Mode of action of VegT in mesoderm and endoderm formation

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SUMMARY

mRNA encoding the T-box transcription factor VegT is located throughout the vegetal pole of the *Xenopus* egg and is believed to play an important part in endoderm and mesoderm formation. We find that VegT generates endoderm both by cell-autonomous action and by generating TGF-β family signals, the latter being entirely responsible for its mesoderm-inducing activity. Signalling molecules induced cell-autonomously by VegT include derrière, Xnr4 and activin B. Xnr1 and Xnr2 are also induced, but primarily in a non-autonomous manner. All of these signalling molecules are found in the blastula and gastrula vegetal pole and induce both endoderm and mesoderm in the animal cap assay, and hence are good candidates both for the endogenous zygotic mesoderm-inducing signal and for reinforcing the vegetal expression of endoderm markers.

Key words: VegT, Mesoderm induction, Endoderm, TGF-β, *Xenopus*

INTRODUCTION

The amphibian embryo develops into the three germ layers in a predictable fashion, the pigmented animal pole forming the ectoderm, the yolky vegetal pole forming the endoderm and the mesoderm developing from an annulus at the equator. Mesoderm formation is believed to require a TGF-β family signal originating from vegetal cells because a truncated dominant negative activin receptor, promiscuous in inhibiting TGF-β family receptors, inhibits mesoderm formation in the intact embryo (Kessler and Melton, 1994; Slack, 1994; Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994). The main candidate for this signal has been Vg1, since its mRNA is located throughout the vegetal pole of the *Xenopus* egg and is believed to play an important part in endoderm and mesoderm formation. However, the processing of the Vg1 precursor has not been detected in the egg or embryo, experiments with dominant negative Vg1 ligands suggested that Vg1 was essential for dorsal mesoderm formation (Joseph and Melton, 1998). Recently, however, the effects of ablating maternal stores of the T-box transcription factor variously known as Antipodean (Stennard et al., 1996), Xombi (Lustig et al., 1996), VegT (Zhang and King, 1996) and Brat (Horb and Thomsen, 1997) were described (Zhang et al., 1998). Late blastula vegetal masses depleted of VegT mRNA were unable to induce mesoderm in wild-type animal caps, suggesting that the mesoderm-inducing signal is predominantly zygotic, and is a target of VegT (although it is possible that a zygotic component is required for the processing of a maternal signal, or that a maternal signal acts earlier than the late blastula stage). The depleted embryos still form mesoderm, but its appearance is very considerably delayed – perhaps because there is a second slower mesoderm-inducing pathway, or because 5-10% of maternal VegT remains (Zhang et al., 1998).

What are the candidates for a zygotic mesoderm-inducing signal? The experiments of Zhang et al. (1998) imply that it is generated by VegT in the vegetal pole, but to date only one TGF-β family member, Derrière, has been shown to be inducible by VegT (Sun et al., 1999). Derrière is present in the vegetal pole, and induces mesoderm and endoderm in the animal cap assay. A dominant inhibitory version severely reduces trunk and tail mesoderm, but leaves heads relatively unaffected (Sun et al., 1999).

A cleaved version of Cerberus protein, Cerberus-short, has been shown to inhibit Xnr1 signalling, but not activin or Vg1, and injection of cer-S mRNA eliminates expression of the pan-mesodermal marker Xbra in whole embryos, implicating Xnr1 as a major component of the signal (Piccolo et al., 1999). Xnr1 has been shown to be transcribed in the blastula vegetal pole (Jones et al., 1995). This would imply a conserved role for nodal-related proteins, since Nodal is needed to make the node and primitive streak in the mouse (Conlon et al., 1994; Zhou et al., 1993) and a double nodal-related mutant in the zebrafish lacks most mesoderm and endodermal gene expression is disrupted (Feldman et al., 1998). However, the specificity of cer-S against other members of the Xnr family and its effect on Derrière have not yet been ascertained, and Hsu et al. (1998) reported that Cerberus also inhibits activin signalling. Finally, experiments with *Xenopus* follistatin were taken to indicate that activin is needed to form the ventral mesoderm (Marchant et al., 1998). However, this could be an effect of follistatin on BMP4 and it has also been reported that mammalian follistatin does not block the vegetal pole signal (Slack, 1991). Nevertheless, Dyson and Gurdon (1997) primarily detected
head defects with a specific dominant negative activin Type II receptor.

