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Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFβ growth factors

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SUMMARY

The maternal transcription factor VegT is important for establishing the primary germ layers in *Xenopus*. In previous work, we showed that the vegetal masses of embryos lacking maternal VegT do not produce mesoderm-inducing signals and that mesoderm formation in these embryos occurred ectopically, from the vegetal area rather than the equatorial zone of the blastula. Here we have increased the efficiency of the depletion of maternal VegT mRNA and have studied the effects on mesoderm formation. We find that maternal VegT is required for the formation of 90% of mesodermal tissue, as measured by the expression of mesodermal markers *MyoD*, *cardiac actin*, *Xbra*, *Xwnt8* and *alphaT4 globin*. Furthermore, the transcription of FGFs and TGFβs, *Xnr1, Xnr2, Xnr4* and *derrière* does not occur in VegT-depleted embryos. We test whether these growth factors may be endogenous factors in mesoderm induction, by studying their ability to rescue the phenotype of VegT-depleted embryos, when their expression is restricted to the vegetal mass. We find that *Xnr1, Xnr2, Xnr4* and *derrière* mRNA all rescue mesoderm formation, as well as the formation of blastopores and the wild-type body axis. *Derrière* rescues trunk and tail while *nr1*, *nr2* and *nr4* rescue head, trunk and tail. We conclude that mesoderm induction in *Xenopus* depends on a maternal transcription factor regulating these zygotic growth factors.

Key words: Mesoderm, *Xenopus*, VegT, Induction, Growth factor

INTRODUCTION

The mechanism of mesoderm formation has been more closely studied in the *Xenopus laevis* embryo than in any other vertebrate. The morphological event establishing mesodermal tissue is the process of gastrulation, but the molecular determination of mesoderm begins earlier in development. Explants of the marginal zone dissected from blastula stage embryos differentiate into mesodermal derivatives (Nakamura et al., 1970), and cell transplantation experiments indicate that groups of cells are determined to form mesoderm by the early gastrula stage (Kato and Gurdon, 1993). Explant recombination experiments suggest that mesoderm forms in the marginal zone in response to inductive signals passing from adjacent vegetal cells (Boterenbrood and Nieuwkoop, 1973; Nieuwkoop, 1969). However, there is also evidence that equatorial cells may be specified autonomously to form mesoderm, without requiring inducing signals (Gimlich, 1986; Sakai, 1996). Several members of the FGF and TGFβ family of growth factors are expressed in early *Xenopus* embryos, and have been implicated in mesoderm formation. *eFGF*, *bFGF*, *activin B*, *Xnr1, Xnr2, Xnr4*, *derrière* and *bVg1* can all act as mesoderm inducers (reviewed in Harland and Gerhart, 1997; Slack, 1994), in that their expression in animal caps causes animal cells to adopt mesodermal fates. Furthermore, the expression of dominant negative forms of the receptors for FGF or activin disrupt gastrulation and inhibit mesoderm formation (Amaya et al., 1991, 1993; Dyson and Gurdon, 1996; Hemmati-Brivanlou and Melton, 1992) and dominant inhibitory forms of derrière (Sun et al., 1999), Vg1 (Joseph and Melton, 1998), and Xnr2 (Osada and Wright, 1999) also affect gastrulation and both mesodermal and endodermal development. However, the relative importance of each of these candidate growth factors in mesoderm formation and the location of their activity remains to be established.

It has been generally assumed that mesoderm-inducing signals are maternal growth factors, regulating mesodermal specification before the onset of zygotic transcription. This view has been challenged, however, by two recent observations. Firstly, heterochronic Nieuwkoop recombination experiments indicate that vegetal cells are competent to induce mesodermal tissue only after the mid-blastula transition, not before zygotic

transcription starts (Wylie et al., 1996; Yasuo and Lemaire, 1999). Secondly, loss-of-function experiments have identified the maternal transcription factor VegT as a key initiator of mesoderm induction and endoderm formation (Zhang et al., 1998). Since VegT has been shown to act as a transcriptional activator (Zhang and King, 1996), it is likely to regulate the expression of zygotic genes, including growth factors. Overexpression experiments and dominant negative studies also show the likely involvement of VegT in blastopore formation and mesodermal patterning (Horb and Thomsen, 1996; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996).

In this paper, we aimed to establish whether this latter view is correct. In previous work, we showed that depletion of maternal *VegT* mRNA from *Xenopus* embryos affected mesoderm formation. Mesodermal markers were delayed in their expression, mesodermal differentiation occurred ectopically, in cells derived from the vegetal mass of the blastula rather than from the equatorial origin, and the vegetal cells lost their ability to produce mesoderm-inducing signals as measured by Nieuwkoop assays (Zhang and King, 1996). Here we have asked, firstly, to what extent maternal VegT is essential for mesoderm formation. Secondly, we establish which of the candidate growth factors are downstream of VegT, by studying the initiation of their expression in VegT-depleted embryos. Although mesoderm formation may be the result of both cell autonomous and inducing activity, we chose to concentrate on the latter aspect, asking the question 'which growth factors downstream of VegT are responsible for mesoderm induction by the vegetal mass?' We test this by injecting the candidate mRNAs into vegetal cells of the 32-cell stage VegT-depleted embryos, such that they are expressed only in the vegetal mass at the blastula stage. We then assess their ability to rescue the VegT-depleted phenotype and to rescue mesoderm formation. We find that maternal VegT is responsible for the formation of 90% of mesodermal tissue as measured by the expression of *MyoD*, *cardiac actin*, *Xbra*, *Xwnt8* and *alphaT4 globin*. *eFGF*, *FGF3*, *FGF8*, and the TGFβs, *Xnr1, Xnr2, Xnr4* and *derrière*, are directly or indirectly downstream of VegT, while *BMP4*, *BMP7*, *Xnr3* and *activin B* are not. *Xnr1* has potential VegT-binding sites in its promoter, and VegT activates the expression of an *Xnr1* promoter-Luciferase construct, suggesting it is directly regulated by VegT. mRNAs for *Derrière*, *Xnr1*, *Xnr2* and *Xnr4* rescue blastopore formation and mesodermal tissue. *Derrière* rescues trunk and tail formation, while *Xnr1*, *Xnr2* and *Xnr4* rescue head, trunks and tails. Furthermore, *Xnr1* expression rescues the mesoderm-inducing activity of VegT-depleted vegetal masses. This establishes that mesoderm formation in *Xenopus* depends on zygotic rather than maternal growth factors, and that mesoderm induction involves the TGFβ growth factors, *Xnr1, Xnr2, Xnr4* and *derrière*.

MATERIALS AND METHODS

Oocytes and embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium, as described in Zuck et al. (1998). Oocytes were injected at the vegetal pole with oligo using a Medical Systems picoinjector, in oocyte culture medium (OCM) and cultured a total of 24-48 hours 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 12 hours. Oocytes were then labelled with vital dyes and fertilized using the host-transfer technique described previously (Zuck et al., 1998). 3 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 coloured, 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 1% Ficoll in 0.5× MMR at the 16-cell stage. mRNAs were 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 antisense oligodeoxynucleotide (oligo) used was an 18 mer: C*A*G*CAGCATGTACTT*G*G*C where * indicates a phosphorothioate bond and was HPLC purified before use (Genosys/Sigma). Oligos were resuspended in sterile, filtered water and injected in doses of 5-8 ng per oocyte, and cultured immediately at 18°C. Capped RNAs were synthesized using the mMessage mMachine kit (Ambion), ethanol precipitated and resuspended in sterile distilled water for injection. Isolation of the *Xnr1* genomic DNA, construction of Pro-Luc, and methods for luciferase assays are described elsewhere (S.-I. Osada et al., unpublished data).

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 probe was synthesized by random priming of the excised insert of *VegT* (*Eco*RI). Blots were stripped and rehybridized with a probe for *plakoglobin* as a loading control.

Analysis of gene expression using real time RT-PCR

Total RNA was prepared from oocytes and embryos using the proteinase K method and treated with RNase-free DNase 1 (10 μg/μl Boehringer Mannheim) prior to cDNA synthesis. cDNA was synthesized from 0.5 to 1.0 μg RNA according to Zhang et al. (1998) in a volume of 20 μl. After reverse transcription, 1 μl 0.5 M EDTA, 30 μl H_2O, 1 μl glycogen (20 μg/μl) and 10 μl 5 M ammonium acetate, were added to each RT-reaction. Each sample was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated overnight at −20°C with 2.5 volumes 100% EtOH. Samples were centrifuged at 4°C, 16,000 ***g*** for 15 minutes, washed with 70% EtOH, dried in a speedvac and resuspended in 150 μl H_2O per 1/6th embryo equivalent of RNA used for cDNA synthesis.

