* and *GATA-4* were not activated by the early gastrula stage.

Conclusions: Our results support a two-step model for endoderm determination between fertilisation and the onset of gastrulation. The initial cell-autonomous activation of early endodermal genes by maternal determinants including, but not limited to, VegT is relayed by the action of zygotic TGFβs such as Xnr1, Xnr2 and Derrière.

Address: Laboratoire de Génétique et Physiologie du Développement, Institut de Biologie du Développement de Marseille, CNRS-INSERM-Université de la Méditerannée-AP de Marseille, Campus de Luminy, Case 907, F-13288 Marseille Cedex 9, France.

Correspondence: Patrick Lemaire
E-mail: lemaire@lgpd.univ-mrs.fr

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Background

Formation of the three germ layers, namely ectoderm, mesoderm and endoderm, is an important event during the early stages of the development of triploblasts (that is, most animals). In amphibian embryos, these three layers are established along the animal–vegetal axis. The endodermal germ layer derives from the vegetal pole region of the embryo. During the course of gastrulation, this layer invaginates through the blastopore and becomes completely internalised. The involuted endoderm extends along the body axis and finally differentiates into the respiratory and gastrointestinal systems in association with adjacent mesoderm (the development of the gut of *Xenopus* embryos is described in [1]). In addition, dorso-anterior endoderm may be required during gastrulation for the induction of anterior head structures [2,3].

In spite of the importance of the endodermal layer during embryogenesis, relatively little is known about the mechanisms that are involved in its formation. The fate of the vegetal blastomeres becomes restricted to endoderm around the mid-blastula transition (MBT). At this stage, vegetal blastomeres transplanted to a foreign environment will adopt the fate of the surrounding cells, indicating that they have not yet acquired a fixed endodermal fate. During the late blastula stages, however, vegetal cells gradually lose their sensitivity to their surroundings and still follow an endodermal differentiation pathway when transplanted in an ectopic position [4,5]. These experiments can be interpreted in two ways. Blastula vegetal cells may need to communicate with one another to differentiate into endoderm, an effect similar to the community effect [6]. Alternatively, non-endodermal cells may emit signals that prevent vegetal cells from adopting an endodermal fate. A precise description of the effect of the disruption of cell–cell contacts on endoderm differentiation should make it possible to distinguish between these two models. These studies should be made easier by the recent identification of maternal and early zygotic markers of endoderm formation.

VegT (also known as *Antipodean* [7], *Xombi* [8] and *Brat* [9]) encodes a maternally derived T-box transcription

factor; its transcripts are tightly associated with the vegetal cortex of *Xenopus* oocytes [10]. Depletion of the maternal store of *VegT* transcripts from oocytes results in a disruption of the correct pattern of germ-layer specification in the resulting blastulae. The endoderm does not form and mesoderm is displaced towards the vegetal pole [11]. This finding indicates that the maternal VegT protein is necessary for the formation of the endoderm in the vegetal region. In addition, *VegT* transcripts, when misexpressed, can ectopically induce some endodermal marker genes, implying that VegT is sufficient for endoderm differentiation [9,12].

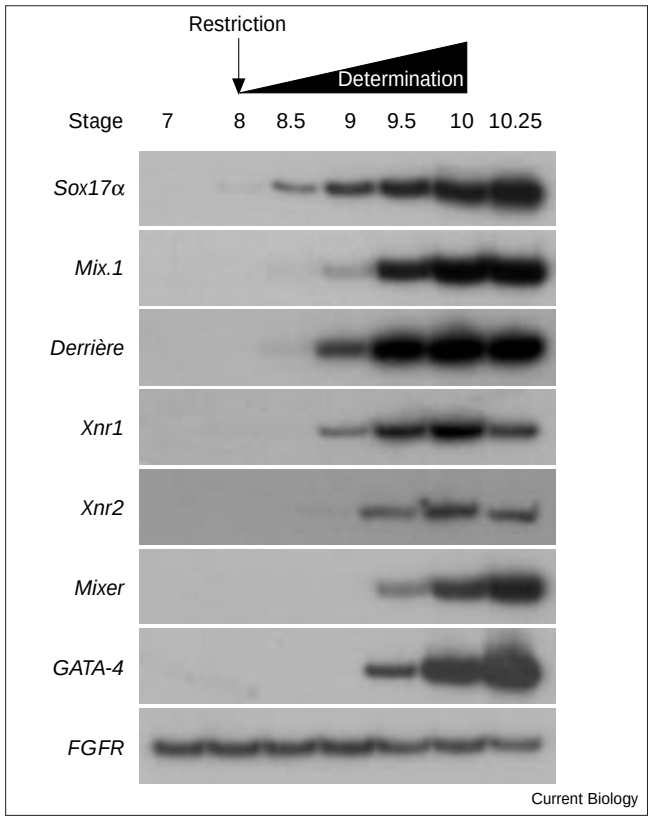
Several zygotic genes have been shown to be expressed in the presumptive endoderm region during the blastula and early gastrula stages and may constitute direct or indirect targets of VegT. *Sox17α* and *β* encode HMG-domain-containing proteins [13], Mix.1 and Mixer are homeobox proteins [3,14] and *GATA-4*, which is also expressed in the endoderm (J. Broadbent and R. Patient, personal communication), encodes a protein with zinc-finger motifs [15]. These proteins probably act as transcription factors. Their overexpression in animal caps causes ectopic expression of late endodermal marker genes such as *endodermin*, *Xlhbox8* and *IFABP* ([3,13] and H. Weber, C. Symes, M. Walmsley, A. Rodaway, R. Patient, unpublished observations); Mix.1 does so synergistically with another homeobox protein, Siamois [2]. Furthermore, it has also been shown that dominant-negative forms of Mixer, Mix.1 and Sox17 perturb endoderm formation [2,3,13]. Zygotic transcription of these genes may therefore cause the determination of the endodermal fate. To shed light on the early steps of endoderm formation, we have analysed in this study the type of cell-communication events required for determination of presumptive endodermal cells, using *Sox17α*, *Mix.1*, *Mixer* and *GATA-4* as markers. In addition, we have tried to analyse more precisely the relationship between *VegT* and these four genes.