Thus although there are several candidates for the endogenous mesoderm inductor, there has been no conclusive identification of this signal.

The experiments of Zhang et al. (1997) also revealed a role for VegT in forming the endoderm. The ability of vegetal explants to express endoderm markers autonomously suggests that endodermal fate is specified by a maternally deposited factor (Gamer and Wright, 1995). VegT has been shown to induce endoderm markers in animal cap explants and, as well as losing the vegetal mesoderm-inducing signal in Nieuwkoop grafts, embryos depleted of maternal VegT fail to form endoderm. We originally set out to determine how VegT induces endoderm, expecting that it would do so in a cell-autonomous manner. We found that, once initiated, the maintenance of endoderm was dependent on a TGF-β signal, and that VegT generated a complex signal — comprising Xnr1, Xnr2, Xnr4, activin B and Derrière. All of these molecules are found in the blastula vegetal pole and we propose that together they constitute the zygotic mesoderm-inducing signal and also maintain vegetal expression of endoderm markers.

MATERIALS AND METHODS

Biological methods
Embryos were cultured and dissected by standard methods (Wilson et al., 1986). All RNA injections were performed bilaterally at the 2-cell stage, unless stated otherwise. For animal cap explants, injection was into the animal pole, for vegetal explants, injection was into the vegetal pole. Injections were performed in 3% Ficoll in 0.1× Barth’s saline and embryos cultured at 13°C, 18°C or 23°C. For dissection, embryos were transferred to 0.1× Barth’s saline and embryos cultured at 13°C, 18°C or 23°C. For dissection, embryos were transferred to 0.1× Barth’s saline at stage 8.5, and dissected fragments were cultured in 0.5× Barth’s medium supplemented with 1 mM CaCl₂ and 10 units/ml gentamycin.

For the disaggregation experiment, animal cap explants or whole embryos were dissociated in calcium- and magnesium-free Barth’s medium, and cultured at 23°C with frequent agitation. The outer layer of cells, which did not dissociate efficiently, was discarded.

RNA analysis
Total RNA was prepared from embryos and explants as described previously (Richardson et al., 1995; Hudson et al., 1997). Quantitative RT-PCR analysis of mRNAs was based on the method of Rupp and Weintraub (1991) as detailed in Hudson et al. (1997). All PCRs were carried out at 62°C.

Visualisation and quantitation were performed using a Molecular Dynamics phosphorimager with ImageQuant software.

Recloning of VegT cDNA
In order to improve the efficiency of translation of mRNA transcribed in vitro, the VegT coding region was recloned from PCS2-Brat (Horb and Thomsen, 1997) into pSPJC2L (Cook et al., 1993), removing 5′ and 3′ UTR sequences. To do this, the resulting PCR product was digested with HindIII sites. The resulting PCR product was cloned into the HindIII site of pSPJC2L, and sequenced using Expand High Fidelity polymerase (Boehringer) with 3′ and 5′ primers, which were tagged with HindIII sites. The resulting PCR product was digested with HindIII and cloned into the HindIII site of pSPJC2L to create pSPVegT.

In situ hybridisation
Albino embryos were injected in a A-tier single blastomere at the 32-cell stage with VegT and β-galactosidase mRNAs. The embryos were cultured to stage 10.5, then the membranes were manually removed and the embryos fixed for 30 minutes in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde). β-galactosidase expression was detected by incubation in β-gal buffer (0.1 M NaPi pH 7.2, 2 mM MgCl₂, 0.02% NP-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM EGTA) with 0.4 mg/ml X-gal at 37°C for a maximum of 2 hours. The embryos were then refixed in MEMFA at 4°C overnight and the embryos fixed for 30 minutes in MEMFA (0.1 M MOPS pH 7.2, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde). β-galactosidase expression was detected by incubation in β-gal buffer (0.1 M NaPi pH 7.2, 2 mM MgCl₂, 0.02% NP-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM EGTA) with 0.4 mg/ml X-gal at 37°C for a maximum of 2 hours. The embryos were then refixed in MEMFA at 4°C overnight and in situ hybridisation carried out essentially as described by Harland (1991).

