Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*

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SUMMARY

During cleavage stages, maternal VegT mRNA and protein are localized to the *Xenopus* embryo's vegetal region from which the endoderm will arise and where several zygotic gene transcripts will be localized. Previous loss-of-function experiments on this T-box transcription factor suggested a role for VegT in *Xenopus* endoderm formation. Here, we test whether VegT is required to initiate endoderm formation using a loss of function approach. We find that the endodermal genes, *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2*, *Mixer*, *Xsox17α*, *Gata4*, *Gata5*, *Gata6* and *endodermin*, as well as the anterior endodermal genes *Xhex* and *cerberus*, and the organizer specific gene, *Xlim1*, are downstream of maternal *VegT*. We also find that the TGFβs, *Xnr1*, *Xnr2*, *Xnr4* and *derrière* rescue expression of these genes, supporting the idea that cell interactions are critical for proper endoderm formation. Additionally, inhibitory forms of Xnr2 and Derrière blocked the ability of VegT mRNA injection to rescue VegT-depleted embryos. Furthermore, a subset of endodermal genes was rescued in VegT-depleted vegetal masses by induction from an uninjected vegetal mass. Finally, we begin to establish a gene hierarchy downstream of *VegT* by testing the ability of *Mixer* and *Gata5* to rescue the expression of other endodermal genes. These results identify VegT as the maternal regulator of endoderm initiation and illustrate the complexity of zygotic pathways activated by VegT in the embryo's vegetal region.

Key words: Endoderm, *Xenopus*, VegT, Mixer, GATA factor, TGFβ

INTRODUCTION

Previous studies have shown that tailbud stage *Xenopus* embryos depleted of the maternal mRNA encoding the T-box transcription factor VegT do not express the endodermal genes, *endodermin*, *Xsox17α*, *Xlhbox8*, *insulin* and *Ifabp*, suggesting that *VegT* plays a role in endoderm formation (Zhang et al., 1998). However, the effect of depleting VegT mRNA on early endoderm formation was not examined. Several other approaches to determine the role of *VegT* in germ layer formation have been used. Studies employing a VegT repressor construct (Horb and Thomsen, 1997) and overexpressing VegT mRNA in animal cap cells (Clements et al., 1999; Yasuo and Lemaire, 1999) also point to a role for *VegT* in endoderm formation. Here, we aimed first to define the importance of maternal *VegT* for initiating and maintaining endodermal fates using a loss-of-function approach (Zuck et al., 1998). Since our initial study on the effects of depleting VegT mRNA, many zygotic transcription factors have been identified that are expressed in the vegetal region of the late blastula and early gastrula, from which the endoderm will arise. These genes include the homeobox genes *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2* and *Mixer*, and genes encoding the HMG box proteins *Xsox17α* and *Xsox17β* (Ecochard et al., 1998; Henry

and Melton, 1998; Hudson et al., 1997; Rosa, 1989; Tada et al., 1998; Vize, 1996), all being expressed with no apparent dorsoventral or anteroposterior pattern. Overexpression of *Mixer* and *Xsox17* in animal caps induces ectopic endodermal gene expression and dominant negative versions of Mixer, Xsox17 and Mix.1 result in block of endodermal gene expression (Henry and Melton, 1998; Hudson et al., 1997; Lemaire et al., 1998). In contrast to the expression pattern of these early endodermal genes, several genes show localized vegetal expression. The mRNAs coding for the homeobox protein Xhex (Newman et al., 1997) and the secreted protein Cerberus (Bouwmeester et al., 1996) are restricted to the dorsal-vegetal region at the early gastrula stage, a region that has been called the anterior endomesoderm (Zorn et al., 1999b). The region constituting the organizer overlaps a portion of anterior endomesoderm and is where organizer genes such as *Xlim1*, a gene encoding a LIM class homeodomain protein, are expressed (Taira et al., 1992). The gene encoding the transcription factor *Xlhbox8* (Wright et al., 1988) shows restricted expression to anterior endoderm at the tailbud stage. Recently, a closer examination of the GATA factors, GATA4/5/6, in *Xenopus* revealed that *Gata5* expression is also localized to the future endoderm in the early embryo (Weber et al., 2000), and like *Gata5*, *Gata4* and *Gata6*

have been implicated in endoderm formation (Jiang and Evans, 1996; Laverriere et al., 1994; reviewed in Rehorn et al., 1996). *Endodermin* expression is commonly used as a marker of endoderm in *Xenopus* (Sasai et al., 1996). We have examined the expression of all these genes in maternal VegT-depleted embryos to determine the extent to which *VegT* regulates early endodermal gene expression.

An important question that has received much attention recently is whether endoderm formation is a cell autonomous phenomenon, or involves cell interactions. Vegetal explants have been shown to express endodermal markers cell autonomously (Gamer and Wright, 1995; Henry et al., 1996). However, cell signaling interference in the vegetal mass, either with dominant negative Nodal (Osada and Wright, 1999), Derrière (Sun et al., 1999) or Activin receptors (Chang and Hemmati-Brivanlou, 2000; Clements et al., 1999; Yasuo and Lemaire, 1999), reduces endodermal gene expression in the embryo, suggesting that cell signaling via the TGFβ class of growth factors plays an important role in endoderm formation. In a previous paper, we showed that VegT regulates expression of genes encoding TGFβ growth factors, *Xnr1*, *Xnr2* (Jones et al., 1995), *Xnr4* (Joseph and Melton, 1997) and *derrière* (Sun et al., 1999), but not expression of *activin* and *Bmp* (Kofron et al., 1999). Here, we examine the relative roles of these signaling molecules in endoderm formation downstream of *VegT* by testing their ability to rescue endodermal genes in VegT-depleted embryos. We further confirmed their importance for endoderm formation by testing whether VegT mRNA could rescue endoderm initiation in VegT-depleted embryos in the presence of the TGFβ signaling inhibitors, cmXnr2 and cmDerrière (Osada and Wright, 1999; Sun et al., 1999).