Results

Sequential activation of the early endodermal genes after the MBT

We first wanted to precisely map the temporal expression profiles of the early endodermal genes *Sox17α*, *Mix.1*, *Mixer* and *GATA-4*. For this purpose, whole embryos were cultured at 23°C, collected from stage 7 onwards at intervals of 1 hour, and gene expression was analysed by reverse-transcriptase PCR (RT–PCR; Figure 1). Soon after the MBT (stage 8), *Sox17α* and *Mix.1* started to be expressed and their transcripts steadily accumulated up to the early gastrula stage. Transcripts for *Mixer* and *GATA-4* were first detected around stage 9.5, 2–3 hours later than those of *Sox17α* and *Mix.1*, and rapidly accumulated in the embryo. By stage 10, when the cell fate of the presumptive endoderm blastomeres is determined, all the endoderm genes tested were fully activated.

Figure 1



Expression profile of early endodermal genes. The temporal gene-expression profiles of *Sox17α*, *Mix.1*, *Derrière*, *Xnr1*, *Xnr2*, *Mixer*, *GATA-4* and the fibroblast growth factor (FGF) receptor gene (*FGFR*) were analysed by RT–PCR. *FGFR* is ubiquitously expressed and served as a loading control. Expression of *Sox17α* starts at stage 8; that of *Mix.1* and *Derrière* starts at stage 8.5; expression of *Mixer* and *GATA-4* is first detected at stage 9.5; transcripts for *Xnr1* and *Xnr2* are detected from stage 9. The timing of restriction and gradual determination of the fate of presumptive endodermal blastomeres is shown at the top.

Two lessons can be learned from these results. First, the progressive accumulation of early endodermal transcripts parallels the progressive fate determination of the presumptive endodermal blastomeres, pointing to a possible causal relationship between these two events. Second, the temporal difference observed between the time of activation of *Sox17α* and *Mix.1* on the one hand, and *Mixer* and *GATA-4* on the other, suggests that these two groups of genes may be activated by different mechanisms. We have concentrated on the study of the regulation of these genes.

Post-MBT cell contacts are required for expression of early endodermal genes

We next addressed whether cell–cell interactions are required for the accumulation of early endodermal transcripts. Three different manipulations were carried out (Figure 2a): embryos were dissociated just after the first cleavage, then reaggregated at stage 8, when the MBT

occurs, and cultured until stage 10; embryos were kept dissociated from the first cleavage until stage 10; or embryos were dissociated at stage 8 and kept dissociated until stage 10. In these experiments, the efficiency of the dissociation procedure was monitored by analysing the expression of *Xbra*, an early mesodermal gene the expression of which is strictly dependent on cellular interactions [16,17]. Expression of all the endodermal markers tested was downregulated in embryos dissociated throughout the whole period of investigation or solely after the MBT (Figure 2b). In embryos dissociated until the MBT and then reaggregated, however, the level of expression of the genes tested was not significantly different from the level in control embryos. Thus, post-MBT cell contacts are necessary for the expression of endoderm marker genes, whereas pre-MBT cell contacts are dispensable. Interestingly, a similar result was obtained with *Xbra*, strengthening the proposition that mesoderm formation mainly relies on post-MBT signalling events [18,19]. Although the expression of all genes tested was reduced in dissociated embryos, the expression of *Sox17α* and *Mix.1* was not completely abolished under these experimental conditions. A similar result has been reported for *Sox17β* [12]. Furthermore, this cell-autonomous expression of *Sox17α* and *Mix.1* was confined to the vegetal hemisphere cells, as it was never observed in dissociated animal cap cells (data not shown).

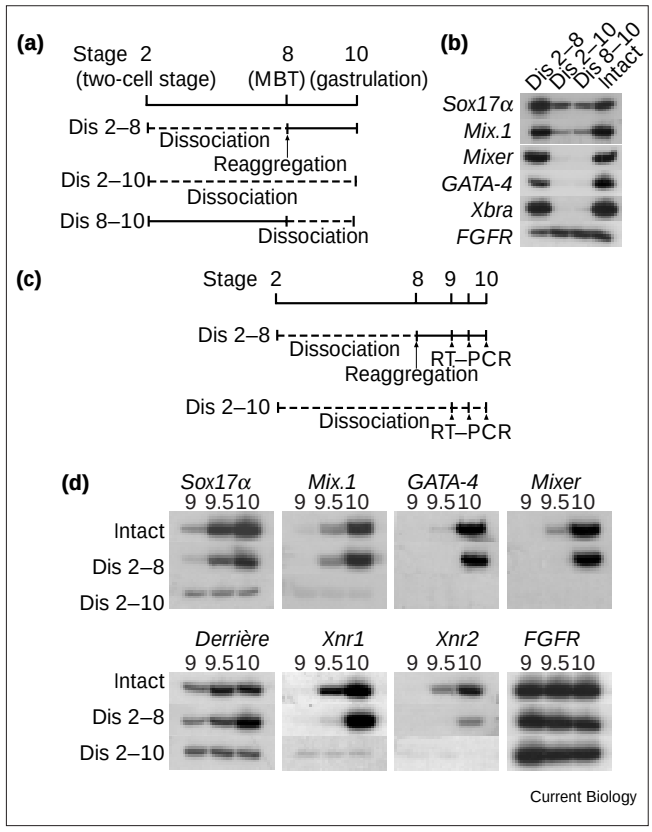
The reduction in gene expression observed in dissociated embryos could be due to a delayed activation of the genes, to a reduced rate of accumulation of the transcripts, or to a poor maintenance of their expression. To discriminate between these possibilities, we analysed the kinetics of transcript accumulation in control or continuously dissociated late blastula embryos (stages 9,10; Figure 2c,d). In continuously dissociated embryos, expression of *Mixer* and *GATA-4* was observed neither at stage 9.5, at which stage these genes are first activated, nor at stage 10. In contrast, inhibition of cellular communication had no effect on the expression of *Sox17α* and *Mix.1* at stage 9 but prevented any further accumulation of their transcripts during the late blastula stages.

These results therefore establish that two different mechanisms are at work during the determination of endoderm during the blastula stages. Cell-autonomous events lead to the activation of *Sox17α* and *Mix.1* around the MBT, whereas cell communication is required for the activation of the later markers *Mixer* and *GATA-4* and for the accumulation of all four marker transcripts during the late blastula stages.

When is cell communication required after the MBT?