RT-PCR was carried out using a LightCycler™ System (Roche), which allows amplification and detection (by fluorescence) in the same tube, using a kinetic approach. LightCycler PCR reactions were set up in microcapillary tubes using 5 μl cDNA with 5 μl of a 2× SYBR Green I (Roche Molecular Biochemicals, Wittwer et al., 1997) master mix containing upstream and downstream PCR primers, $MgCl_2$ and SYBR Green. The final concentrations of the reaction components were 1.0 μM each primer, 2.5 μM $MgCl_2$, and 1× SYBR Green master mix. The primers used and cycling conditions are listed in Table 1. In order to compare expression levels of depleted and rescued embryos relative to controls, a dilution series of uninjected control cDNA was made and assayed in each LightCycler run. Undiluted control cDNA = 100%, 1:1 cDNA: H_2O = 50% and 1:10 cDNA: H_2O = 10% (shown only in Fig. 2). In experiments where multiple embryonic stages were examined, the dilution series was used from cDNA of the uninjected control stage of development predicted to give the highest expression of the gene product being amplified. These values were entered as concentration standards in the LightCycler sample input screen. Other controls included in each run were −RT and water blanks. These were negative in all cases but not included in the figures for lack of space.

After each elongation phase, the fluorescence of SYBR green (a

Table 1. Primers used and cycling conditions for their use with LightCycler™ real-time PCR

PCR primer pair		Sequence	Melting temp. °C	Annealing temp. °C/ time (sec)	Extension temp. °C/ time (sec)	Acquisition temp. °C/ time (sec)
Activin B	new	U: 5-CAA CCT GTG GCT GTA CCT GAA G-3 D: 5-GCA CTC GAG GCC TCT CTT ACG GA-	95	55/5	72/15	88/3
3BMP4	Fainsod et al., 1994	U: 5-GCA TGT AAG GAT AAG TCG ATC-3 D: 5-GAT CTC AGA CTC AAC GGC AC-3	95	56/10	72/17	83/3
BMP7	new	U: 5-GGA TGG CTG ACG TTT GAT-3 D: 5-GCT CTT TCC TGA TTC CAG-3	95	57/5	72/12	81/3
Chordin	XMMR	U: 5-AAC TGC CAG GAC TGG ATG GT-3 D: 5-GGC AGG ATT TAG AGT TGC TTC-3	95	55/5	72/12	81/3
Cardiac actin	Rupp and Weintraub, 1991	U: 5-TCC CTG TAC GCT TCT GGT CGT A-3 D: 5-TCT CAA AGT CCA AAG CCA CAT A-3	95	55/5	72/12	83/3
Derrière	Sun et al., 1999	U: 5-TGG CAG AGT TGT GGC TAT CA-3 D: 5-CTA TGG CTG CTA TGG TTC CTT-3	95	55/5	72/18	82/3
eFGF	Casey et al., 1998	U: 5-CTT TCT TTC CAG AGA AAC GAC ACC G-3 D: 5-AAC TCA CGA CTC CAA CTT CCA CTG-3	95	60/5	72/12	83/3
FGF3	new	U: 5-GTC ATT TGT TTC CAG ACT TC-3 D: 5-TAT CTG TAG GTG GTA CTT AG-3	95	55/5	72/12	85/3
FGF8	new	U: 5-CTG GTG ACC GAC CAA CTA AG-3 D: 5-ACC AGC CTT CGT ACT TGA CA-3	95	55/5	72/14	86/3
MyoD	Rupp and Weintraub, 1991	U: 5-AGC TCC AAC TGC TCC GAC GGC ATG AA-3 D: 5-AGG AGA GAA TCC AGT TGA TGG AAA CA-3	95	55/5	72/18	86/3
Plakoglobin	new	U: 5-GCT CGC TGT ACA ACC AGC ATT C-3 D: 5-GTA GTT CCT CAT GAT CTG AAC C-3	95	60/10	72/16	85/3
VegT	Zhang et al., 1998	U: 5-CAA GTA AAT GTG AGA AAC CGT G-3 D: 5-CAA ATA CAC ACA CAT TTC CCG A-3	95	55/5	72/18	82/3
Xbra	Sun et al., 1999	U: 5-TTC TGA AGG TGA GCA TGT CG-3 D: 5-GTT TGA CTT TGC TAA AAG AGA CAG G-3	95	55/5	72/8	75/3
Xnr1	new	U: 5-TGG CCA GAT AGA GTA GAG-3 D: 5-TCC AAC GGT TCT CAC TTT-3	95	55/5	72/12	81/3
Xnr2	new	U: 5-GTC TTC TAT ATC CAG CAG CAA T-3 D: 5-TTG ATG GAG ATA ATA CTG GAG C-3	95	55/5	72/11	81/3
Xnr3	new	U: 5-CCA TGT GAG CAC CGT TCC-3 D: 5-GAG CAA ACT CTT AAT GTA G-3	95	57/5	72/10	82/3
Xnr4	Sun et al., 1999	U: 5-ACT TGG CTG CTC TAC CTC-3 D: 5-CAG CAA GTT GAT GTT CTT CC-3	95	55/5	72/12	82/3
Xwnt8	Ding et al., 1998	U: 5-CTG ATG CCT TCA GTT CTG TGG-3 D: 5-CTA CCT GTT TGC ATT GCT CGC-3	95	58/6	72/14	85/3
alphaT4 Globin	new	U: 5-AGC TGC CAA GCA CAT CGA-3 D: 5-GTG AGC TGT CCT TGC TGA-3	95	56/5	72/12	86/3

dye that binds double-stranded DNA giving a fluorescent signal proportional to the DNA concentration) was measured at a temperature 1°C below the determined melting point for the PCR product being analyzed. This excluded primer-dimers, which melt at a lower temperature, from the measurement. The fluorescence level is thus quantitated in real-time, allowing the detection and display of the log-linear phase of amplification as it happens. LightCycler quantification software v1.2 was used to compare amplification in experimental samples during the log-linear phase to the standard curve from the dilution series of control cDNA. The comparisons are displayed as histograms (Figs 2, 3, 7, 9 and 10).

For each primer pair used, we optimized conditions so that melting curve analysis showed a single melting peak after amplification, indicating a specific product. Some published primers used for radioactive PCR always gave multiple peaks in all conditions tried and so were not used.

Fixation and histology

For X-gal staining, embryos were fixed in MEMFA for 2 hours, rinsed in PBS and stained using X-gal (Hemmati-Brivanlou and Harland, 1989). Embryos were washed in PBS after staining and photographed before clearing with Murray's clear (2:1 butyl alcohol and butyl butyrate).

Explant culture

Mid-blastula stage uninjected, VegT-depleted and VegT-depleted/Xnr1 mRNA-injected embryos were devitellined and dissected on agar-coated dishes in 1× MMR, into animal, equatorial and vegetal segments. The VegT-depleted embryos used to provide either equators or vegetal masses were dyed different colors as oocytes, so that they could be distinguished and separated after recombination. After washing away dead cells, equatorial pieces were placed, each over two vegetal masses, and cultured on agar in OCM for 3 hours. The recombinants were separated using tungsten needles and stray vegetal cells were identified by their different vital dye colouring and removed from the equators. The equators were cultured in OCM until sibling embryos reached stage 21 and frozen for analysis.

RESULTS

Maternal VegT is required for mesoderm formation

To study further the role of maternal VegT in mesoderm formation, we enhanced the effectiveness of the antisense oligo used previously, by both increasing the dose and by HPLC purifying the oligo (Fig. 1a,b). Embryos derived from oocytes injected with 5-7 ng of this HPLC-purified oligo have a more severe phenotype than in the previous study (Fig. 1c,d), that is specifically due to the loss of maternal *VegT* mRNA, since it is rescued by the reintroduction of synthetic *VegT* mRNA (Fig. 1c,d). The VegT-depleted embryos do not form blastopores and remain only with an animal/vegetal axis when siblings are at the swimming tadpole stage (Fig. 1d).