The classical approach to demonstrate inductive interactions is to co-culture explants of inducing and responding tissue. Such co-cultures have been used to demonstrate the importance of the vegetal mass in inducing mesoderm formation in the equatorial region of *Xenopus* blastulae (Nieuwkoop, 1969; Smith, 1989), and to identify the TGFβ growth factors involved (Kofron et al., 1999). Here we tested whether uninjected vegetal explants could induce VegT-depleted vegetal explants to restore the expression of endodermal genes.

Lastly, we wished to begin to establish the gene hierarchy downstream of *VegT* by testing whether individual genes expressed in the future endoderm that are not expressed in VegT-depleted embryos could rescue the expression of the other endodermal genes. This approach has been used in zebrafish studies where *Mixer* has been placed downstream of TGFβ signaling and upstream of *sox17* (Alexander and Stainier, 1999).

Our studies demonstrate that *VegT* initiates most known early endodermal gene expression, and that all but a basal level of expression is dependent on *Xnr1*, *Xnr2*, *Xnr4* and *derrière* expression downstream of *VegT*. However, only a subset of endodermal genes are rescued in co-cultures of uninjected and VegT-depleted vegetal masses, suggesting that these signals act over a very short range, perhaps via autoregulatory loops. Finally, we demonstrate that endodermal genes downstream of *VegT* have different functions during gastrulation. *Gata5* causes *Xhex* and *Xlim1* expression; *Mixer* causes the expression of *Xsox17α*, *Gata4* and *Gata6*; and *Gata5* and *Mixer* act synergistically to enhance expression of these genes. Our results illustrate the complexity of zygotic pathways activated by *VegT* in the vegetal region of the embryo.

MATERIALS AND METHODS

Oocytes and embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM), as described previously (Zuck et al., 1998). Oocytes were injected at the vegetal pole with oligo in OCM using a Medical Systems picoinjector, and cultured a total of 24-48 hours at 18°C before fertilization. In preparation for fertilization, oocytes were stimulated to mature by the addition of 2 µM progesterone to the OCM and cultured for 12 hours. Oocytes were then colored with vital dyes and fertilized using the host-transfer technique described previously (Zuck et al., 1998). Three hours after being placed 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 experimental embryos were sorted from host embryos. Unfertilized eggs and abnormally cleaving embryos were removed from all batches.

For oocyte rescue experiments described in Fig. 4A, 100 pg VegT mRNA was injected into the vegetal pole of the oocyte and then 200 pg cmXnr2 mRNA and/or 200 pg cmDerrière mRNA was injected. For rescue experiments in the embryo, a total of 600 pg Mixer mRNA and/or 600 pg GATA5 mRNA was injected into four D-tier cells at the 16-32-cell stage. For injections of mRNAs except cmXnr2 and cmDerrière, 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 18 mer antisense oligodeoxynucleotide (oligo) complementary to VegT was C*A*G*C*AGCATGTACT*T*G*G*C, where * indicates a phosphorothioate bond and was HPLC purified before use (Genosys/Sigma). Oligos were resuspended in sterile, filtered water, injected at 7 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 filtered water for injection.

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. (Zhang et al., 1998) in a volume of 20 µl. After reverse transcription, 1 µl 0.5 M EDTA, 30 µl H$_2$O, 1 µl glycogen (20 µg/µl) and 10 µl 5M ammonium acetate, were added to each RT-reaction. *Gata4* required gene-specific priming in order to recover sufficient cDNA for PCR. The *Gata4* downstream primer was added to the random hexamers at a concentration of approximately 15 µM. Each sample was precipitated overnight at −20°C with 2.5 volumes 100% ethanol. Samples were centrifuged at 4°C 16,000 ***g*** for 15 minutes, washed with 70% ethanol, dried in a speedvac and resuspended in 200 µl H$_2$O per 1/6th embryo equivalent of RNA used for cDNA synthesis.

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$_2$O=50% and 1:10 cDNA:H$_2$O=10% (shown only in Fig. 1). 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 all the figures, owing to lack of space.

After each elongation phase, the fluorescence of SYBR green (a 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 quantified in real time, allowing the detection and display of the log-linear phase of amplification as it happens. LightCycler quantification software v3 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 1-3, 5-7). 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 were not used.

For easier comparison, histograms in Figs 5, 6 were normalized to the loading control, ODC (ornithine decarboxylase), expression levels. For each primer pair, gene expression levels were determined as a percentage of ODC expression. To determine severity of reduction with cmXnr2 and cmDerrière, expression levels were first normalized to ODC and then expressed as a percentage VegT-depleted embryos injected with VegT mRNA (see Table 2). Less than or equal to 30% was classified as a severely reduced rescue, between 30% and 80% a reduced rescue, between 80% and 120% unaffected, and greater than 120% overexpressed with cmXnr2 and cmDerrière mRNA injections.

Explant culture

Mid-blastula stage 8 uninjected and VegT-depleted embryos were devitellined, and vegetal masses were dissected on agar coated dishes in 1× MMR. After washing away dead cells, vegetal pieces were placed together in the combinations described in Fig. 6A-C and cultured on agar in OCM for 1 hour. The recombinants were separated using tungsten needles and stray vegetal cells were identified by their different vital dye coloring and removed from the vegetal explants. Explants were cultured until sibling uninjected embryos were stage 11 (3 hours).