For Xsox17β, antisense riboprobe in situ hybridisation carried out essentially as described by Harland (1991). The original probe was generated by EcoRI digestion of pBS-Xsox17β (Hudson et al., 1997), followed by in vitro transcription using T7 RNA polymerase and DIG DNA labelling mix (Boehringer).

Transcriptions for injection
All transcriptions were carried out using mMessage mMachine (Ambion). β-galactosidase transcripts were generated by XhoI digestion of SP6-nuc-Beta-gal (Smith and Harland, 1991) and transcribed with SP6 transcription. pSPVegT was linearised with XhoI and transcribed with SP6. Other expression clones were processed according to the authors’ instructions.

RESULTS
Non-cell-autonomous induction of mesoderm and endoderm by VegT
It has been demonstrated that VegT induces both mesoderm (Stennard et al., 1996; Lustig et al., 1996; Zhang and King, 1996) and endoderm (Horb and Thomsen, 1997) in animal cap.
explants. It could do this in a cell-autonomous way, acting directly as a transcription factor, since it has been shown to have an activation domain (Zhang and King, 1996); alternatively, or additionally, it might act non-cell autonomously by generating signalling molecules. To distinguish these possibilities, we examined the spatial configuration of gene expression induced by VegT when its mRNA was injected into single animal hemisphere blastomeres at the 32-cell stage and the resulting clone marked by co-injecting nuclear β-galactosidase mRNA (Smith and Harland, 1991). The embryos were fixed in early gastrulation (stage 10.5), stained for β-galactosidase and hybridised with either Xsox17β or Xbra (Fig. 1), which have been shown to be pan-endodermal and pan-mesodermal, respectively, at this stage (Hudson et al., 1997; Smith et al., 1991).

The marked clones are largely coherent at the gastrula stage. Typically Xsox17β is strongly expressed in the centre of the group of cells (Fig. 1A) and clearly there are cells expressing β-galactosidase that are not expressing detectable levels of Xsox17. This is not consistent with a strictly cell-autonomous induction by VegT, since in this case one would expect all cells to express, or not express, the marker. Either Xsox17 expression is produced by very intense signalling, the ligand only building up to high enough levels in the centre of the clone, or, alternatively, signalling reinforces a cell-autonomous effect (Fig. 1C). Xbra is expressed (Fig. 1B) around and outside the periphery of the clone. Since there are cells expressing Xbra which are not expressing β-galactosidase, mesoderm is also formed, at least in part, by signalling.

To support these findings, we investigated the effects of cell disaggregation on the VegT induction of endoderm and mesoderm markers, since this should disrupt cell-cell signalling (Fig. 2). In order to observe the effect of dissociation on the onset of expression, caps were harvested at late stage 8 to early stage 9, within 1 hour of MBT, and also at stage 10. Xsox17 upregulation is characteristic of endoderm induction (Hudson et al., 1997). Eomes is the earliest mesoderm marker, although some expression may also be in anterior endoderm (Ryan et al., 1996), while Mix.1 is reported to be in both nascent mesoderm and endoderm at this stage (Rosa, 1989; Lemaire et al., 1998). mRNA encoding the TGF-β signalling molecule BVg1 was used as a control for the efficacy of disaggregation.

We found that BVg1 induces Xsox17, Mix.1 and Eomes at both stages, and these inductions are suppressed by disaggregation, as expected. In contrast, VegT induces Xsox17 and Mix.1, but not Eomes at stage 8/9. The induction of Xsox17 and Mix.1 is not suppressed, but rather is slightly increased by disaggregation, supporting a cell-autonomous induction of

![Fig. 1. Relationship between domains of induced Xbra and Xsox17 expression. Albino embryos were injected with 50 pg VegT and 200 pg β-galactosidase mRNA into a single A tier blastomere at the 32-cell stage. The embryos were stained for β-galactosidase activity at stage 10.5, then induced Xbra or Xsox17 expression was detected by in situ hybridisation. (A) Xsox17 expression (purple/brown stain) is seen in the centre of a cluster of β-galactosidase-expressing cells (blue nuclear stain). (B) The expression of Xbra (purple/brown) extends beyond the cells expressing β-galactosidase, but is absent from some β-galactosidase-expressing cells, particularly those in the centre of the clone of labelled cells. (C) Model for the action of injected VegT in an animal hemisphere clone.](image)