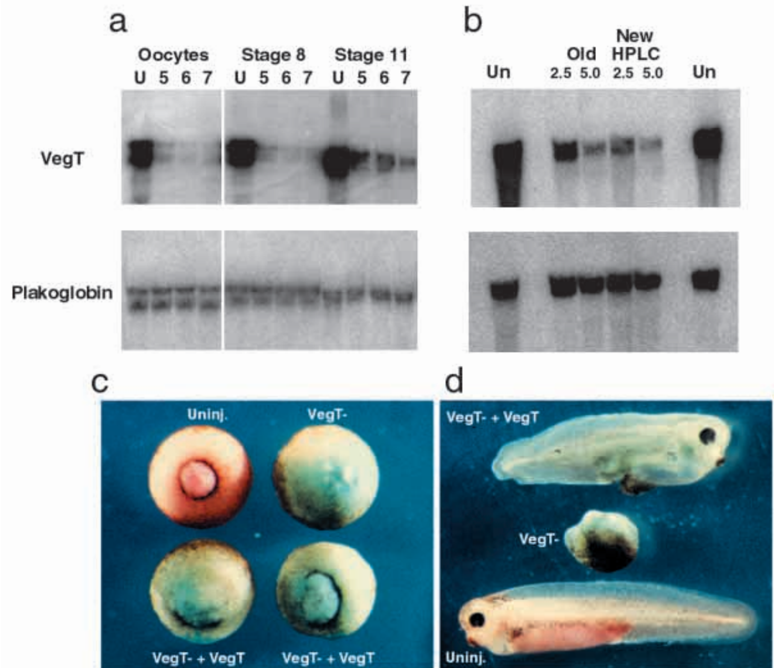


Fig. 1. Increasing the dose and purification of an antisense oligo complementary to VegT causes an extreme phenotype that is rescued by VegT mRNA. (a) Developmental northern blot of control uninjected (U) and increasing doses (5, 6 and 7 ng) of *VegT* antisense oligo-injected oocytes and embryos, derived from the same batch of oocytes, at the blastula (stage 8) and gastrula (stage 11) stage, probed for *VegT*. The blot was stripped and reprobed for a loading control, *plakoglobin*. (b) Northern blot of oocytes, either uninjected (Un) or injected with 2.5 or 5 ng of the antisense oligo used in Zhang et al. (1998) (old), or the same oligo HPLC purified (new). The HPLC-purifed oligo depletes the mRNA more substantially than the non-purified oligo. (c,d) Embryos derived from uninjected (red) or HPLC-purified *VegT* oligo-injected (blue) oocytes. (c) Top row shows one control and one sibling VegT-depleted embryo at the mid-gastrula stage of development, and the lower row shows two VegT-depleted embryos, treated identically to the blue embryo above, except that 200 pg of *VegT* mRNA was injected into their vegetal poles, 24 hours after the oligo, and before maturation. The injection of *VegT* mRNA rescues the formation of the blastopore. (d) The same embryos as in c at the tadpole stage. The upper embryo is depleted of VegT and then injected with *VegT* mRNA, the middle embryo is VegT-depleted, and the lower (red) embryo is an uninjected control from the same batch of oocytes. While the upper embryo is rescued compared to the VegT-depleted embryo, some cells of pigmented animal origin remain in the ventral belly region, indicating that the rescue is not complete.

We studied the effect of maternal VegT depletion on mesoderm formation, by comparing the expression of mesodermal markers *MyoD* (dorsal mesoderm), *cardiac actin* (dorsal mesoderm), *Xwnt8* (ventral mesoderm), *T4 globin* (ventral mesoderm) and *Xbra* (early general mesoderm) in a developmental series of control and experimental embryos, using RT-PCR. To make these comparisons as accurate as possible and to ensure they were made in the linear range, we used the LightCycler™ (Roche) amplification and detection system for real-time RT- PCR. The program compares the degree of amplification of each sample with that of a dilution series (100%, 50% and 10%) of uninjected control cDNA in each RT-PCR run (see Materials and Methods). While control embryos expressed increasing amounts of *MyoD*, *cardiac actin* and *alphaT4 globin* over time, VegT-depleted embryos expressed very low levels (10% or less of control) at all stages (Fig. 2). The early mesodermal markers, *Xbra* and *Xwnt8* were similarly reduced to below the 10% level in VegT-depleted embryos compared to uninjected controls. The experiment was repeated, with the same result (data not shown). This indicates that maternal VegT regulates the formation of mesodermal tissue in the embryo.

Maternal VegT is required for the expression of zygotic FGFs and TGFβs

Members of the *FGF* and *TGFβ* families have been implicated in mesoderm formation in *Xenopus* embryos. We tested whether maternal VegT is necessary for the initiation of expression of these genes by comparing their zygotic expression in a staged series of control and maternal VegT-depleted embryos. The effect of VegT depletion on early endoderm formation will be considered elsewhere (J. X. et al., unpublished data). The expression of *Xnr1*, *Xnr2*, *Xnr4* and *derrière*, as well as *FGF3* and *FGF8* was dramatically reduced to below the 10% level in VegT-depleted embryos compared to controls (Fig. 3). In contrast, *BMP4* and *BMP7* expression was either unaffected or increased in VegT-depleted embryos compared to control levels (Fig. 3a and b). *eFGF*, *activin B* and *Xnr3* expression was reduced but only to 30-50% of control levels. These results show that maternal VegT regulates, either directly or indirectly, the transcription of several TGFβ and FGF class growth factors.

To study whether VegT activates *TGFβ* genes directly, we scanned the regulatory regions of *Xnr1* (Fig. 4a; S.-I. O. et al., unpublished data) for potential VegT binding sites (CTTCACACCT; Tada et al., 1998; Smith, 1999). Two such possible sites were found, a distal 10/10 match in reverse orientation and proximal 7/10 match (underlined in Fig. 4a).

A promoter-Luciferase construct containing these sites was injected either alone or together with VegT mRNA into both cells of 2-cell stage wild-type embryos. VegT dose-dependently activates the reporter gene expression from the *Xnr1* promoter (Fig. 4b) suggesting that *Xnr1* is directly regulated by VegT. Injection of reporter construct alone into vegetal regions led to activation of luciferase by endogenous factors, with a higher activity in vegetal compared to animal injections (data not shown).

Xnr1, *Xnr2*, *Xnr4* and *derrière* rescue the VegT-depleted phenotype

To test the likely importance of each of these factors downstream of VegT in mesoderm formation, we studied their ability to rescue VegT-depleted embryos. Since mesoderm-inducing signals are known to be produced by vegetal masses, we injected *Xnr1, Xnr2, Xnr4*, *derrière*, *eFGF* and *VegT* mRNAs into four of the D tier cells of VegT-depleted embryos at the 32-cell stage. We confirmed that this site of injection restricted the injected mRNAs to cells of endodermal fate, by injecting the lineage label, *β-galactosidase* mRNA along with the growth factor

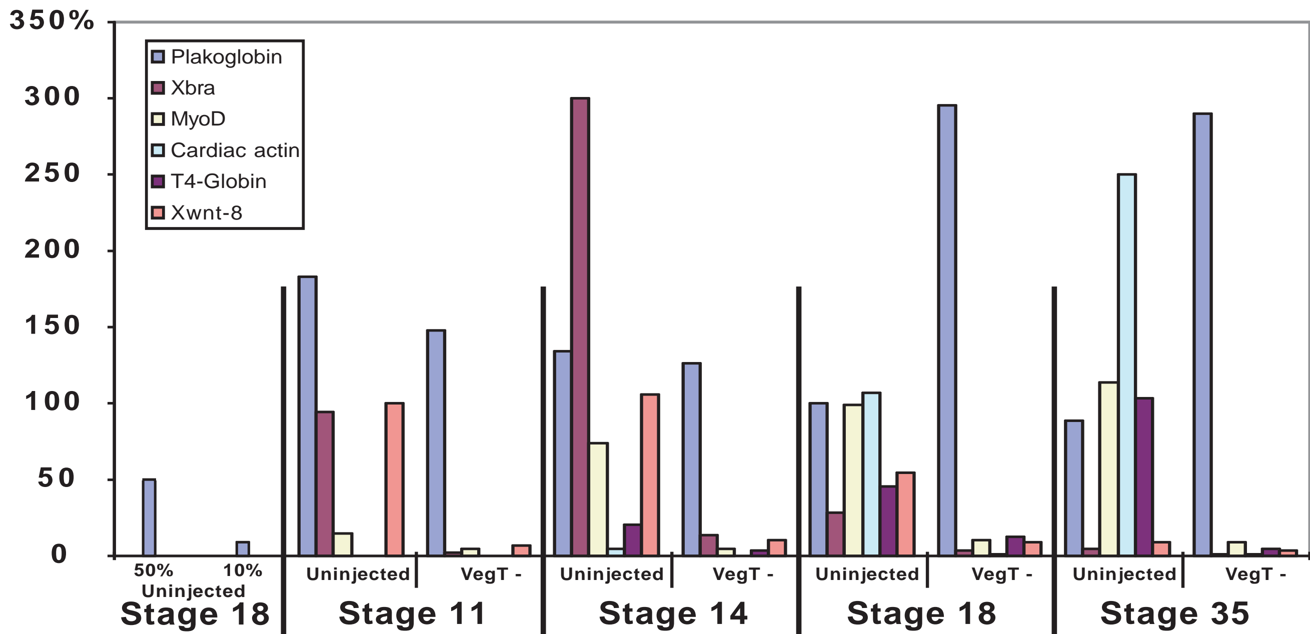


Fig. 2. Mesodermal markers are not expressed in VegT-depleted embryos. Real-time RT-PCR analysis of the relative gene expression of mesodermal markers in uninjected control and VegT-depleted embryos at the gastrula (stage 11), early (stage 14) and late (stage 18) neurula and late tailbud (stage 35) stages. The cDNA prepared from these samples was tested sequentially in LightCycler runs using *plakoglobin*, *Xbra*, *MyoD*, *cardiac actin*, *alphaT4 globin*, and then *Xwnt8* primers. Thus each colour represents one LightCycler run. In every run, a dilution series of uninjected controls, as well as –RT and water controls was included (although not shown here for lack of space). For *plakoglobin*, *Xbra* and *Xwnt8*, the standard curve was generated from a dilution series of stage 11 uninjected control cDNA. For *MyoD*, *cardiac actin* and *alphaT4 globin*, stage 18 uninjected control cDNA was used to make the standard curve.

mRNAs. Fig. 5 shows a representative experiment in which the embryos were cleared and stained for X-gal. Control embryos have X-gal staining in the embryonic endoderm and not in mesodermal structures (Fig. 5a). VegT-depleted embryos injected with *VegT* (Fig. 5b), *Xnr1* (Fig. 5c) and *derrière* mRNA (Fig. 5d) also have staining in the rescued gut region, while VegT-depleted embryos have blue cells in one hemisphere (Fig. 5e).