Table 1. PCR primers

PCR primer pair	Reference	Sequence	Melting temp. (°C)	Annealing temp. /time*	Extension temp. /time*	Acquisition temp. /time*
Bix1	New	U: 5-AGA GAC TCC CAG TTC ATC TGA-3 D: 5-GGT AGG TGG GAA GTT GCT AAT-3	95	60/5	72/8	83/3
Bix3	New	U: 5-TGC TCG AGT CAC GCA TAC AG-3 D: 5-TGG ATG TCC TGG GAG TCT CTG GC-3	95	60/5	72/11	83/3
Bix4	New	U: 5-AGA TGC TAC AGG CTG GAG CAA-3 D: 5-GTG TGT AAG GGG TGA GTC ATA-3	95	60/5	72/11	84/3
cerberus	Darras et al., 1997	U: 5-GCT TGC AAA ACC TTG CCC TT-3 D: 5-CTG ATG GAA CAG AGA TCT TG-3	95	60/5	72/20	81/3
endodermin	Sasai et al., 1996	U: 5-TAT TCT GAC TCC TGA AGG TG-3 D: 5-GAG AAC TGC CCA TGT GCC TC-3	95	55/5	72/6	81/3
Gata4	New	U: 5-AGT GCT ACT GCT GCT ACC TC-3 D: 5-ACT GTA GGA GAC CTC TCT GC-3	95	54/5	72/15	87/3
Gata5	New	U: 5-ACC TTC AGA GCT GCG ACA CT-3 D: 5-CAG TGT ATT GCC ATA CTG GTC-3	95	60/5	72/20	86/3
Gata6	New	U: 5-CCA ACC GGG AGC CCC GAT A-3 D: 5-GCT GCT GTA GCC TGT ATC C-3	95	60/5	72/26	88/3
Milk (Bix2)	New	U: 5-TCT CGC ATT CAG GTT TGG TTC C-3 D: 5-ATC TCC TTG TTA GGG ATC ATA C-3	95	55/5	72/22	85/3
Mix.1	New	U: 5-GCA GAT GCC AGT TCA GCC AAT G-3 D: 5-TTT GTC CAT AGG TTC CGC CCT G-3	95	55/5	72/8	83/3
Mix.2	New	U: 5-TGC AAG CCA TCA TTA TTC TAG C-3 D: 5-AGG AAC CTC TGC CTC GAG ACA T-3	95	55/5	72/11	83/3
Mixer	Henry and Melton, 1998	U: 5-CAC CAG CCC AGC ACT TAA CC-3 D: 5-CAA TGT CAC ATC AAC TGA AG-3	95	55/5	72/12	83/3
ODC	Heasman et al., 2000	U: 5-GCC ATT GTG AAG ACT CTC TCC AAT C-3 D: 5-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
Xbra	Sun et al., 1999 Kofron 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
Xhex	Chang and Hemmati-Brivanlou, 2000	U: 5-AAC AGC GCA TCT AAT GGG AC-3 D: 5-CCT TTC CGC TTG TGC AGA GG-5	95	60/5	72/13	87/3
Xlhbox8	New	U: 5-GAA ATC CAC CAA ATC CCA CAC C-3 D: 5-TCC TTC TTC CAC TTC ATT CTC CG-3	95	55/5	72/10	82/3
Xlim1	New	U: 5-GAA GGA TGA GAC CAC TGG TGG-3 D: 5-CAC TGC CGT TTC GTT CAT TTC-3	95	55/5	72/14	83/3
Xsox17α	New	U: 5-GCA AGA TGC TTG GCA AGT CG-3 D: 5-GCT GAA GTT CTC TAG ACA CA-3	95	58/5	72/8	85/3

*Temperature in °C, time, in seconds.

RESULTS

Maternal VegT is required for the initiation of early endodermal gene expression.

To test whether maternal VegT is required for the initiation of endoderm formation we carried out RT-PCR analysis on cDNA from a staged series of VegT-depleted embryos, using primers specific for the endodermal genes shown in Table 1. By analyzing gene expression of mid blastulae (stage 8), late blastulae (stage 9) and early gastrulae (stage 10), the effect of depleting VegT mRNA on the intitiation of gene expression was determined. *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2*, *Mixer*, *Gata4/5/6*, *endodermin*, *Xhex*, *cerberus* and *Xlim1* were all expressed at less than 10% of control levels in VegT-depleted embryos, indicating that proper initiation of endodermal gene expression, as well as the organizer gene Xlim1, did not occur in embryos lacking maternal VegT

mRNA (Fig. 1A-C). Xsox17α expression was reduced to 31% and 23% of control levels in the VegT-depleted late blastulae and early gastrulae, respectively (Fig. 1B). Fig. 2A-C show that as development continues through gastrulation, the expression levels of *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2*, *Mixer*, *Xsox17α*, *Gata4/5/6*, *endodermin*, *Xhex*, *cerberus* and *Xlim1* were reduced in VegT-depleted early and late gastrulae to less than 10% of control levels. Xsox17α was expressed at less than 10% of control levels at stages 10.5, 12 and 32, (Fig. 2B,D), indicating that the initial level of *Xsox17α* expression is not maintained in VegT-depleted embryos. In a previous paper, we examined the expression of several late endodermal genes (Zhang et al., 1998). Here we extended this study to include *Gata4/5/6* and *Xlim1*. All these genes were severely under-expressed in VegT-depleted embryos at stage 32 (Fig. 2D).

We established that the lack of endodermal gene expression was specifically due to the absence of maternal VegT, as expression of all the genes studied was restored when *VegT* mRNA was injected into the vegetal region of VegT-depleted oocytes, or injected into the embryo's vegetal region at the 32-cell stage (see Fig. 5A-C and data not shown). These data show that VegT initiates most known endodermal gene expression.