To define precisely when the cell contacts are required for the accumulation of endodermal transcripts, we dissociated blastula embryos for different time periods: stages 8–8.5, 8–9, 8–9.5, 8–10, 8.5–10, 9–10 and 9.5–10 (Figure 3a). As shown in Figure 3b, a 2 hour dissociation between stages 8 and 9 had little effect on *Mixer*, *Xbra*, *Sox17α* and *Mix.1* expression but led to a downregulation of *GATA-4*. Dissociation for the same length of time between stages 9 and 10 had a more severe effect and led to a strong reduction in the level of expression of all genes tested. We conclude that cell communication is mainly needed during the late blastula stages for the expression of endodermal and mesodermal (*Xbra*) markers in early gastrulae.

Figure 2

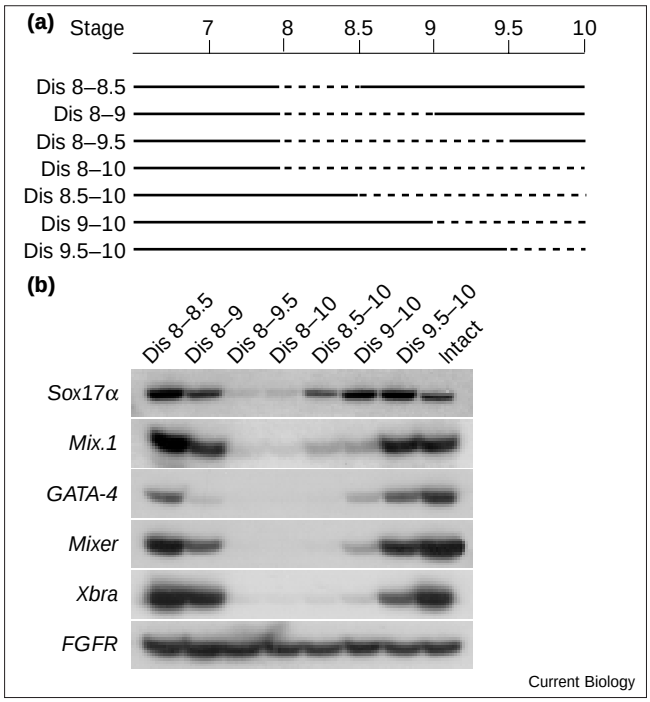


Cellular communication after the MBT is required for the activation or full expression of early endodermal genes. Embryos were dissociated (dis) at the stages indicated in (a,c) and, in some cases, reaggregated at later stages as shown. (b,d) The indicated transcripts were then detected by RT–PCR at (b) stage 10 or (d) stages 9, 9.5 and 10. As a positive control, intact embryos of the appropriate stage were analysed in parallel.

Timing of the secretion of endoderm-inducing molecules by vegetal poles

The results above do not necessarily mean that signalling molecules are active only after the MBT. An alternative possibility is that the signals are present around the cells throughout the cleavage stages but the vegetal blastomeres only become competent to read these signals after the MBT. In support of the former mechanism, Wylie and colleagues [19] previously showed that the

Figure 3



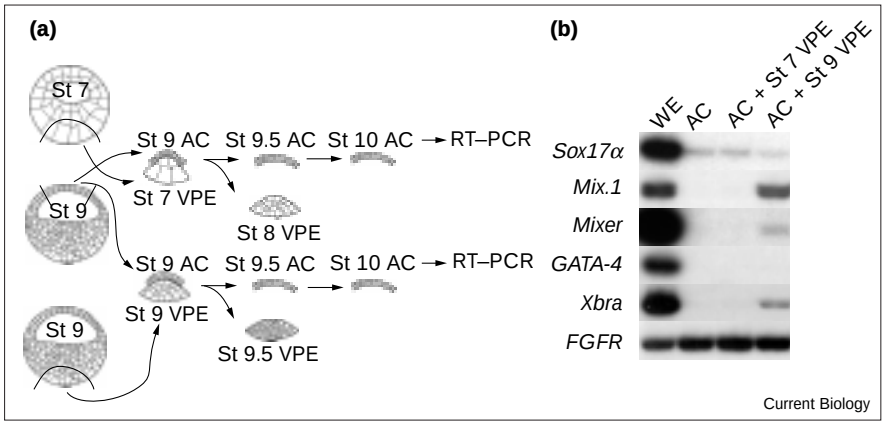
Post-MBT cellular communication occurs during the late blastula stage. Embryos were dissociated (dis) and reaggregated at the stages indicated in (a); dotted lines show the period of dissociation. (b) The indicated transcripts were then detected by RT–PCR at stage 10, with intact stage 10 embryos as a positive control.

secretion of muscle-inducing signals mainly occurs post-MBT. To test whether this was also true for other mesodermal types and for endoderm, we compared the abilities of pre- and post-MBT vegetal pole explants to secrete signals that activate early pan-mesodermal and pan-endodermal markers in animal caps. This was carried out by conjugating stage 7 or 9 vegetal pole explants to stage 9 animal caps [19]. After 1 hour of contact, the ectodermal part of the conjugate was then dissected and cultured in isolation for an additional hour before analysing its gene expression programme (Figure 4a). By the equivalent of stage 10, animal caps expressed *Xbra*, *Mix.1* and *Mixer* in response to stage 9 vegetal pole explants, but not in response to stage 7 vegetal pole explants (Figure 4b). Activation of *GATA-4* and *Sox17α* was not observed in either experiment. This shows that vegetal pole explants emit more endoderm- and mesoderm-inducing molecules after the MBT and that 1 hour of cell contact is sufficient to induce two endodermal genes (*Mix.1* and *Mixer*) and a mesodermal gene (*Xbra*). We next investigated the nature of the signals involved.