The degree to which VegT-depleted embryos were rescued by growth factor mRNAs was examined by three criteria: by the formation of the blastopore, by the rescue of mesodermal markers using RT-PCR and by the phenotypic rescue of head, trunk and tail. Rescue of head organizer and endoderm specific genes will be considered elsewhere (J. X. et al., unpublished data).

Rescue of blastopore formation

Embryos severely depleted of maternal VegT do not form blastopores or undergo gastrulation movements (Fig. 6a,b; data not shown).

The expression of all of the TGFβ class mRNAs, with the exception of *Xnr3*, rescued the formation of blastopores in VegT-depleted embryos (Table 2). *Xnr2* and *Xnr1* were most effective in this respect (95% of VegT-depleted embryos have blastopores after *Xnr2* injection, 85% for *Xnr1*), while *derrière* and *Xnr4* were less active (68% for *derrière*, 43% for *Xnr4*). Blastopores were generally delayed in their appearance, and frequently more vegetally placed than those of control embryos, but bottle cell formation was followed by tissue involution (Fig. 6c), as in control embryos. For all these mRNAs (except *Xnr4*) an upper limit of concentration was found (*activin B*: 4 pg, *Xnr1*: 600 pg, *Xnr2*: 150 pg, *derrière*: 400 pg), above which the blastopores formed were extremely large and the embryos developed with the same abnormal appearance. *eFGF* did not rescue blastopore formation over a range of doses (0.5-24 pg), and higher doses (12 pg) caused cell disaggregation at the gastrula stage (data not shown).

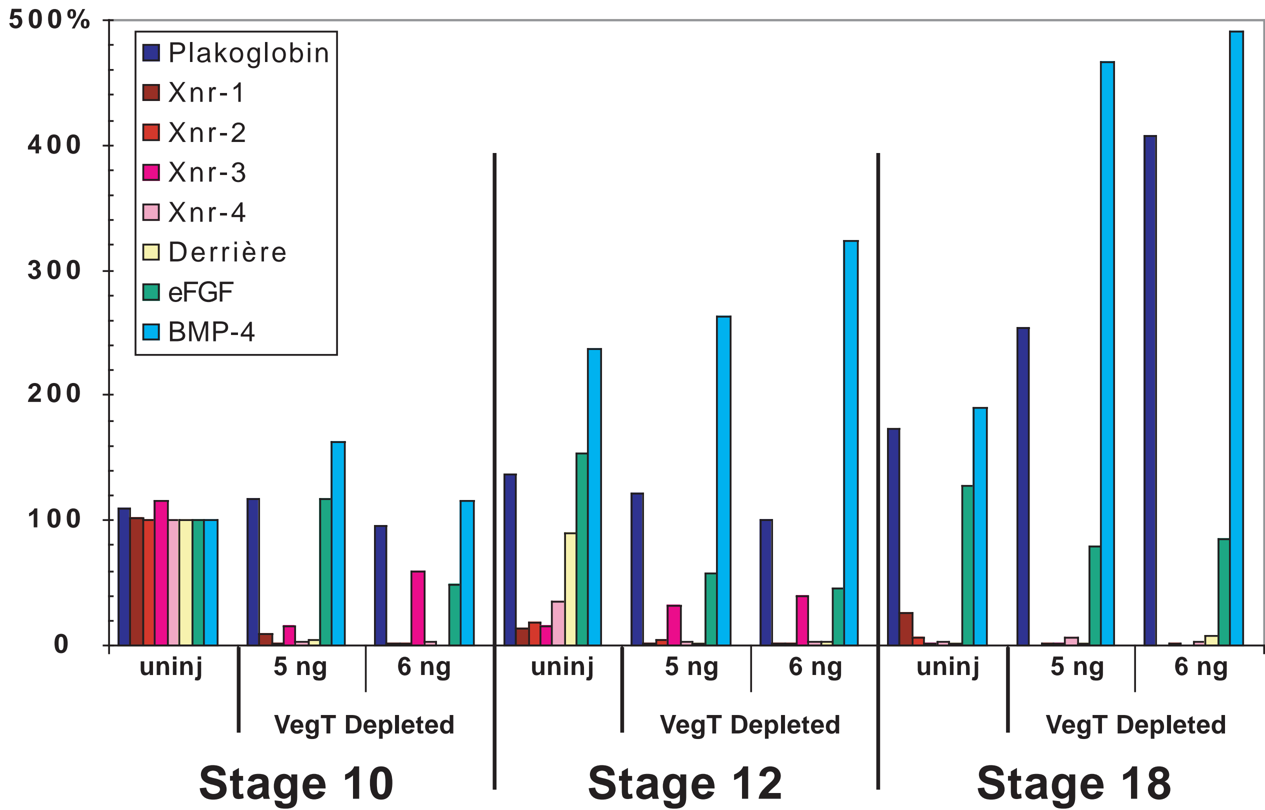
Embryos from these experiments were frozen at the mid-gastrula stage and analysed by RT-PCR for the expression of mesodermal markers, *chordin*, *Xbra* and *Xwnt8* (Fig. 7). The expression of *VegT* mRNA itself, as well as *Xnr1*, *Xnr2* (Fig. 9), *Xnr4* and *derrière* in vegetal cells of VegT-depleted embryos caused a partial rescue of these markers.

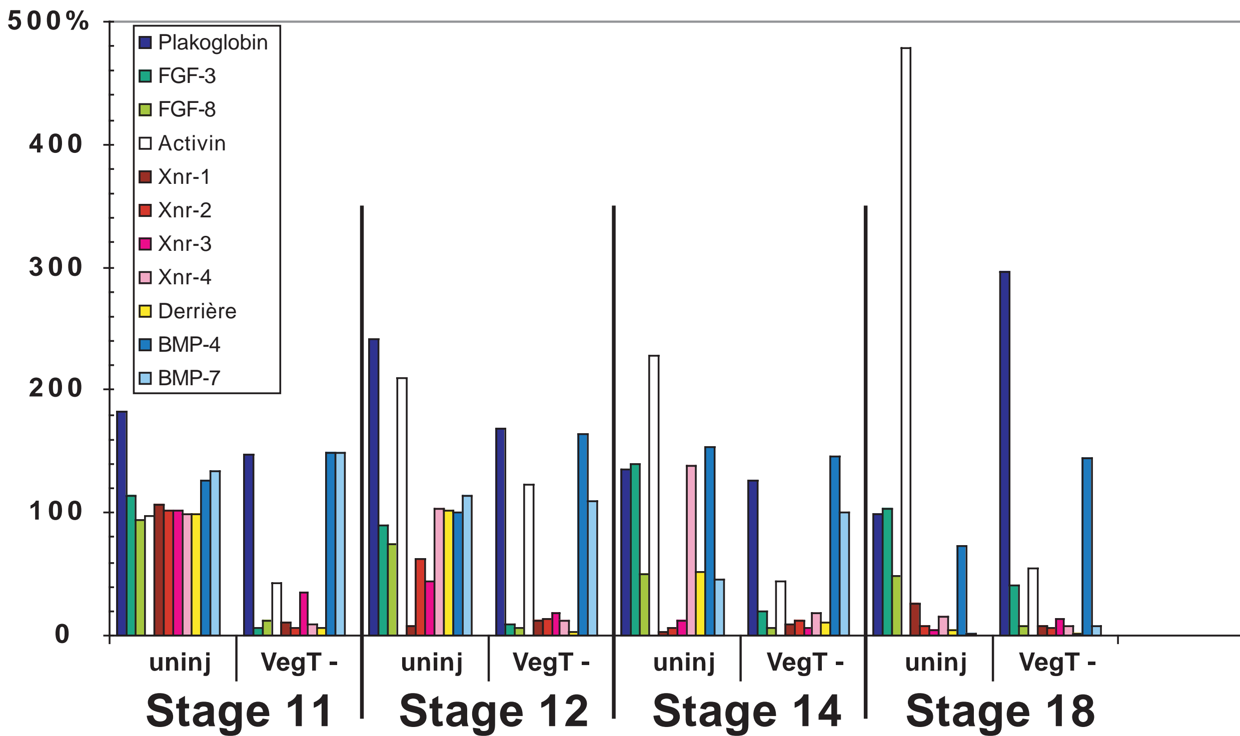
Rescue of the VegT-depleted phenotype

Since previous studies have shown that there is a dose-dependent activity of TGFβs and FGFs, we carried out rescue experiments using a dose range of each of the growth factors regulated by VegT. Fig. 8a-d shows a typical experiment, where embryos were either VegT-depleted (Fig. 8a), or VegT-depleted and injected with *derrière* RNA at the 32-cell stage in doses of 200 (Fig. 8b), 400 (Fig. 8c) and 800 pg (Fig. 8d). In three