Vegetally expressed TGFβs act downstream of VegT to specify endoderm.

Next we studied whether TGFβs shown previously to be downstream of *VegT* were able to rescue endodermal gene expression. Previously, we have shown
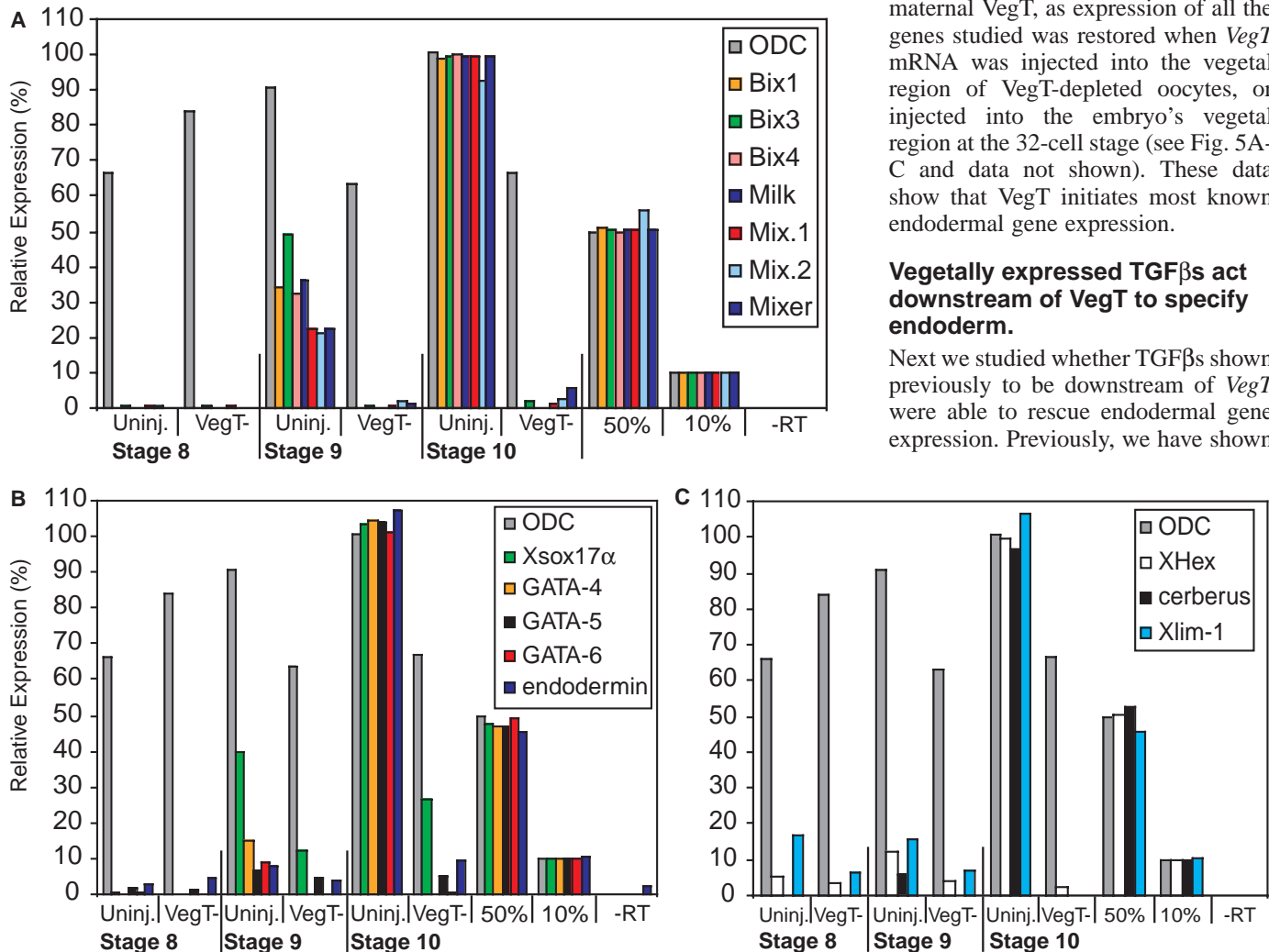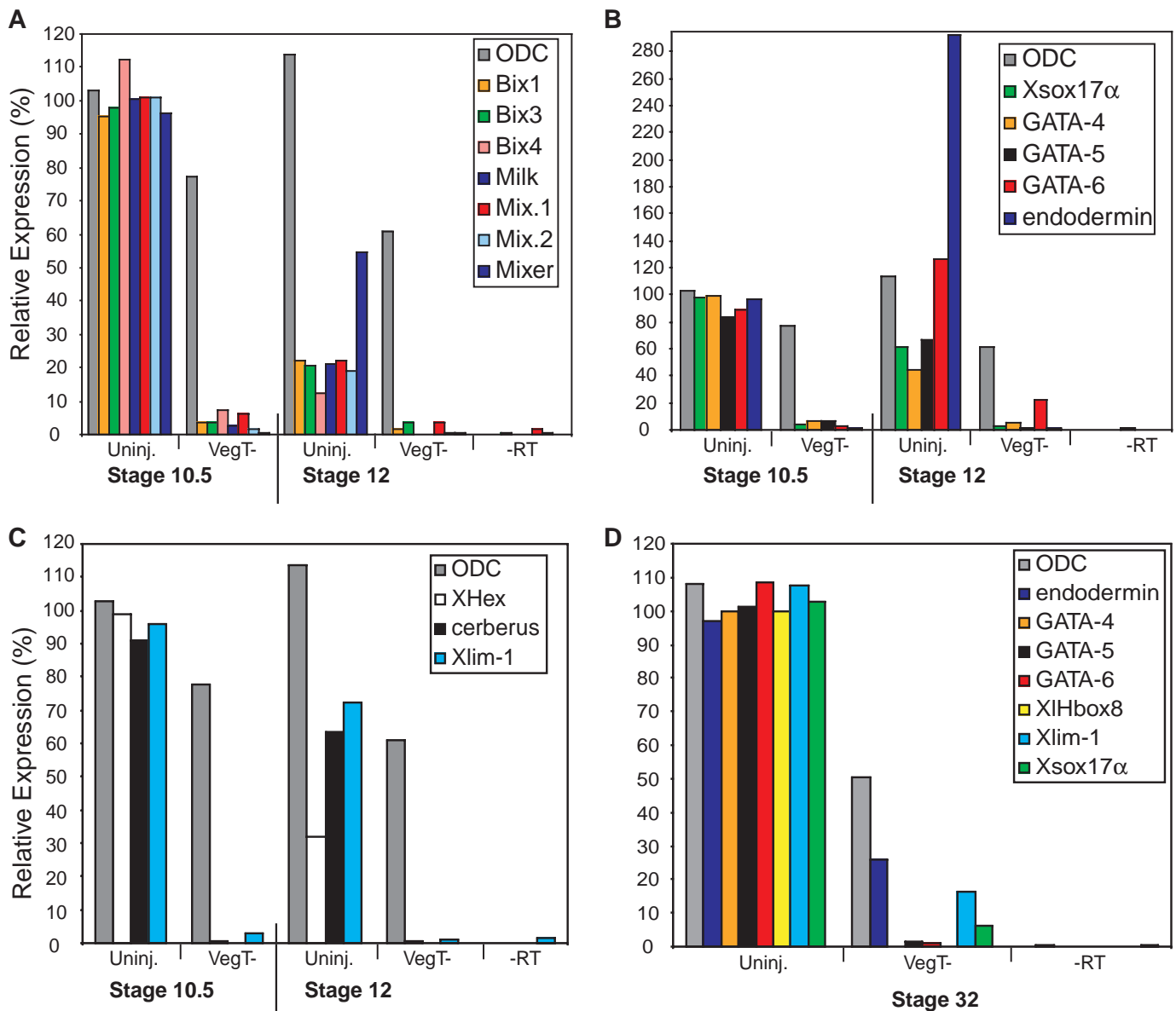