TGFβ signals are involved in endoderm determination

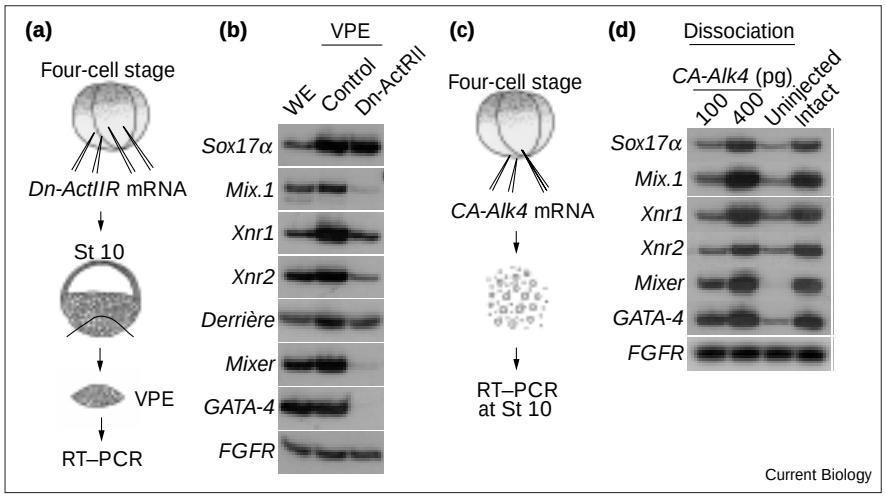
It has been shown that transforming growth factor β (TGFβ) family signals are required for the expression of several endodermal markers in *Xenopus* and zebrafish [12,20–26]. We therefore tested whether this family of signalling molecules is involved in the post-MBT cell–cell contacts necessary for the expression of the early endoderm genes studied here. The mRNA encoding a dominant-negative type-II activin receptor (DnActRIIB; also called tAR [27]) was injected into the vegetal poles of four-cell embryos. Control or injected vegetal poles were dissected at stage 10 and analysed for gene expression by RT–PCR (Figure 5a). Expression of all endodermal transcription factors tested was downregulated to a variable extent in the *DnActRIIB*-injected vegetal poles (Figure 5b). As in cell-dissociation experiments, expression of *Mixer* and *GATA-4* was completely abolished, whereas expression of *Mix.1* was only decreased. Although Figure 5b suggests that the expression of *Sox17α* is unaffected, this gene was also partially downregulated in other repetitions of this experiment (data not shown). Also, consistent with a role for TGFβ signalling in endoderm determination, injection of RNA encoding a constitutively active type I activin receptor (CA-Alk4) in vegetal poles rescued expression of endoderm genes in continuously dissociated

Figure 4



Signalling molecules in vegetal pole explants are more active after the MBT. (a) Stage 9 (St 9) animal caps (AC) were cultured in contact with stage 7 or stage 9 vegetal pole explants (VPE) for 1 h. Animal caps were then removed and cultured alone until the equivalent of stage 10. (b) The indicated transcripts were then detected by RT–PCR, with whole stage 10 embryos (WE) as a positive control and unconjugated animal caps as a negative control.

Figure 5



TGFβ like signals are involved in the post-MBT cellular communication. (a) Dominant-negative ActIIR mRNA (*DnActIIR*, 4 ng RNA per blastomere) was injected vegetally into four-cell embryos. At stage 10 (St 10), vegetal pole explants (VPE) from injected or control, uninjected embryos were removed and analysed. (b) The indicated transcripts were then detected by RT–PCR, with whole stage 10 embryos (WE) as a positive control. Although, in the experiment shown here, expression of *Sox17α* is not affected in the *DnActIIR*-injected vegetal pole explants, this gene was also partially downregulated in other repetitions of this experiment (data not shown). (c) Constitutively active *Alk4* RNA (*CA-Alk4*, 100 or 400 pg per embryo) was injected vegetally into four-cell embryos, which were immediately dissociated and cultured until the equivalent of stage 10. (d) The indicated transcripts were then detected by RT–PCR, with intact stage 10 embryos as a positive control and uninjected dissociated embryos as a negative control.

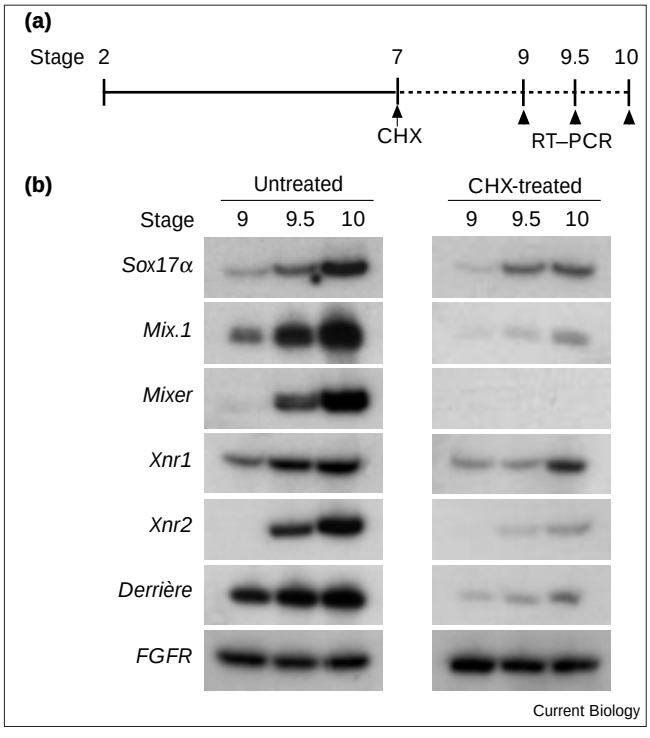
embryos (Figure 5c,d). These results indicate that TGFβ signals constitute an important component of the post-MBT cell contacts required for the fate determination of presumptive endoderm blastomeres.

Sox17α and *Mix.1* are direct targets of maternal factors, but *Mixer* is a target of zygotic factors

To gain further understanding of the nature of the molecules responsible for the autonomous activation of *Sox17α* and *Mix.1* and for the cell-contact-dependent activation of *Mixer* and *GATA-4*, we treated embryos with the protein synthesis inhibitor cycloheximide (CHX) from stage 7–10 and analysed the expression of these genes (Figure 6a,b). In this experiment, post-MBT protein synthesis is blocked so that a gene will only be activated if its positive regulators are of maternal origin. Expression of *Sox17α* and *Mix.1* in CHX-treated embryos, though reduced, was still easily detectable. In contrast, *Mixer* transcripts were never detected. *GATA-4* could not be analysed in this type of experiment, as treatment of animal caps with CHX is sufficient to activate this gene (data not shown). We conclude that *Sox17α* and *Mix.1* are partially under the control of maternal factors, whereas activation of *Mixer* requires the presence of a molecule translated after the MBT.