Table 2. The effect on blastopore formation of depletion of maternal VegT and its rescue by specific growth factors

Sample	Blastopore present	Blastopore absent
Uninjected	82	0
VegT–	0	95
VegT– +Xnr1	68	12
VegT– +Xnr2	38	2
VegT– +Xnr4	21	27
VegT– +derrière	61	29
VegT– +eFGF	0	24
VegT– +VegT	45	5


a
500%
400
300
200
100
0
Plakoglobin
Xnr-1
Xnr-2
Xnr-3
Xnr-4
Derrière
eFGF
BMP-4
uninj
5 ng
6 ng
VegT Depleted
Stage 10
Stage 12
Stage 18


b
500%
400
300
200
100
0
Plakoglobin
FGF-3
FGF-8
Activin
Xnr-1
Xnr-2
Xnr-3
Xnr-4
Derrière
BMP-4
BMP-7
uninj
VegT -
Stage 11
Stage 12
Stage 14
Stage 18

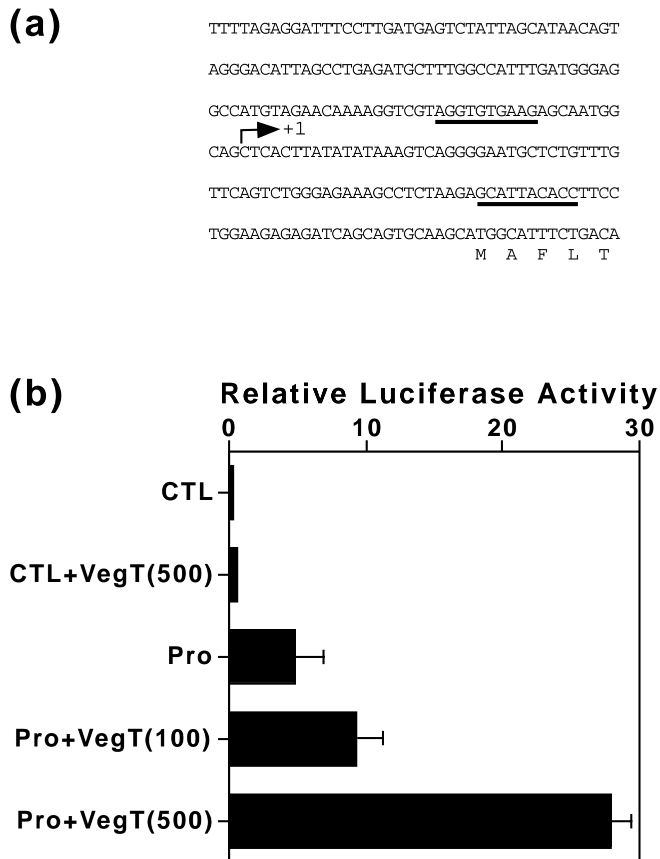


Fig. 4. Putative T-box-binding sites in the *Xnr1* promoter. (a) Partial sequence of the *Xnr1* promoter region. The transcription start site is indicated by the arrow and the beginning of the protein sequence is shown. Putative 10 bp T-box response elements are underlined. (b) VegT dose-dependently activates the reporter gene expression from the *Xnr1* promoter. Amounts of injected RNA (pg) is in parentheses. CTL, the promoterless control vector GL3; Pro, Pro-Luc plasmid

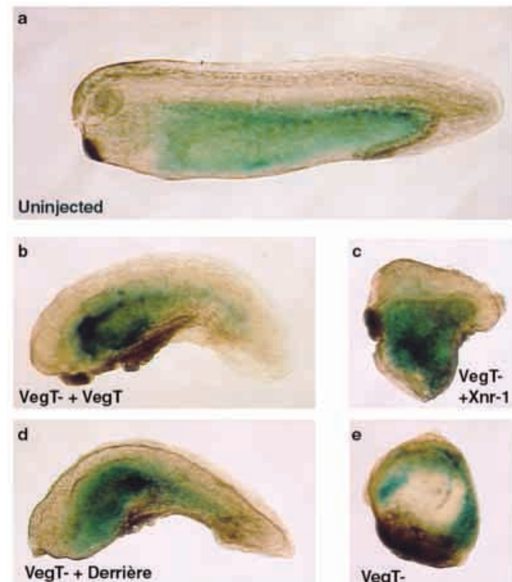


Fig. 5. Lineage tracer injected into vegetal cells at the 32-cell stage is expressed in endoderm of control and rescued embryos. Control (a) and VegT-depleted (e) embryos were injected with a total of 200 pg β-galactosidase mRNA into four D tier (vegetal) cells at the 32-cell stage and stained for X-gal at the swimming tadpole stage. (b-d) Injected with a mixture of 200 pg of X-gal and 200 pg of *VegT* (b), *Xnr1* (c) or *derrière* (d) mRNA.

experiments, *derrière* RNA rescued the formation of trunk and tail at doses above 200 pg but did not rescue head formation. 800 pg caused abnormalities in control embryos.

Similar experiments with *Xnr1* (Fig. 8e), *Xnr2* (Fig. 8f) and *Xnr4* (Fig. 8g), showed that all these nodal-related proteins rescued head as well as trunk and tail formation. Each showed a dose-responsiveness of activity, with *Xnr2* having most rescue activity at lowest doses (60 pg), while *Xnr1* was most active at 100-200 pg and *Xnr4* showed the least rescuing activity, particularly of head formation. In higher doses of *Xnr1* (200 pg), embryos often had enlarged heads and reduced tails (e.g. Fig. 5c). In comparison, *eFGF* was unable to rescue formation of the normal body axis, even at doses (3 pg) that caused tail abnormalities in control embryos (Fig. 8h). Higher doses of *eFGF* caused abnormality and cell disaggregation in both control and experimental embryos. Even though *activin* expression is not solely regulated by VegT (Fig. 3), we also tested its ability to rescue the VegT-depleted phenotype. Doses above 3 pg caused abnormalities in both experimental and control embryos, while lower doses rescued trunk and tail structures but not heads (data not shown). Perhaps not surprisingly, given our limited way of introducing the rescuing mRNAs compared to endogenous mechanisms, VegT-depleted embryos never developed completely normally when rescued in this way, with either VegT or the growth factor mRNAs.

To study the extent to which the growth factor mRNAs were able to rescue mesodermal markers in these embryos, sibling embryos were frozen at the gastrula and neurula stages and analysed by RT-PCR. Fig. 9 shows the rescue of the expression of *MyoD*, *Xwnt8*, *Xbra*, *eFGF*, *chordin* and *cardiac actin* mRNAs in VegT-depleted embryos with increasing doses of *Xnr2* mRNA. Doses of 300-600 pg caused overexpression of all of these markers in VegT-depleted embryos (with the exception of *Xbra*) compared to wild-type levels. *Xnr1*, *Xnr4* and *derrière* rescued mesodermal markers in a similar fashion (data not shown). For all the TGFβ growth factors, there was a close correlation between the degree of phenotypic rescue and the degree of rescue of mesodermal markers.

Fig. 3. Growth factors regulated by maternal VegT. Two experiments in which the relative gene expression of zygotic growth factors is compared in uninjected control and VegT-depleted embryos by real-time RT-PCR analysis. Each colour represents one LightCycler run. In both figures, the levels of expression of each growth factor cDNA were compared to a standard curve generated from stage 10 uninjected control cDNA. (a) Control uninjected and VegT-depleted embryos (injected as oocytes with 5 or 6 ng of oligo) are compared for the expression of the growth factors, *Xnr1*, *Xnr2*, *Xnr3*, *Xnr4*, *derrière*, *eFGF* and *BMP4*, at the early (stage 10), late gastrula (stage 12) and late neurula (stage 18) stage. (b) Control uninjected and VegT (5 ng)-depleted embryos are compared for the expression of *plakoglobin*, *FGF3*, *FGF8*, *activin B*, *Xnr1-4*, *derrière*, *BMP4* and *BMP7*.

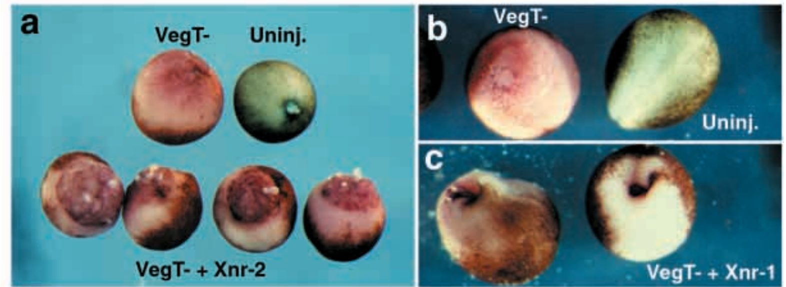


Fig. 6. Blastopores do not form inVegT-depleted embryos and are rescued by the expression of *Xnrs 1* and *2* in vegetal cells. (a) Top row shows a VegT-depleted (red) and uninjected control embryo (blue) at the late gastrula stage (stage 12). Bottom row shows four sibling VegT-depleted embryos, whose blastopores have been rescued by the injection of *Xnr2* mRNA (150 pg) in vegetal cells at the 32-cell stage. (b) Top row shows VegT-depleted (red) and uninjected control (blue) embryos at the neurula stage. Bottom row shows sibling VegT-depleted embryos injected with *Xnr1* (100 pg) mRNA tilted to show the site of gastrulation movements at their blastopores.