Fig. 1. Initiation of early endodermal gene expression is blocked in VegT-depleted embryos. Histograms depict relative gene expression (*y*-axis) measured by RT-PCR. Expression levels of (A) the homeobox genes *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2* and *Mixer*; (B) of *Xsox17α*, *Gata4/5/6* and *endodermin*; and (C) of *Xhex*, *cerberus* and *Xlim1* in uninjected and VegT-depleted embryos in mid blastula (Stage 8), late blastula (Stage 9) and early gastrula (Stage 10) embryos. Depleting VegT mRNA reduces expression of all these genes except *Xsox17α* to less than 10% of control levels in all stages the genes are expressed. *Xsox17α* expression level is reduced to 31% and 23% of control levels in late blastula and early gastrula, respectively. ODC, ornithine decarboxylase and is used as a loading control. See Materials and Methods for a description of relative gene expression quantification.

that *VegT* specifies mesoderm via TGFβs expressed in the embryo's vegetal region (Kofron et al., 1999). Here, we have studied the ability of the TGFβs *Xnr1*, *Xnr2*, *Xnr4* and *derrière* to rescue endoderm initiation in VegT-depleted embryos. VegT-depleted embryos were injected with different doses of mRNA for each growth factor. To restrict their expression to the vegetal mass, the mRNAs were injected into four D-tier cells at the 16-32 cell stage.

Fig. 3 shows that Xnr2 mRNA injected at doses that previously rescued mesodermal gene expression (Kofron et al., 1999), also rescued endodermal gene expression in gastrula and neurula stage embryos. Remarkably, Xnr1, Xnr2, Xnr4 and Derrière mRNA expression (Fig. 3 and data not shown) rescued all of the endodermal genes tested. All genes shown here

(*Mixer*, *Mix.2*, *Xsox17α*, *Gata6*, *Xhex* and *Xlim1*) showed rescued expression by Xnr2 mRNA injection (Fig. 3A). *Xsox17α*, *Gata6* and *Xlim1*, genes normally expressed in neurulae, showed continued rescued expression (Fig. 3B). Some endodermal genes, including *Mix.2*, *Gata6*, *Xhex* and *Xlim1* were restored to near control levels with the lowest dose of rescuing mRNA (60 pg; Fig. 3A,B). Interestingly, the organizer gene *Xlim1* was particularly sensitive to Xnr2 mRNA injection and was overexpressed fourfold in response to 300 pg of Xnr2 mRNA (Fig. 3A). In contrast, even the highest Xnr2 mRNA dose did not return *Xsox17α* expression to controls levels in the late gastrula stage (Fig. 3A). Xnr1, Xnr4 and Derrière mRNAs similarly rescued gene expression (data not shown).

Fig. 2. Early endodermal gene expression is severely reduced in VegT-depleted embryos throughout gastrulation. Again, histograms depict relative gene expression. Expression levels (A) of the homeobox genes *Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*), *Mix.1*, *Mix.2* and *Mixer*; (B) of *Xsox17α*, *Gata4/5/6* and *endodermin*; and (C) of *Xhex*, *cerberus* and *Xlim1* in uninjected and VegT-depleted embryos in early gastrula (Stage 10.5) and late gastrula (stage 12) embryos. Depleting VegT mRNA reduces expression of these genes to at least 10% of control levels. (D) Relative gene expression of endoderm genes expressed in tailbud stage embryos (stage 32) show continued reduction in gene expression.