The picture that emerges from this and the previous sections is therefore that *Sox17α* and *Mix.1* are first activated at the MBT by maternal cytoplasmic determinants acting in a cell-autonomous manner. The accumulation of their transcripts as well as the activation of *Mixer* and *GATA-4* is then due to the subsequent action of zygotic TGFβ molecules.

Figure 6



Sox17α and *Mix.1* are direct targets of maternal factors, whereas expression of *Mixer* requires zygotic proteins. (a) Embryos were treated continuously with CHX from stage 7 onwards and analysed at stages 9, 9.5 and 10 in parallel with control, untreated embryos. The dotted line shows the period of treatment with CHX. (b) The indicated transcripts were then detected by RT–PCR of untreated and CHX-treated embryos.

Xnr1, Xnr2 and Derrière are candidates for the post-MBT signals

Xnr1, Xnr2 and Derrière are potential candidates for the zygotic TGFβs necessary for endoderm determination. They belong to the TGFβ family and are expressed in the presumptive endodermal blastomeres before (*Xnr1*, *Derrière*) or around (*Xnr2*) the time of the activation of *Mixer* (Figures 1,6b) [28,29]. It has been shown that these three factors have mesoderm-inducing properties and are able to activate at least some endodermal markers [26,28,29]. To characterise in greater detail the endoderm-inducing properties of these factors, we injected a range of concentrations of their mRNAs in the animal pole of two-cell embryos, isolated the animal caps around the MBT and analysed the expression of endodermal marker genes at the equivalent of stage 10 (Figure 7). In *Xnr1*-, *Xnr2*- or *Derrière*-expressing animal caps, *Mixer* and *Mix.1* were induced (Figure 7b,c). In contrast, *GATA-4* was strongly activated in animal caps overexpressing *Xnr1* but barely detectable in *Xnr2*-expressing caps and not activated in response to *Derrière*. *Sox17α* was strongly activated by Xnr1 and Derrière but could only occasionally be weakly activated by Xnr2. The differential ability of Xnr1 and Xnr2 to activate *GATA-4* did not reflect a generally higher activity of Xnr1 as injection of low doses (50 pg) of mRNA for *Xnr2* led to a more robust activation of *Xbra* than the injection of a similar amount of *Xnr1* mRNA (Figure 7b). Likewise, lack of activation of *GATA-4* by Derrière was unlikely to be accounted for by a weaker activity of Derrière as activation of *Sox17α* by Derrière was comparable to that obtained with Xnr1 (Figure 7c).

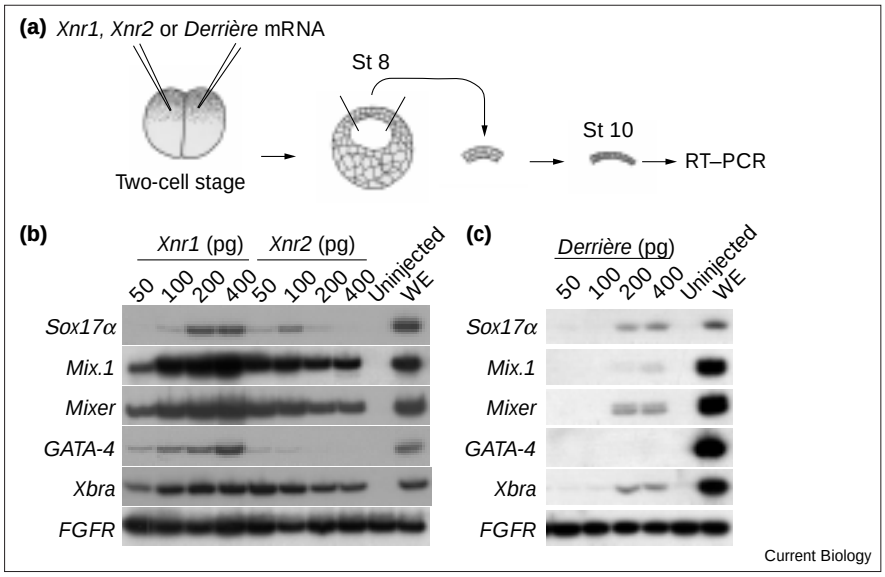
In conclusion, the expression patterns and overlapping, yet distinct, endoderm-inducing properties of Xnr1, Xnr2 and Derrière suggest that they constitute part of the post-MBT signals required for endoderm formation. Although all three factors induce *Mix.1* and *Mixer* in early gastrula animal cap cells, only Xnr1 is able to induce *GATA-4* by this stage.

Regulation of the expression of *Xnr1, Xnr2* and *Derrière* during the blastula stages

We next tested whether the *Xnr1*, *Xnr2* and *Derrière* genes were, like *Sox17α* and *Mix.1*, directly activated by maternal factors or whether they were targets of early zygotic proteins. We therefore analysed their expression in embryos in which post-MBT protein synthesis had been blocked. Treatment of embryos with CHX from stage 7 onwards caused partial reduction of the expression level of *Xnr1*, *Xnr2* and *Derrière* (Figure 6b), indicating that at least part of the expression of these genes is due to direct action of maternal factors. In the case of *Xnr1* and *Derrière*, these maternal factors act at least in part cell-autonomously, as the levels of expression of these genes at stage 9 was not greatly affected by continuous cell dissociation from the two-cell stage onwards (Figure 2d). At stage 9, the level of expression of *Derrière* was much stronger than that of *Xnr1* and *Xnr2*. By stage 10, continuous cell dissociation prevented the accumulation of *Xnr1* and *Xnr2* transcripts but had a less severe effect on the level of expression of *Derrière* (Figure 2d).