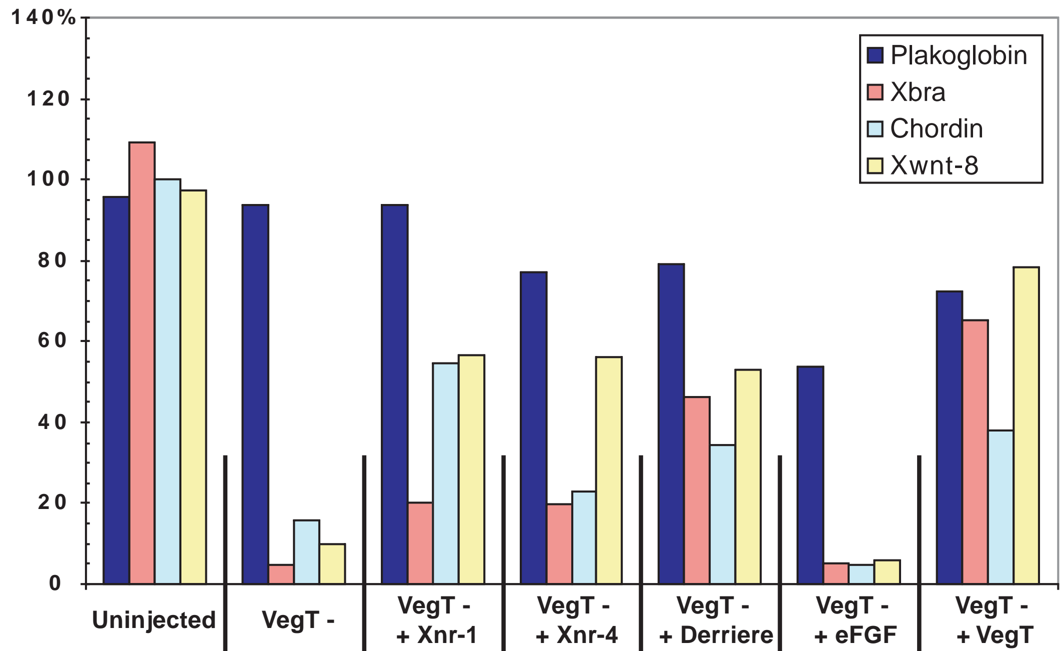


Fig. 7. Early mesodermal markers are not expressed in VegT-depleted embryos and are rescued by TGFβ growth factors. Real-time RT-PCR analysis of the relative expression of early mesodermal markers (*Xbra*, *chordin*, *Xwnt8* and control *plakoglobin*) in uninjected and VegT-depleted embryos and VegT-depleted embryos injected with *Xnr1* (240 pg), *Xnr4* (240 pg), *derrière* (300 pg), *eFGF* (6 pg) or *VegT*(240 pg) mRNAs at the 32-cell stage. Analysis was carried out at the mid-gastrula stage (stage 11). Each colour represents one LightCycler run. The standard curve was produced from a dilution series of control uninjected stage 11 cDNA.

Xnr1 rescues the mesoderm-inducing property of VegT-depleted vegetal masses

The experiments described above suggest that TGFβs downstream of VegT are released by vegetal cells to induce mesoderm. To confirm this, we tested whether one of the factors with most rescuing ability, *Xnr1*, was able to rescue the mesoderm-inducing activity of VegT-depleted vegetal masses in recombination experiments. In previous experiments, we showed that VegT-depleted equatorial regions are unable to form mesoderm (Zhang et al., 1998; Fig. 10). Here we co-cultured such equatorial regions with the vegetal masses from wild-type, or VegT-depleted embryos, or with VegT-depleted/*Xnr1*-expressing vegetal masses, where *Xnr1* mRNA had been injected into four vegetal cells at the 32-cell stage. Recombinants were combined at stage 8 (Fig. 10a), separated after 3 hours and cultured until stage 21. While control VegT-depleted equators cultured alone or in combination with VegT-depleted vegetal masses failed to elongate or express mesodermal markers, those cultured with wild-type vegetal masses or VegT-depleted/*Xnr1*-rescued vegetal masses underwent shape changes (Fig. 10b) and formed mesoderm, as evidenced by the expression of *Xbra*, *Xwnt8*, *MyoD*, *cardiac actin*, *VegT* and *eFGF* (Fig. 10c). Thus *Xnr1* rescues the ability of VegT-depleted vegetal masses to induce mesoderm in the equatorial region and to initiate the expression of *eFGF* in this region.

DISCUSSION

Does mesoderm formation rely solely on inducing signals downstream of VegT?

The loss-of-function approach used here allowed us to study the importance of maternal VegT for mesoderm formation in *Xenopus* development and to identify zygotic mesoderm-inducing growth factors regulated by VegT. We find that when VegT is depleted, ventral, general and dorsal mesodermal markers are reduced to less than 10% of control uninjected levels at gastrula and neurula stages. It is unclear whether residual mesoderm formation occurs because there is some VegT protein still active in these embryos or whether a second pathway is involved. Other studies and reviews have suggested the existence of a second “early weak mesoderm-inducing signal”(Clements et al., 1999; Kimelman and Griffin, 1998; Yasuo and Lemaire, 1999). If this signal exists, it is unable to cause blastopore formation and can only induce mesodermal markers to the 10% level, in VegT-depleted embryos.

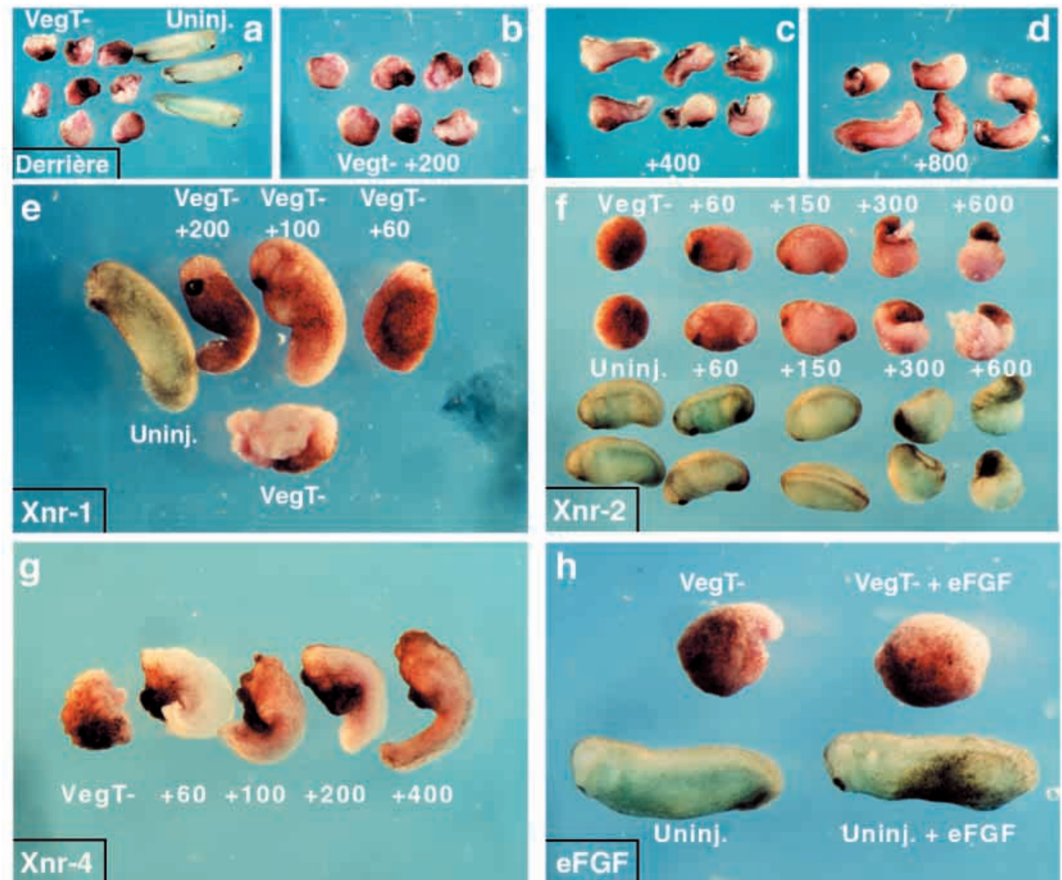


Fig. 8. TGFβ growth factors rescue VegT-depleted embryos. (a-d) Control tailbud (stage 32), and VegT-depleted embryos (a), or sibling VegT-depleted embryos that were injected with derrière RNA at the 32-cell stage in doses of 200 (b), 400 (c) and 800 pg (d). (e-h) Similar rescue experiments using mRNAs for *Xnr1* (e), *Xnr2* (f) and *Xnr4* (g) at the doses shown in pg amounts. (h) 3pg of *eFGF* mRNA causes tail abnormality in a control embryo (lower right), but does not rescue the VegT phenotype (upper right).