A

B
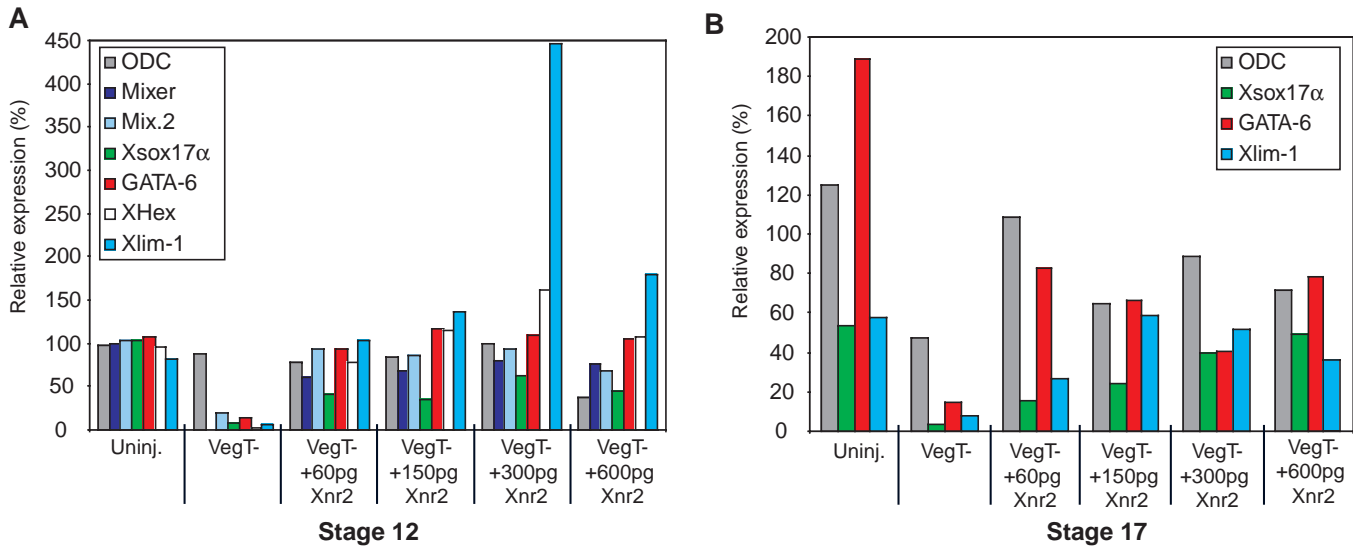
Fig. 3. *Xnr2* rescues endoderm formation in VegT-depleted embryos. Histograms depict relative gene expression measured by RT-PCR. (A) Late gastrula embryos (stage 12) show rescued expression of *Mixer*, *Mix.2*, *Xsox17α*, *Gata6*, *Xhex* and *Xlim1*, which are representative genes from different categories of endodermal genes. (B) This rescue is also evident later in development at stage 17 with *Xsox17α*, *Gata6* and *Xlim1* showing continued expression in VegT-depleted embryos rescued with Xnr2 mRNA.
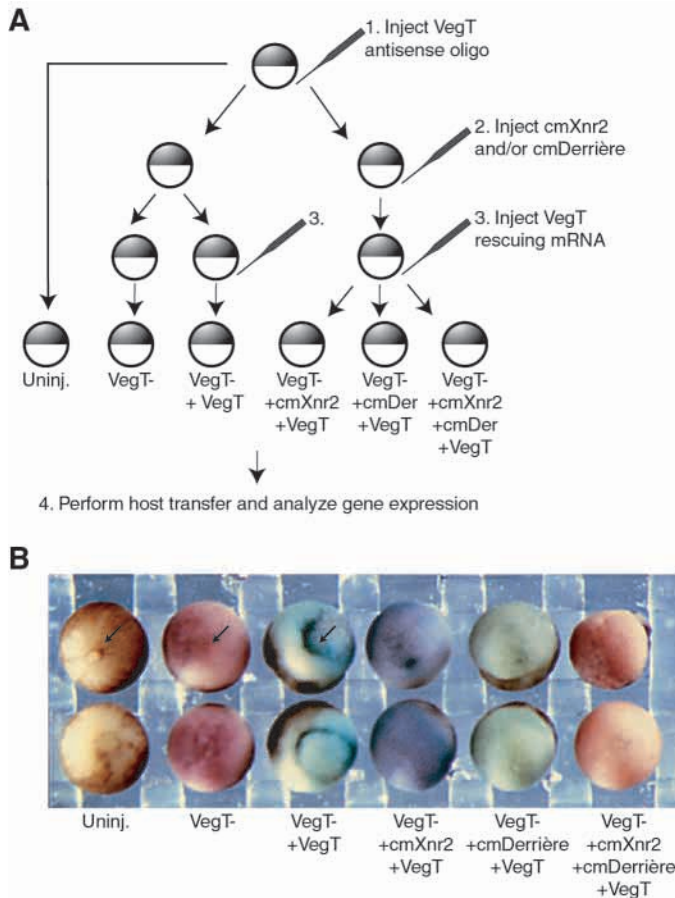
TGFβ signaling is required for the expression of genes downstream of *VegT*