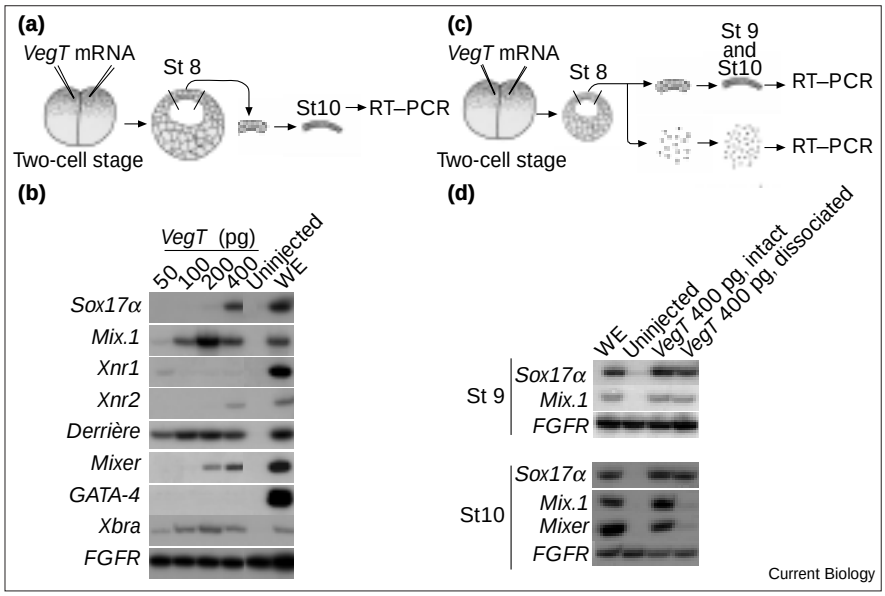
Overexpression of the dominant-negative type-II activin receptor in vegetal cells (Figure 5b) had the same effect as cell dissociation (Figure 2d), strongly affecting *Xnr1* and *Xnr2* but not *Derrière*. Conversely, the reduced expression of *Xnr1* and *Xnr2* in dissociated embryos was rescued by overexpression of constitutively active Alk4 (Figure 5d). This indicates that following their initial activation by

Figure 7



Overexpression of *Xnr1, Xnr2* or *Derrière* induces prospective ectoderm to express endodermal genes. (a) *Derrière, Xnr1* or *Xnr2* mRNA was injected into the animal part of both cells of two-cell embryos, which were cultured until stage 8 (St 8). Animal caps were then dissected, cultured until the equivalent of stage 10 and analysed. (b,c) The indicated transcripts were then detected by RT–PCR from embryos injected with the indicated amounts of (b) *Xnr1* and *Xnr2* mRNA and (c) *Derrière* mRNA. Whole stage 10 embryos (WE) acted as a positive control and uninjected animal caps as a negative control.

Figure 8



VegT triggers a two-step partial endodermal program in animal caps. (a) *VegT* mRNA was injected into the animal part of both cells of two-cell embryos, which were cultured until stage 8 (St 8). Animal caps were then dissected, cultured until the equivalent of stage 10 and analysed. (b) The indicated transcripts were then detected by RT–PCR from embryos injected with the indicated amounts of *VegT* mRNA. A very weak induction of *Xnr1* in the *VegT*-injected animal caps at stage 10 was only observed in two out of four repetitions of this experiment. (c) *VegT* mRNA was injected into two-cell embryos as in (a). At stage 8, animal caps were then dissected and either dissociated or left intact, cultured until the equivalent of stage 9 or stage 10 and analysed. (d) The indicated transcripts were then detected by RT–PCR. Whole stage 10 embryos (WE) acted as a positive control and uninjected animal caps as a negative control.

maternal factors, the expression of *Derrière* rapidly and cell-autonomously reaches its maximum, whereas expression of *Xnr1* and *Xnr2* increases progressively in response to TGFβ signals. Interestingly, these three genes can activate each other in the animal-cap assay (data not shown), suggesting the establishment of a positive regulatory loop during the late blastula stages.

Taken together, these results indicate that the regulation of *Xnr1* and *Xnr2* is similar to that of *Sox17α* and *Mix.1*, whereas *Derrière* appears to be mainly under the control of factors that act cell-autonomously.

Overexpression of *VegT* in animal cells triggers a two-step partial endodermal programme

Our data indicate the existence of maternal factors that are able to cell-autonomously activate *Sox17α*, *Mix.1*, *Xnr1*, *Xnr2* and *Derrière*. *VegT* is a good candidate for such a maternal factor. It encodes a transcription factor and its maternal product is required for generation of the endodermal germ layer [11]. In addition, overexpression of *VegT* in animal caps leads to the activation of *cerberus*, which encodes an extracellular head-inducing protein, and *Sox17β* [12]. It is not clear, however, whether VegT is the sole maternal endodermal determinant [11,30]. To resolve this issue, we asked whether overexpression of *VegT* in animal cap cells is sufficient to trigger an endodermal pathway identical to that observed in vegetal cells during normal development.

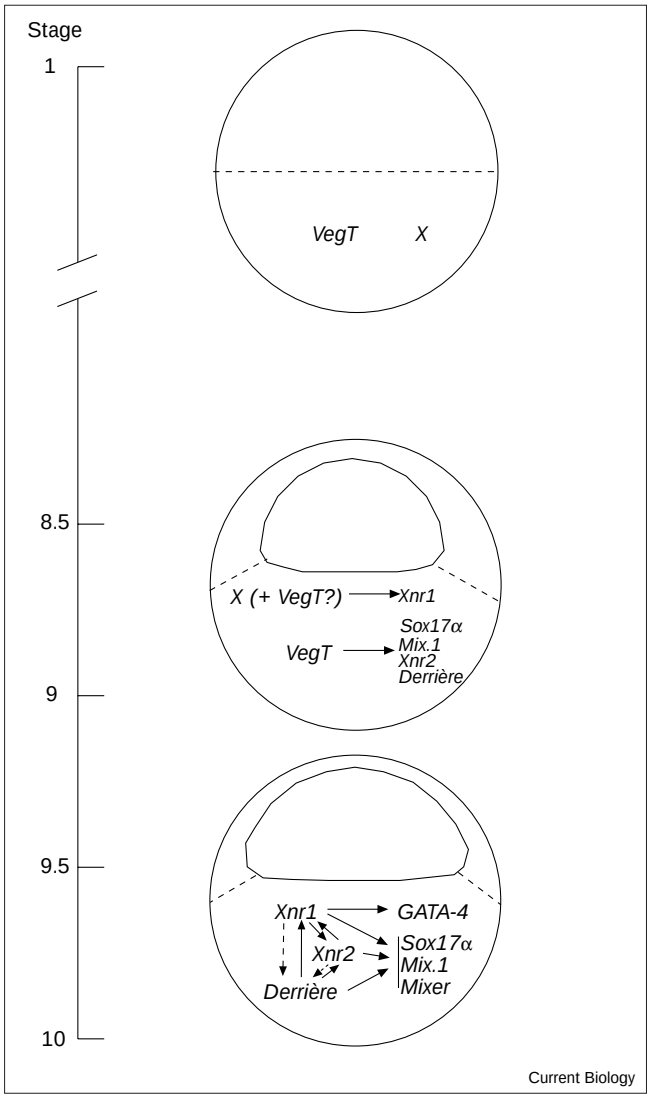
Different amounts of *VegT* mRNA were injected in the animal pole of two-cell embryos, the animal caps were dissected around the MBT and gene expression was analysed in the explants at the equivalent of stages 9 or 10 (Figure 8). By stage 10, VegT-injected animal caps expressed *Sox17α*, *Mix.1*, *Mixer*, *Xnr2* and *Derrière* but failed to activate *GATA-4*. *Xnr1* was only very weakly activated in two out of four experiments performed. This result indicates that the overexpression of *VegT* in animal caps is not sufficient to trigger a full endodermal pathway in animal caps. Although the programme triggered by VegT in animal caps was incomplete, it retained the two-step characteristics of the endogenous endodermal programme. Activation of *Sox17α* and *Mix.1* occurred early and was independent of cell communication up to stage 9. As in normal embryos, by stage 10, cell dissociation led to reduced expression of these genes (Figure 8c,d). Activation of *Mixer* was not observed in the *VegT*-expressing animal caps by stage 9 (data not shown). By stage 10, the expression of this gene in the *VegT*-injected animal caps depended strongly on cell communication (Figure 8d, bottom panel). Thus, as in vegetal cells during normal development, the early cell-autonomous activation of *Sox17α* and *Mix.1* is followed by a second, cell-contact-dependent phase of gene expression. These results indicate that overexpression of *VegT* in animal cap cells mimics the endogenous events leading to the activation of *Derrière*, *Xnr2*, *Mix.1*, *Sox17α* and *Mixer* but is not sufficient to activate *Xnr1* and *GATA-4* by the early gastrula stage. The presence of additional maternal determinants must therefore be postulated.