![Fig. 2. Effect of cell disaggregation on the induction of endoderm and mesoderm markers by VegT. Embryos were injected at the 2-cell stage with 100 pg of VegT RNA or 25 pg of BVg1 RNA. Animal caps were then dissected at stage 6.5-7 and either disaggregated in Ca²⁺/Mg²⁺-free medium or maintained intact in culture. Cells/explants were harvested at late stage 8/early stage 9 and stage 10 for analysis of markers by RT-PCR. BVg1 is a control for the efficacy of the disaggregation, since one would expect all inductions by BVg1 to be disrupted by disaggregation. At stage 8/9, the induction of Mix.1 and Xsox17 by VegT is increased slightly in the disaggregated samples relative to the intact caps, whereas by stage 10 disaggregation reduces expression levels. Note that control levels of Xsox17 in animal caps are normally relatively high at these early stages. Eomes is not induced by VegT at stage 8/9 and it is not induced in disaggregated caps at stage 10, although it is induced in intact caps at this stage.](image)
endoderm by VegT at this stage. By stage 10 VegT induces Eomes, as well as Xsox17 and Mix.1. These inductions are sensitive to disaggregation – Xsox17 and Mix.1 are strongly downregulated in dissociated caps, while Eomes is reduced to control level. Note that there is often a low level of expression of Xsox17 and Eomes in control caps – this is a natural phenomenon and is not a consequence of mesodermal or endodermal contamination.

These data support a model in which endoderm (as defined by Xsox17 and Mix.1 expression) is induced by VegT in a cell-autonomous manner from MBT. However, this induction must rapidly be reinforced by signalling for expression to be autonomous manner from MBT. However, this induction must be reinforced by signalling for expression to be maintained. Mesoderm induction, i.e. Eomes expression, is delayed relative to its induction by BVg1, and also relative to its appearance in the whole embryo, this is discussed further below. Eomes is induced by VegT at stage 10, but this induction is wholly sensitive to disaggregation, and so is a consequence of cell-cell signalling initiated by VegT.

**VegT generates a TGF-β family signal**

So far we have shown that signalling is involved in VegT induction of both mesoderm and endoderm. We tested if VegT induced endoderm and mesoderm via a TGF-β family signal. To do this, we used the dominant negative truncated activin receptor tAR (Hemmati-Brivanlou and Melton, 1992; also called Δ1XAR1), which has wide specificity against TGF-β family signals, including activin, BMP and Vg1 (Kessler and Melton, 1994; Schulte-Merker et al., 1994). It also blocks Xnr1, Xnr2 and Xnr4 (data not shown).

The induction of most mesodermal markers is efficiently inhibited by tAR at all VegT concentrations (Fig. 3). As well as pan-mesodermal markers, like early Xbra and Eomes, this includes the dorsal marker Xnot and the ventral marker Xvent1 (not shown). An exception is the signalling molecule Xwnt8, which is expressed in the ventrolateral mesoderm of gastrulae.

However, Xwnt8 is also expressed in the vegetal cells (Lemaire and Gurdon 1994), so it could also be classed as an endodermal molecule. These data are consistent with the data from the disaggregation experiment, in that mesoderm induction is totally dependent on a VegT-generated TGF-β signal.

The induction of endoderm markers is more complex. Xsox17 expression is variably reduced by tAR, implying some dependence on a TGF-β signal, and there may in addition be cell-autonomous effects, or signals that are not blocked by tAR. Mix.1, however, is completely sensitive to tAR, whereas Xwnt8, as noted above, is insensitive.

We conclude that maternal VegT has the potential to induce mesoderm in the marginal zone by generating a TGF-β family signal. Indeed, VegT induction of mesoderm is entirely dependent on this signal. In the vegetal pole, where VegT mRNA is naturally located, it may induce endoderm formation by a combination of signalling and cell-autonomous action.

**The nature of the VegT signal**

To identify the signal induced by VegT, we first asked which of the known secreted mesoderm inducers it generated when expressed in animal caps; derrière has already been identified as a target of VegT (Sun et al., 1999).