One question is the degree to which mesoderm formation is an autonomous property of cells of the equatorial region. Although *VegT* mRNA is located throughout the vegetal hemisphere of oocytes (Zhang et al., 1996; Stennard and Gurdon, 1996), recent studies on VegT protein localization show that it is concentrated in cells of the vegetal mass at the gastrula stage, and not in equatorial cells (Stennard et al., 1999). This suggests that the principle mechanism by which mesoderm forms is one of induction by zygotic growth factors initiated by vegetally localized VegT. It is also possible that low levels of VegT protein could activate mesoderm formation autonomously in equatorial cells (eg. by initiating *FGF* expression directly in this region).

Growth factors regulated by VegT

The lack of expression of mesodermal markers in VegT-depleted embryos is explained here by the fact that many of the known candidate zygotic mesoderm inducers are regulated by maternal VegT. As a result, VegT-depleted embryos have a more extreme phenotype than that caused by the inhibition of *FGF*, *Xnrs* or *derrière* individually (Amaya et al., 1991; Osada and Wright, 1999; Sun et al., 1999). Since the expression of *Xnr1, Xnr2, Xnr4*, *derrière*, *FGF3* and *FGF8* are almost completely absent in VegT-depleted embryos from the early gastrula stage, it is likely that maternal VegT is the sole transcription factor initiating their expression whether directly or indirectly. We disagree with previous overexpression studies that conclude that the primary signal downstream of VegT consists of *activin B*, *derrière* and *Xnr4* (Clements et al., 1999), since *activin B* is still expressed in VegT-depleted embryos, and *Xnr1* and *Xnr2* are at least as active in germ layer formation as *Xnr4* and *derrière*. Similarly, our work does not support an overexpression study suggesting that VegT is insufficient to activate *Xnr1* expression (Yasuo and Lemaire, 1999).

VegT might regulate this gene expression directly, or initiate a chain, or, more likely, a network of signals. For *Xnr1* at least, there is evidence from the work presented here (Figs 4a, 9) for both direct activation by VegT and indirect regulation via an Xnr2-initiated pathway. Also we show that *eFGF* expression is initiated by the reintroduction of *Xnr1* and *Xnr2* mRNAs into VegT-depleted embryos (Figs 9, 10c), while others have found T-box response elements in the promoter of *eFGF* (Casey et al., 1998). Verification of the direct targets of VegT may require their functional analysis by the transgenic approach, as recently carried out for the *Bix 4* gene (Casey et al., 1999), a direct target of VegT involved in endoderm formation.

Although *activin B* is induced by VegT in animal cap assays (Clements et al., 1999), we find here that *activin* is not solely regulated by VegT in the embryo. It is present at 50% control levels in VegT-depleted embryos, but this level of expression does not allow embryos to form mesoderm or to undergo gastrulation. This supports the body of evidence that *activin* does not play a major role in mesoderm formation (Schulte-Merker et al., 1994). In contrast, when we inject synthetic *activin* mRNA into VegT-depleted embryos, it has rescuing ability, similar to that of *derrière*. One interpretation of this is that all Smad2-activating TGFβs can mimick the endogenous pathway when overexpressed.

This work adds further evidence to the loss-of-function studies

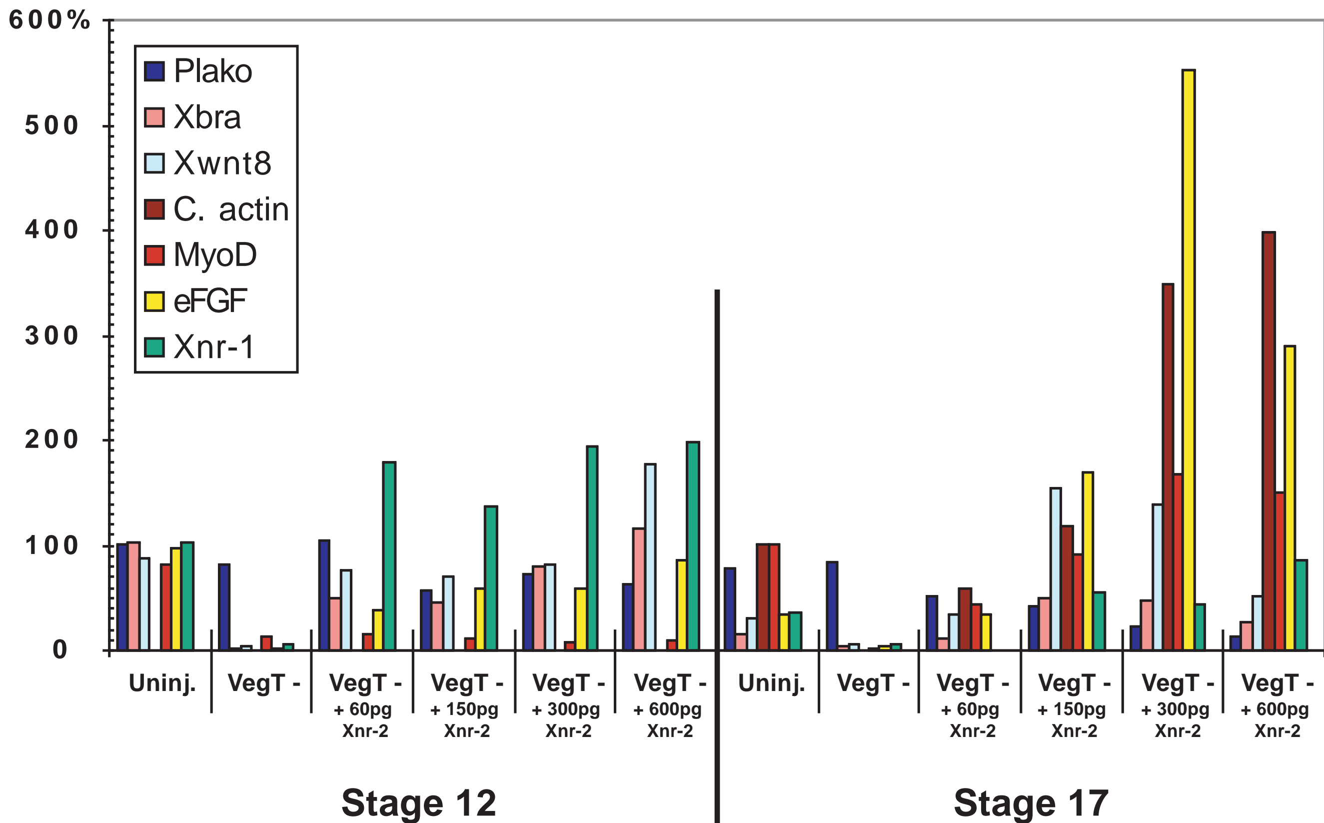


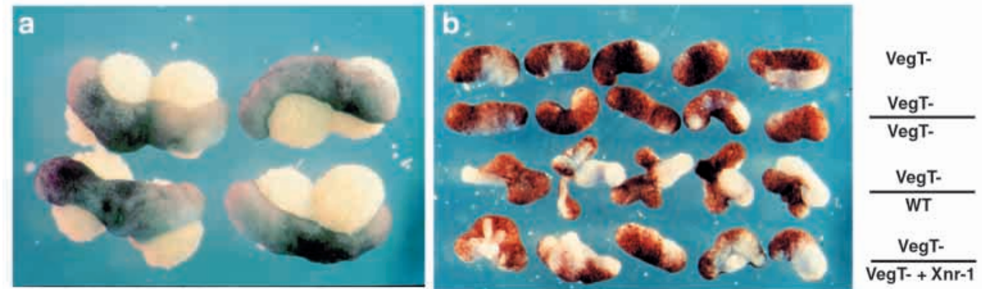
Fig. 9. Xnr2 rescues mesodermal markers and the expression of *Xnr1* and *eFGF* in VegT-depleted embryos in a dose-dependent manner. Real-time RT-PCR analysis of the relative expression of early (*Xbra*, *Xwnt8*) and late (*MyoD*, *cardiac actin*) mesodermal markers, and growth factors *eFGF* and *Xnr1* in uninjected and VegT-depleted embryos and in VegT-depleted embryos injected with increasing doses of *Xnr2* mRNA into the vegetal cells at the 32-cell stage. Each colour represents one LightCycler run. In every run, a dilution series of uninjected controls, as well as –RT and water controls were included but not shown. For *plakoglobin*, *Xbra*, *Xwnt8*, *eFGF* and *Xnr1*, the standard curve was generated from a dilution series of stage 12 uninjected control cDNA. For *cardiac actin* and *MyoD*, stage 17 uninjected control cDNA was used to make the standard curve.

on mouse *nodal* (Conlon et al., 1994) and zebrafish *nodal*-related genes *Znr1* and *Znr2* (Erter et al., 1998; Feldman et al., 1998; Sampath et al., 1998), and to *Xenopus* overexpression and dominant negative studies (Jones et al., 1995; Osada and Wright, 1999; Clements et al., 1999; Yasuo and Lemaire, 1999), indicating that the *nodal*-related genes are essential for both gastrulation movements and mesodermal tissue formation. The fact that *Xnr1*, *Xnr2* and *Xnr4* mRNAs show similar abilities to rescue mesoderm, makes it difficult to assess whether each has separate roles in the embryo; this could only be assessed by loss-of-function studies on each gene. Zebrafish *nodal*-related genes have been shown to have functional redundancy (Erter et al., 1998; Feldman et al., 1998; Sampath et al., 1998).