Discussion

In this study, we have initiated a description of the early events leading to the determination of the endodermal fate of vegetal blastomeres. Our results can be summarised

in the model presented in Figure 9, which shares similarities with that recently proposed by Griffin and Kimelman [30]. Two phases can be distinguished in the early endodermal programme. In phase 1, which occurs between the MBT and stage 9, maternal cytoplasmic determinants cell-autonomously activate early zygotic genes such as *Sox17α*, *Mix.1*, *Derrière*, *Xnr1* and *Xnr2*. The effect of overexpression of VegT in animal cap cells indicates that this transcription factor is sufficient to activate *Sox17α*, *Mix.1*, *Derrière* and *Xnr2* but not *Xnr1*. Other maternal factors must therefore exist that complement the action of VegT in vegetal cells. The second phase of the early endodermal programme takes place during the late blastula stages and leads to the activation of genes such as *Mixer* and *GATA-4*, and to a reinforcement of the expression of *Sox17α*, *Mix.1*, *Xnr1* and *Xnr2*. This phase requires that endodermal cells signal to each other using post-MBT TGFβ molecules such as Xnr1 and Xnr2 and Derrière, three factors with overlapping but distinct endoderm-inducing activities. This model raises several issues that we will discuss below.

Figure 9



A model for the cell-fate determination of vegetal pole blastomeres to endoderm. For simplicity, only molecules that have been analysed in this study are considered in this model, which is therefore necessarily incomplete. *X* represents an additional maternal endoderm determinant acting upstream of *Xnr1* and *GATA-4*. Dotted lines indicate the upper boundary of the presumptive endodermal region. Levels of gene expression are represented by the size of the lettering. See text for details.

Identity of the maternal endodermal determinants

Our study shows that VegT is not sufficient to activate *Xnr1* and *GATA-4* by the early gastrula stage, indicating the presence of additional maternal determinants of the endodermal fate. It has been proposed that an activin-like activity might act upstream of the Xnr proteins [26], and a possible candidate for this activity is Vg1, a TGFβ family member the maternal mRNA of which is localised in the vegetal cells of early embryos [31]. Our finding that *Xnr1* is initially activated in dissociated vegetal cells and in vegetal cells overexpressing *DNActRIIB*, however, suggests that at least some of the missing endodermal determinants are molecules unrelated to activin, Xnr proteins or Vg1. It will be interesting to determine whether the additional endoderm determinants act as cofactors for VegT or trigger an independent pathway that includes *Xnr1* and *GATA-4*. The analysis of the early activation of *Xnr1* and *GATA-4* in embryos depleted of maternal *VegT* mRNA should enable discrimination between these two possibilities.

Identity of the zygotic TGFβs involved

In this study, we have analysed the potential role of Xnr1, Xnr2 and Derrière in the second phase of endoderm determination. Another nodal-related factor, Xnr4 [32], displays mesoderm-inducing activity and it will also be interesting to test its endoderm-inducing potential, as well as to study its regulation. Activin, which is also transcribed post-MBT [33] and induces endoderm [20,21], *Xnr1* and *Xnr2* [28] and *Derrière* [29], may also play a role in step 2 of endoderm determination.

So far, Xnr1 is the only factor able to strongly activate *GATA-4* in stage 10 animal caps. It is interesting to note that, in spite of their high sequence similarities, Xnr1 and Xnr2 have different inducing properties: Xnr2 can only induce some of the endodermal genes. It was previously reported that Xnr2 is a more powerful mesoderm inducer than Xnr1 [28]. As endodermal cells also act as a source of mesoderm inducers, it is tempting to propose that secretion of Xnr1 by vegetal cells is important for their fate determination, whereas Xnr2 may have a more prominent role in mesoderm induction.

Because of the overlapping domains of expression and biological activities of these TGFβ molecules, it may be

difficult to precisely define their individual roles in endoderm (and mesoderm) determination. Dominant-negative mutants for Derrière (Cm-derrière) and Xnr2 (Cm-Xnr2) have been generated [26,29] and overexpression of Cm-Xnr2 leads to a delayed activation of *Mixer* and *cerberus*, [26]. The action of these mutant proteins, however, is unlikely to be specific to one TGFβ. For example, a dominant-negative form of Derrière was also reported to partially inhibit Xnr1 signalling [29], and Cm-Xnr2 blocked the action of Xnr1, Xnr2 and Xnr4 [26]. The impossibility of genetic loss-of-function experiments in *Xenopus* may therefore make it difficult to precisely determine the function of individual TGFβ molecules in this system. The zebrafish system may be more suitable. As early endodermal markers start to be cloned in this system [25,34], it will be interesting to test whether the two-step characteristic of the early endodermal pathway has been conserved between zebrafish and *Xenopus*.