Figs 3 and 4A show that VegT induces animal caps to express several mesoderm-inducing signalling molecules, specifically derrière, Xnr1, Xnr2, Xnr4, Activin B and eFGF. Activin A and Vg1 were not induced above control levels by gastrula stages, and we also examined BMP4, but control caps contain high levels of BMP4 that are not altered by VegT (not shown). Xwnt8 is induced (Fig. 3), but Wnts do not induce mesoderm. eFGF is induced at all VegT concentrations tested (Fig. 3). Here we focus on the TGF-β family, since the induction of mesoderm by VegT is sensitive to tAR.

Analysis of the time course over which these signalling molecules are induced suggests that Xnr4 and Activin B are good candidates for a primary signal, since their induction is rapid, coincident with that of Xsox17, and precedes that of the mesoderm markers Xbra and Eomes (Fig. 4A). However, Xnr1 and Xnr2 show a more gradual accumulation from stage 9. derrière shows the same gradual accumulation, but this PCR was carried out for 25 cycles rather than 27 or 29 in order to keep within the linear range, and thus the level of derrière at stage 9 is actually much higher than that of the other markers.

In drawing several of the conclusions outlined above, it is assumed that the amounts compared for a particular marker fell within the linear range of the RT-PCR; this was ensured in each experiment for the samples compared. There is also implicit in our arguments a comparison of different markers and it is essential that there should not be great disparities in the RT-PCR amplification efficiency for different transcripts. In fact we find that the absolute amplification of different transcripts is remarkably similar, when the cycle number was taken into account. Table 1 shows a quantification of certain of the data in Fig. 4A with respect to Xnr1, Xnr2, Xnr4, derrière, activin B and VegT. These data bear out the slowness of induction of Xnr1 and Xnr2 by VegT and the rapidity of that of derrière, activin B and Xnr4.

The amounts of each signal transcript, except derrière, induced in a cap by VegT are small. However, 50 fg of activin B transcript will induce Xbra (not shown), whereas at least 10 pg of derrière mRNA are required (Sun et al., 1999), even...
though the transcript from this construct is efficiently translated in oocytes over a 24 hour period (not shown). Thus the abundance of the derrière transcript in these caps (and also in whole embryos, see later) is not necessarily a reflection of its importance in vivo, since its activity in the animal cap assay is apparently lower than that of activin B and the Xnrs.

Could these molecules account for the properties of the VegT-derived signal, i.e. induction of mesoderm and maintenance of endoderm? All have already been shown to induce mesoderm in the animal cap assay; Derrière, activin and Xnr1 have been shown also to induce endoderm markers (Sun et al., 1999; Henry et al., 1996; Piccolo et al., 1999), but this has not been tested for Xnr2 and Xnr4.

We analysed the induction of Xsox17β and Xbra over a concentration range of the inducer (Fig. 4B). We find that, although their activities vary, they all induce mesoderm and endoderm to some extent, with endoderm generally induced at higher injected doses of mRNA than mesoderm. We also find that Xnr4 is a relatively weak inducer of the endodermal marker Xsox17 over all concentration ranges tested, despite being a potent inducer of the mesodermal marker Xbra.

Potentially there would seem to be a high degree of redundancy in a signal composed of Xnr1, Xnr2, Xnr4, Derrière and activin B, since they appear to have overlapping activities. This may be the situation in vivo, or other factors such as distribution within the vegetal pole, or diffusibility – may determine whether one molecule predominates as the functional mesoderm-inducing signal, or in the maintainance of endoderm.

**VegT initiates a signalling relay**

In order to determine whether the induction of any of these signalling molecules is a secondary effect of a primary signal, we analysed their expression in intact and disaggregated animal cap explants expressing VegT (Fig. 5). The induction of Xnr4, derrière and activin B is weak in intact caps at the earlier stage, but Xnr4, activin B and derrière are strongly induced in disaggregated cells at this stage. This upregulation is also seen at stage 10, relative to intact caps. At this later stage, Xnr1 and Xnr2 are also induced in intact caps; but not in the disaggregated samples.