Because *VegT* is clearly upstream of vegetally expressed transcription factors (this work) and growth factors (Kofron et al., 1999), endoderm initiation could be the result of both cell autonomous and cell non-autonomous events. We next wanted to confirm that cell signaling was required for endoderm formation. To test this we used Xnr2 and Derrière cleavage mutants, previously shown to inhibit TGFβ signaling in early *Xenopus* embryos. cmXnr2 has been shown to block signaling via Xnr1, Xnr2 and Xnr4 (Osada and Wright, 1999), while cmDerrière blocks Derrière activity specifically (Sun et al., 1999). We first confirmed that the constructs were active by showing that normal Xnr2 and Derrière mRNAs were unable to rescue VegT-depleted embryos in the presence of these inhibitors (data not shown). Next, we examined the ability of VegT mRNA to rescue endoderm formation in VegT-depleted embryos in the presence of these TGFβ signaling inhibitors (Fig. 4A). To ensure that both VegT and the dominant negative mRNAs were present throughout the vegetal mass, we injected the mRNAs into oocytes before fertilization, rather than into the vegetal tier at the 32-cell stage. Fig. 4A schematically depicts the experimental design and Fig. 4B the appearance of embryos at the late gastrula stage (stage 12). We have previously shown that VegT-depleted embryos do not form a blastopore or recognizable axes and that this effect can be rescued by reintroducing VegT mRNA (Zhang et al., 1998; Kofron et al., 1999). Injecting either cmXnr2 or cmDerrière mRNAs, or both together, prevented the ability of VegT mRNA to rescue gastrulation movements, as judged by blastopore formation (Fig. 4B, arrows).

Early endodermal gene expression downstream of *VegT* was examined by RT-PCR in these embryos (Fig. 5). For easier comparison, relative gene expression levels were normalized to the loading control, ornithine decarboxylase (ODC). The reintroduction of VegT mRNA rescued the expression of all the endodermal genes and *Xlim1*, as expected (Fig. 5, black bars). VegT mRNA injection caused an overexpression of *Bix1*, *Bix3*, *Bix4*, *Milk*, *Mix.1* and *Mix.2*. It caused a delayed rescue for *Gata5*, *endodermin*, *Xhex* and *cerberus*, since their expression was not restored until the late gastrula stage (stage 12) (Fig. 5, compare gray and black bars). In comparison, the reintroduction of VegT mRNA together with cmXnr2 (blue bar), cmDerrière (red bar) or both (green bar) reduced or severely reduced the expression of all genes tested (*Bix1*, *Bix3*, *Bix4*, *Milk* (*Bix2*) *Mix.1*, *Mix.2*, *Mixer*, *Xsox17α*, *Gata4/5/6*, *endodermin*, *Xhex*, *cerberus* and *Xlim1*). In no case was the rescue of endodermal gene expression blocked completely, suggesting that VegT directly activates a basal level of expression of these genes, or that the dominant negative block to TGFβ activity was incomplete.

A specific profile for each gene emerged when we examined the degree to which cmXnr2 and/or cmDerrière blocked the rescue of endodermal gene expression by VegT in the early gastrula (stage 10.5). cmDerrière (red bar) had more effect on the rescue of *Bix1* than did cmXnr2, and injecting both cmXnr2 and cmDerrière further reduced the *Bix1* rescue (green bar). However, in the case of *Bix3*, all three situations, cmXnr2 alone, cmDerrière alone, or both injected together, were relatively ineffective in blocking the rescue by VegT. For *Bix4*, cmXnr2 had a greater effect in blocking the rescue than did cmDerrière, and again, both dominant negatives injected together had the most severe effect. For *Mix.1* and *Mix.2*, cmXnr2 and cmDerrière had similar effects when injected separately, while co-injection most effectively blocked VegT rescue activity. For *Mixer*, cmXnr2 was more effective than cmDerrière alone, and as effective as both dominant negatives. *Xsox17α*, *Gata4* and *Gata6* all showed reduced expression in the early gastrula when VegT's activity was blocked by cmXnr2 or by the combination of cmXnr2 and cmDerrière, although cmDerrière alone had little effect. cmXnr2 and cmDerrière acted synergistically to inhibit *endodermin*,