A community effect in endoderm determination

Work by Gurdon and colleagues has established that the determination of the muscle and notochord fates requires presumptive muscle or notochord cells to communicate with other cells of the same type [35,36]. Our results indicate that, likewise, presumptive endodermal cells need to communicate with one another during the blastula stages to become determined. In the case of muscle, the community-effect factor has so far remained elusive. In endoderm, however, we could show that the community factor is a TGFβ protein, probably identical to or related to the mesoderm-inducing factors. This illustrates that the same family of signalling factors can act as both classical inducers and community-effect factors.

One could wonder why *Xenopus* embryos make use of a two-step system for the determination of the endoderm lineage. A simpler strategy would have been for the localised maternal determinants to act in one step by directly determining the endodermal fate of the cells that inherit them. Indeed, such a strategy has been adopted by ascidians, the most primitive living chordates [37]. A comparison of the early modes of development of ascidians and *Xenopus* embryos suggests a possible reason for the difference in the way maternal information is read by *Xenopus* and ascidian embryos. Ascidian embryos have an invariant cleavage; the fate of primordial endoderm blastomeres becomes restricted as early as the 16-cell stage and these blastomeres differentiate cell-autonomously [37]. By contrast, *Xenopus* embryos cleave irregularly and the vegetal cells only become determined when the embryo comprises around 10,000 cells. We propose that the type of cleavage pattern and the number of cells at the determination stage may be causally related. In the absence of an invariant cell cleavage in *Xenopus* embryos, the precise boundaries between tissues can only be defined at a stage when cells have become sufficiently small. If maternal determinants acted directly at the MBT to determine cell fate by cell-autonomous activation of zygotic determination genes, the large size of the cells at the MBT, coupled to the irregular cleavage pattern, would hinder a precise definition of the position of the boundary between presumptive endoderm and mesoderm. By contrast, the establishment of a community effect allows the determination of the presumptive endodermal blastomeres to proceed gradually up to the early gastrula stage, a time of development when cells are sufficiently small to define a precise endoderm–mesoderm boundary.

TGFβ signalling and the integration of early maternal patterning along the dorsoventral and animal-vegetal axes

In *Xenopus* embryos, definition of the dorsoventral axis during the blastula stages occurs largely independently of the definition of the animal–vegetal axis and involves the stabilisation and nuclear translocation of maternal β-catenin. Yet, while early dorsal genes such as *Siamois* are activated cell-autonomously around the MBT by the β-catenin pathway [38], the full expression of later genes such as *goosecoid* or *cerberus* requires TGFβ signalling [12,22,38]. A two-step mechanism involving TGFβ signalling during the late blastula stages thus also applies to the patterning of the *Xenopus* embryo along the dorsoventral axis. This joint requirement for TGFβ signalling along both axes may provide a convenient way of integrating and refining the coarse positional information provided by the animal-vegetal and dorsoventral maternal systems. In this view of early *Xenopus* development, maternal determinants that act cell-autonomously have a key instructive role. The function of TGFβ-mediated early embryonic inductions is less to instruct the cells of their fate than to reinforce and integrate the positional values imposed along both axes by localised maternal determinants.

Materials and methods

Embryo injections and treatments

Embryos were fertilised *in vitro*, dejellied, cultivated and injected with synthetic capped mRNA as described [39]. They were staged according to Nieuwkoop and Faber [40]. Capped mRNA was synthesized using an Ambion mMESSAGE mMACHINE kit. The mRNAs for *VegT* [10], dominant-negative ActRIIB [27], constitutively active form of Alk4, and *Xnr1* and *Xnr2* [28] were prepared as described in each reference. Amounts of injected *in vitro*-synthesised RNAs and sites of injection are as described in the Results and figures. Animal caps and vegetal pole explants were dissected at the stages indicated in the Results and cultured as described [39]. Embryos were dissociated following essentially a protocol described previously [17]. When embryos were dissociated from the first cleavage, vitelline membranes were removed at stage 2. For protein-synthesis inhibition experiments, CHX was applied at a concentration of 10 μg/ml continuously from stage 7. In each experiment, animal caps of CHX-treated embryos were dissected at stage 10 and were tested for whether ectopic gene expression was induced by CHX treatment.

RT–PCR assays

RT–PCR assays were performed as previously described [39] with following additional primers: *Sox17α* (forward primer: 5′-GATGGTGGTTACGCCAGCGA-3′; reverse primer: 5′-TGCGGGGTCTGTACTTGTAG-3′;

27 cycles); *Mixer* (forward primer: 5′-ACAGCCAGAACAAGCTG-GAT-3′; reverse primer: 5′-AATTCCATGGTAGCTGCTCC-3′; 26 cycles); *GATA-4* (forward primer: 5′-GTGCCACCTATGCAAGCCC-3′; reverse primer: 5′-TAGACCCACCCGGCGAGAC-3′; 27 cycles); *Xnr1* (forward primer: 5′-CTGCCAACCATATTGGCTTT-3′; reverse primer: 5′-GTGGTGCCTCAAAACAACCT-3′; 27 cycles); *Xnr2* (forward primer: 5′-CAGACCCTGATTTTGGGAAA-3′; reverse primer: 5′-CTGACCTTCCTTGGTGTGGT-3′; 28 cycles); *Derrière* (forward primer: 5′-CGCTCATATCGAGATCAAGG-3′; reverse primer: 5′-TCCTGCAAGTTCACTGCTTG-3′; 23 cycles). These primer pairs were designed to cross intron–exon boundaries in order to avoid detecting any contaminating genomic DNA.

Plasmid construct

To construct the *Derrière* expression vector, the coding region was PCR-amplified from stage 10 cDNA using the primers: 5′-GGAATTCGTCAACATGGCAGAGTTGTG-3′ (in which an *Eco*RI site was added for subsequent cloning, shown in bold) and 5′-GACTCTG-GTGCATTCTGTAG-3′. The amplified fragment was subcloned into pGEM-T (Promega), excised by *Eco*RI and *Not*I digestion and sub-cloned into pBluescript RN3.

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