**Fig. 5.** Effect of disaggregation on the induction of signalling molecules in VegT-expressing animal caps. Embryos were injected at the 2-cell stage with 100 pg of VegT RNA or 25 pg of BVg1 RNA. Animal caps were dissected at stage 6.5-7 and either disaggregated in Ca²⁺/Mg²⁺-free medium or maintained intact in culture. Cells/explants were harvested at stages 8/9 and 10 for analysis of markers by RT-PCR. Inductions by VegT are weak in intact caps at the earlier stage, but Xnr4, activin B and derrière are strongly induced in disaggregated cells at this stage. This upregulation is also seen at stage 10, relative to intact caps. At this later stage, Xnr1 and Xnr2 are also induced in intact caps; but not in the disaggregated samples.
induction is resistant to disaggregation. Both are induced at stage 10 and both inductions are sensitive to disaggregation.

We conclude that the mesoderm-inducing signal produced by VegT is complex, the primary signal comprising mainly Xnr4, Derrière and activin B. Later Xnr1 and Xnr2 are induced by this signal, and constitute part of a secondary signal, or relay, although Xnr2 may also be induced cell-autonomously to some degree. Xnr1 and 2 have been shown to be inducible by activin (Jones et al., 1995), which is consistent with this model.

Properties of the vegetal pole

From the results with injected animal cap explants, we predict that, in normal embryos, VegT could induce endoderm in the vegetal pole directly or by signalling, this signalling also forming the basis of a vegetal mesoderm-inducing signal. Does this accord with the properties of vegetal poles?

We examined whether the signalling molecules induced, directly or indirectly, by VegT are present in vegetal poles. Published reports on mRNA distributions based on whole-mount in situ hybridisation using standard protocols often miss vegetal expression, and this seems to be true of some of the signalling molecules that we have studied. Fig. 6A shows that Xnr1, Xnr2, Xnr4, derrière and activin B are all expressed in the blastula vegetal pole. This has been demonstrated previously for derrière (Sun et al., 1999) and activin B (Dohrmann et al., 1993), has only been shown at stage 9 for Xnr1 and Xnr2 (Jones et al., 1995), and not at all for Xnr4, although it has been demonstrated to be present in the embryo at this stage (Joseph and Melton, 1997). eFGF is not expressed in the vegetal pole until the gastrula stage, which is too late to be relevant to initiation of endoderm and mesoderm formation, and levels are low compared to equatorial regions. Activin B is not specific to the vegetal pole until gastrula, although earlier it is present there at comparable levels.

Levels of these transcripts can be calculated by reference to known standards. There are approximately 2 pg of derrière transcript in a whole embryo at stage 9, compared to 10 fg of activin B; the Xnrs are all present at the 100-500 fg level.

We conclude that any of these signals could be generated in whole or in part by VegT in the vegetal pole, but agents other than VegT are clearly responsible for the wider distribution of activin at earlier stages and the absence of eFGF from the vegetal pole. The eFGF promoter has been shown to contain a T-box half site (Casey et al., 1998), which may account for its induction by VegT in animal cap explants, but in vivo it may predominantly be an Xbra target in the equatorial zone and suppressed vegetally.

Our experiments with animal cap explants suggest that expression of Xnr4, activin B, derrière and to some extent Xnr2 is induced in a cell-autonomous way by VegT, Xnr1 and the
greater part of Xnr2 expression appear to be induced in a signal-dependent manner. Does this correlate with the onset of endogenous expression of these markers in vegetal poles?

Vegetal pole explants were dissected at stage 8, disaggregated and then harvested immediately or at stage 9, after the onset of zygotic transcription. Our aim was to determine whether the initiation of expression of these genes required signalling. Xnr4 expression is upregulated in the disaggregated samples (as is seen for its induction by VegT in animal cap explants), whereas all other markers, with the exception of Xsox17 (see below), are reduced. Least affected is activin B, while Xnr1 is most strongly inhibited (Fig. 6B). This is broadly in agreement with our previous observations, in that Xnr4 and activin B are induced predominantly cell-autonomously by VegT, while Xnr1 requires signalling. The expression of derrière and Xnr2 is partially sensitive to disaggregation, suggesting that signalling accounts for a proportion of the expression of these molecules even at this early stage.