The roles of the other TGFβs, *derrière* and *Xnr3*, are also difficult to interpret. *Derrière* is clearly regulated by VegT but does not rescue head formation, which is consistent with studies using a dominant negative *derrière* construct, where posterior but not head structures were disrupted (Sun et al., 1999). This suggests that *derrière's* function is non-overlapping in this respect with the *Xnrs*, and that it has a divergent signalling pathway, activating axial and tail genes but repressing or not regulating head genes. *Xnr3* differs from the other Xnrs in continuing to be expressed in VegT-depleted embryos, although at lower levels than in controls and in that it is unable to rescue VegT-depleted embryos even in doses that cause abnormalities in control embryos (600 pg-1.2 ng, data not shown). This is consistent with its identification as a target of the *β-catenin/XTcf* signalling pathway (McKendry et al., 1997), its divergent structure and distinct inductive properties compared to other *Xnrs* (Smith et al., 1995), and supports its suggested role in dorsal rather than mesodermal patterning.

The zygotic FGFs, *FGF3* and *FGF8* are also regulated by VegT. The level of *eFGF* expression in VegT-depleted embryos was more variable than any of the other growth factors. For example, in Fig. 3a, *eFGF* is only slightly reduced in VegT-depleted compared to control embryos. However, in Figs 9, 10c, *eFGF* is not expressed in VegT-depleted embryos and is rescued by *Xnr1* and *Xnr2* expression. Radioactive RT-PCR also confirmed that *eFGF* is expressed only at very low levels in VegT-depleted embryos (data not shown). We conclude that *eFGF* is largely regulated by VegT and that the relatively high expression in Fig. 3a is due to a less severe depletion of *VegT* mRNA than in the other experiments.

In rescue experiments, the injection of *eFGF* mRNA into the vegetal masses of VegT-depleted embryos fails to initiate mesoderm induction (Fig. 6). However, *eFGF* is expressed in VegT-depleted equatorial regions in response to mesoderm-inducing signals from the vegetal mass (Fig. 10). This finding correlates with studies in wild-type embryos showing that *eFGF* mRNA is not present in vegetal masses at the blastula



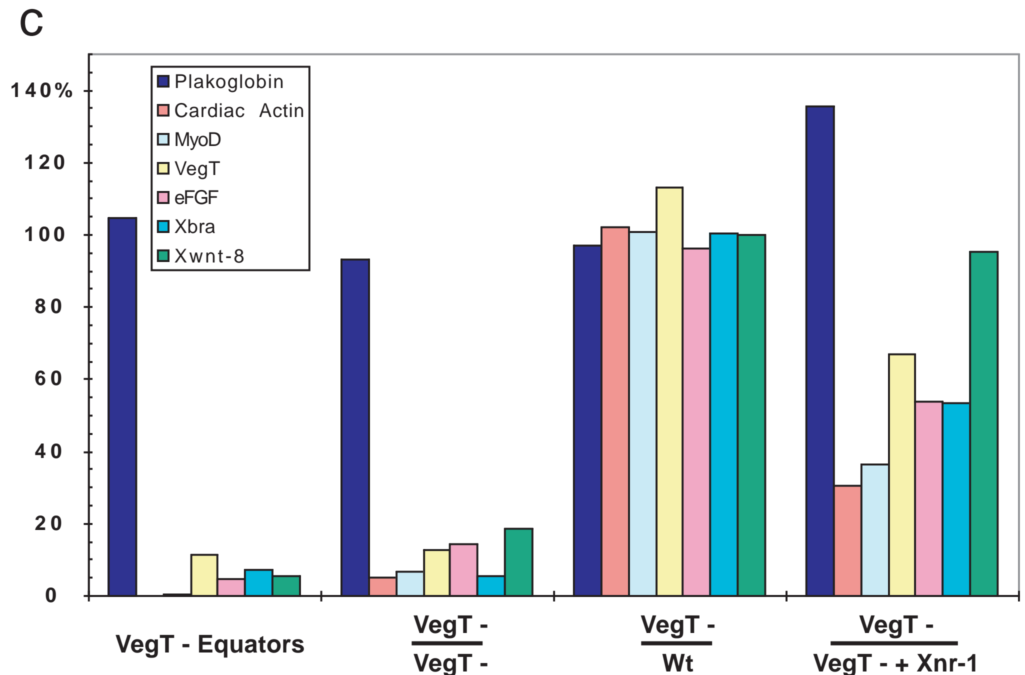


Fig. 10. *Xnr1* expression in VegT-depleted vegetal masses rescues their ability to induce mesoderm. (a) Examples of recombinants of equatorial regions dissected at the mid-blastula stage (stage 8) from VegT-depleted embryos cultured each with two vegetal masses from wild-type embryos. (b) Equatorial explants of VegT-depleted embryos dissected at the mid-blastula stage and shown here at the end of the experiment after overnight culture (sibling stage 21). The top row were cultured alone (VegT–), the second row were cultured with VegT-depleted vegetal masses for 3 hours (from stage 8-10) and then dissected away and cultured alone until stage 21 (VegT–/VegT–), the third row were cultured with wild-type vegetal masses for 3 hours and cultured alone until stage 21 (VegT–/WT), and the fourth row were cultured with VegT-depleted vegetal masses that had been injected with 200 pg *Xnr1* mRNA at the 32-cell stage and then cultured alone until sibling stage 21 (VegT–/VegT–+*Xnr1*). (c) Real-time RT-PCR analysis of the equators shown in b. Relative expression of early (*Xbra*, *Xwnt8*, *VegT*) and late (*MyoD*, *cardiac actin*) mesodermal markers, and the growth factor *eFGF* are shown, as well as control *plakoglobin* levels. Each color represents one LightCycler run. The standard curve for each LightCycler run was generated from a dilution series of VegT-/Wt sample. –RT and water controls were negative in each run but are not shown.

stage, but is expressed in the mesodermal ring of the gastrula (Isaacs et al., 1995). Furthermore, activation of the signalling pathway downstream of eFGF at the gastrula stage, as measured by phosphorylated ERK activity, occurs in cells of equatorial origin, not in vegetal cells (Christen and Slack, 1999). Taken together, these results show that eFGF is not a vegetal mesoderm inducer, but is activated in the equatorial region by Xnrs downstream of VegT.

Other signalling pathways in VegT-depleted embryos

The BMP class of TGFβ growth factors are expressed and even upregulated in VegT-depleted embryos (Fig. 3), and the homeobox gene regulated by BMP4, *Xvent 2* (Friedle et al., 1998), is also maintained (data not shown). It is also likely, since *Xnr3* is still expressed, that the dorsal (*β-catenin/XTcf*) signalling pathway is active. Since mesoderm fails to form in VegT-depleted embryos, it may be that the only cells available to be influenced by the remaining BMP and the Xwnt pathways are of ectodermal origin. Alternatively, the distinction of ectoderm, mesoderm and endodermal lineages may be too simplistic a model of lineage regulation: it seems likely that more than three distinct lineages are activated at the onset of zygotic transcription. We find here and in previous work that both epidermal and neural lineages are still present in VegT-depleted embryos (Zhang et al., 1998; data not shown). The pathways initiating their formation remain uncharacterized.

One unresolved question is the extent to which the VegT-initiated mesoderm-inducing pathway and the dorsal signalling pathway interact. *Xnr1*, *Xnr2* and *Xnr4* are all expressed initally throughout the vegetal mass (Jones et al., 1995; Clements et al., 1999), and are shown here to be regulated by VegT and responsible for mesoderm induction. They have also been implicated in dorsal signalling, since they are expressed later in the Spemann organizer and rescue the dorsal axis of irradiated embryos to various extents (Jones et al., 1995; Joseph and Melton, 1997). It seems likely that the final location of the *Xnrs* is the result of the interplay of the dorsal (*β-catenin/XTcf*), ventral (*BMP*) and vegetal (*VegT*-initiated) signalling pathways. The analysis of the zygotic genes regulated by *β-catenin/XTcf3* and of embryos depleted of both VegT and β-catenin or XTcf3 protein should help to resolve this issue.

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