Shortly after MBT the expression of Xsox17β is unaffected by disaggregation, supporting an initial cell-autonomous induction (Fig. 6B). However, during gastrula stages, the transcript levels diminish in disaggregated cells, but continue to increase in intact embryos (Fig. 6C). This supports the hypothesis that the expression of Xsox17 rapidly becomes dependent on cell-cell signalling. Eomes, however, is not present in disaggregated vegetal cells at stage 9, or in disaggregated whole embryos during gastrula stages, suggesting that the onset of Eomes transcription is signal dependent. This is consistent with our observation that the induction of Eomes by VegT in animal cap explants is sensitive to dissociation (Fig. 2), as well as to inhibition of TGF-β class signalling (Fig. 3).

In animal caps, Mix.1 appears to be initially induced by VegT in a cell-autonomous manner (Fig. 2). However, its endogenous expression is wholly sensitive to disaggregation from early stages (Fig. 6B,C). This result may be explained by the observation that the induction of Mix.1 by VegT in animal cap explants is more sensitive to inhibition of TGF-β signalling by tAR than is the induction of Xsox17 (Fig. 3), implying a greater dependency on cell-cell signalling.

**DISCUSSION**

**VegT generates a signal**

Our results support those of others that VegT induces both mesoderm (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) and endoderm (Horb and Thomsen, 1997). VegT also induces animal cap cells to produce signalling molecules of all families tested. Thus Xwnt8 is induced, as is eFGF. Derrière has already been shown to be induced by VegT (Sun et al., 1999). In the activin signalling group, we checked for induction of Xenopus activin A and activin B. Only activin B was induced at blastula or gastrula stages. Lastly, VegT induces the nodal-related genes Xnr1, Xnr2 and Xnr4 (but not Xnr3 – not shown).

We find that VegT induces mesoderm, defined by a variety of ubiquitous, dorsal and ventral markers, exclusively via a signal of the TGF-β family. This is consistent with the observation that VegT-depleted vegetal poles are incapable of inducing mesoderm in a Nieuwkoop graft at the blastula stage (Zhang et al., 1998). Endoderm induction, defined by the early genes Xsox17α and Xsox17β, is more complex. Initiation of expression appears to occur cell-autonomously. In contrast, a little later a proportion of the endoderm seems to depend on TGF-β family signalling. This suggests that the initial induction is soon reinforced by signalling, and such reinforcement may be relevant to the fact that endoderm cells are not irreversibly determined until gastrulation (Wylie et al., 1987), well after the time that Xsox17 expression is initiated.

The pattern of mesoderm and endoderm induction when VegT is expressed in a small domain of the animal pole fits the model just developed. The centre of a clone of VegT-injected cells expresses endoderm markers, and mesoderm is at or outside the periphery of the clone. This is consistent with VegT generating a signal that induces and maintains endoderm at high levels and mesoderm at lower levels, as has been previously demonstrated for activin, Xnr1 and Derrière, but which we show is also true for Xnr2 and, to some extent, Xnr4. Working out exactly what is happening spatially demands that we have more information on the diffusibility of these signalling molecules.

**Role of VegT in patterning the embryo**

It has been shown that ablation of 90-95% of maternal VegT expression dramatically shifted mesoderm formation from the equator to the vegetal pole and delayed its occurrence until the equivalent of late gastrula stages (Zhang et al., 1998). Gastrulation then occurred at the vegetal pole, instead of the equator. Endodermal markers were absent from these embryos. There must be a little caution in interpreting this result because the markers were not measured until tadpole stages and so these experiments did not distinguish between a role for VegT in the initiation of endoderm, or in its maintenance.

Zhang et al. (1998) interpreted their results to mean that VegT is a key molecule in both endoderm and mesoderm formation. The late appearance of mesoderm might be because there is another inducer at the vegetal pole, strong enough on its own only to make mesoderm, or because the residual 5-10% VegT is only enough to make mesoderm, and perhaps it does it more slowly than normal. Three models of VegT action were proposed by Zhang et al. (1998): (i) the pattern of the Xenopus gastrula depends either on a gradient of VegT, producing endoderm at high concentration and mesoderm at low concentrations; (ii) VegT makes the endoderm at the vegetal pole and generates a signal that forms mesoderm at the equator; (iii) VegT is present in both mesoderm and endoderm-
forming regions and co-factors are invoked to explain the development of either endoderm or ectoderm. Our data fit model (ii), with the addition that autocrine signalling reinforces any cell-autonomous action of VegT in forming endoderm.

All the TGF-βs we have shown to be induced by VegT in animal caps are capable of inducing mesoderm and endoderm, and thus of contributing to the endogenous mesoderm-inducing signal and maintaining endodermal fate. All are present in the blastula and gastrula vegetal pole. Disaggregation of vegetal pole explants strongly reduces Xnr1 expression, consistent with our model in which it is not induced in a cell-autonomous way by VegT. Xnr4 expression is not reduced by disaggregation, and so appears to be induced in a signal-independent manner, as we would predict. Endogenous Xnr2, activin B and derrière expression shows some sensitivity to disaggregation, but a proportion of expression is resistant. This conforms with our model in which they are induced cell-autonomously by VegT, but in vegetal poles the endogenous expression may also be maintained by a VegT-initiated signalling relay, or by an independent signal, such as Vg1. The behaviour of downstream molecules such as Eomes and Xsox17 is also consistent with our model in which their expression is dependent on cell-cell signalling.

Although derrière is the most abundant transcript in vegetal poles, the relative importance of each signal in vivo will depend on several factors – the amount of protein present, its distribution within the vegetal hemisphere, its activity and its diffusibility. The potential complexity of the signal, and the potential degree of redundancy, may explain why signalling inhibitors have failed to reveal a single candidate for it, and currently further progress with inhibitors is limited by their specificity.

It is notable that, in the animal cap assay, the induction of early mesodermal markers (Eomes, Xbra) by VegT is distinctly slower than expected from their natural appearance in the embryo. It is possible that this is a consequence of the use of the animal cap as a model system – disaggregation of animal caps increases the induction of a number of markers by VegT, whereas the same markers do not show the same dramatic upregulation in disaggregated vegetal poles (Xnr4 levels do show a slight increase in disaggregated vegetal explants). Although VegT will induce these markers in intact animal caps, the use of this system may lead to an underestimation of its activity. However, BVg1 induces markers on schedule in the cap, this includes Xnr1, which is present in a stage 8.5-9 embryo, but is not induced by VegT, even in disaggregated animal caps, at this stage. In the experiments of Zhang et al. (1998), the rescue of ablated maternal VegT with injected VegT mRNA also led to delayed appearance of Xbra and Xwnt8, as we would predict (see Zhang et al., 1998, Fig. 5).

These observations suggest that there may be a parallel pathway producing early mesodermal induction in the embryo. In fact, our results best fit the model proposed by Kimelman and Griffin (1998) in which there is an early weak mesoderm-inducing signal at the vegetal pole, in addition to the signal generated by VegT. This has been suggested by grafting experiments (Jones and Woodland, 1987) and also by studies of the fate of cells just above the equator in the Xenopus morula (Ding et al., 1998). Zhang et al. (1998) reported that there was no mesoderm-inducing signal in VegT-depleted late blastulae, but possibly the weak signal is absent by this stage. A good candidate for this weak signal is the TGF-β family member Vg1. It has recently been shown that specific dominant negative variants of Vg1 inhibit dorsal mesoderm development and cause failure of at least one late endoderm marker to appear (Josepah and Melton, 1998). This suggests that Vg1 has an indispensable role in forming the dorsal mesoderm (although it is now necessary to determine the specificity of the Vg1 inhibitors against all Xnr types and Derrière).

Overall the working hypothesis that we propose, to synthesise the results of others with ours is as follows (Fig. 7). The vegetal pole develops into endoderm as a result of VegT action, possibly synergising with Vg1. It does this directly through cell-autonomous effects reinforced by TGF-β class signals, including Xnr1, Xnr2, Xnr4, Derrière and activin B, all of which are expressed in the vegetal pole and are able to induced endoderm in the animal cap assay. VegT also generates a complex signal that is reinforced by secondary signals; these diffuse into the equatorial region generally and produce an annulus of mesoderm. This signal would be reinforced, particularly dorsally, by a maternal TGF-β (possibly Vg1), generating the asymmetrically appearing blastopore lip. There may also be restriction of the effect of the mesodermal signal by inhibitors in the animal cap, and this fits our data that the VegT induction of early targets is markedly increased by cell disaggregation.

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