present together. However, in the case of *Bix1*, *Bix3*, *Mix.1* and *Mix.2* there was increased expression at the late gastrula stage in the presence of the dominant negatives, suggesting cell autonomous maintenance of their expression at this time.
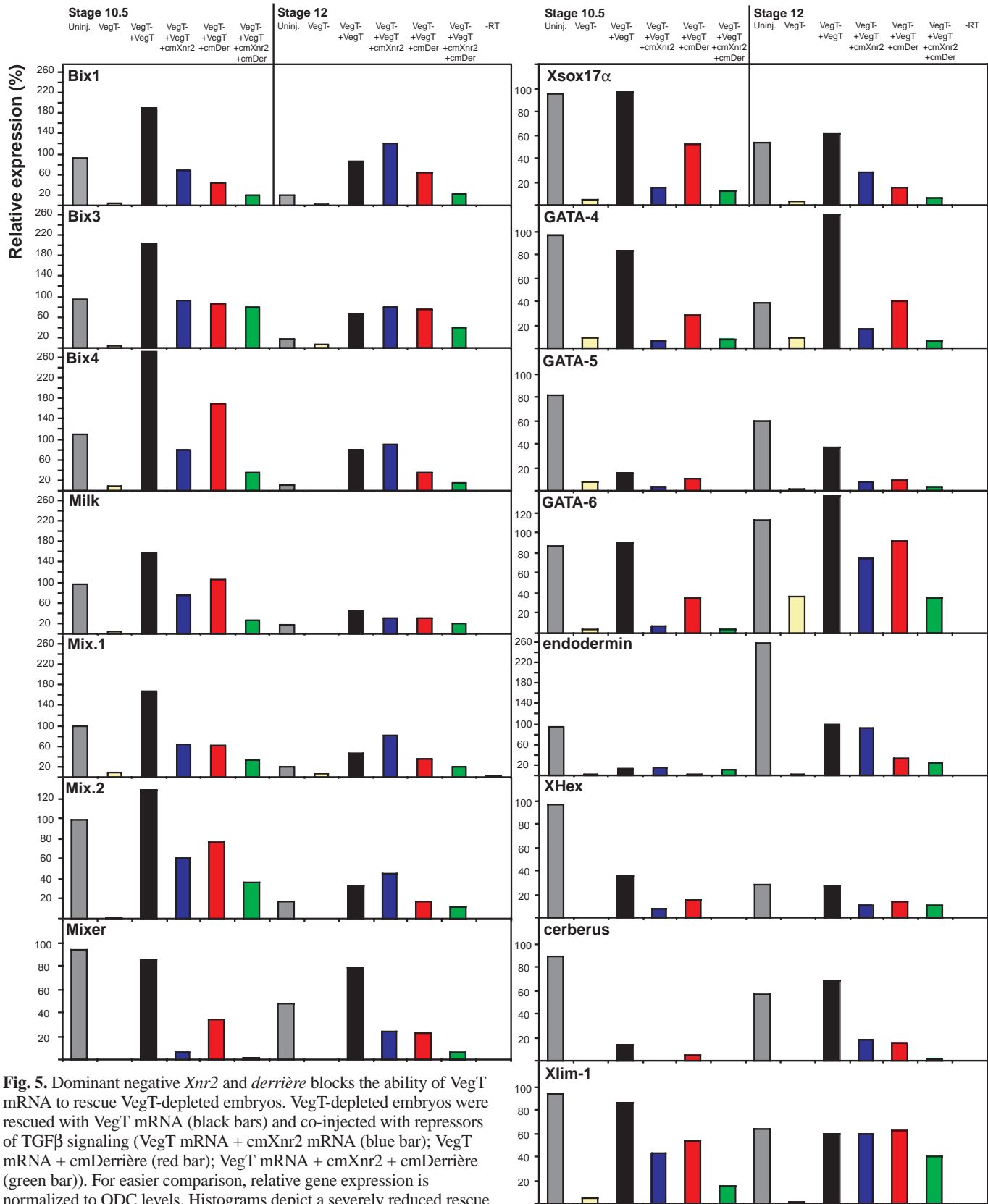
Endodermal cells undergo intercellular communication

These studies confirm previous experiments showing that signaling molecules play a role in endoderm formation (Chang and Hemmati-Brivanlou, 2000; Clements et al., 1999; Osada and Wright, 1999; Sun et al., 1999; Yasuo and Lemaire, 1999). To what extent then is endoderm formation, like mesoderm formation, the product of induction? Previous studies have shown that vegetal masses of wild-type embryos cause the expression of endodermal markers *Mix.1* and *Mixer* in co-cultured animal caps (Yasuo and Lemaire, 1999). Here we tested the ability of uninjected vegetal masses to rescue endodermal markers in co-cultured VegT-depleted vegetal masses. We measured the ability of the cells to signal by co-culturing VegT-depleted vegetal masses (red), which do not express *Xnr1*, *Xnr2*, *Xnr4* and *derrière* or other vegetally expressed genes, with uninjected vegetal masses (blue) (Fig. 6A). Rescue of endodermal gene expression in the VegT-depleted vegetal mass would indicate that the uninjected vegetal mass could signal to the adjacent VegT-depleted vegetal mass. Explants were cut from stage 8 embryos and co-cultured for 1 hour and then separated and cultured until sibling embryos reached mid-gastrula stage (stage 11). The explants were analyzed by RT-PCR for endodermal gene expression. To ensure that there was no contamination of VegT-depleted explants with uninjected cells, blue cells were completely cleaned from red explants and vice versa (Fig. 6B,C). Some rescue of endodermal gene expression was observed, specifically for *Bix4* (rescued to 35%), *Milk* (rescued to 51%), and *Xlim1* (rescued to 20%) (Fig. 6D). The other genes examined (*Mixer*, *Xsox17α*, *Gata5*, *Xhex* and *cerberus*) were not rescued by co-culture. The experiment was repeated using a longer co-culture period (2.5 hours beginning at stage 9), with the same result (data not shown).

Mixer and GATA5 act downstream of VegT and TGFβ signaling in separate pathways

Two vegetally expressed transcription factors downstream of *VegT*, *Gata5* and *Mixer*, were selected for further study. *Mixer* has been identified as an important early gene in endoderm formation. Mixer mRNA overexpression in *Xenopus* animal caps leads to ectopic endodermal gene expression of *Xlhbox8*, *Ifabp*, *Lfabp*, *endodermin* and *Xsox17α*. Conversely, *Xsox17α* overexpression in animal caps does not cause *Mixer* expression (Henry and Melton, 1998). Furthermore, a dominant negative version of Mixer blocks expression of endoderm specific genes and embryos appear abnormal (Henry and Melton, 1998).

GATA proteins are transcription factors implicated in gut formation in many organisms including mammals, *Xenopus*, *Caenorhabditis elegans* and *Drosophila* (Arceci et al., 1993; Gao et al., 1998; Laverriere et al., 1994; Rehorn et al., 1996; Zhu et al., 1997). In *Xenopus*, the *Gata4*, *Gata5* and *Gata6* are expressed in the developing and adult gut (Arceci et al., 1993; Jiang and Evans, 1996; Laverriere et al., 1994). Additionally in *Xenopus*, they have been shown to regulate intestinal epithelium cell differentiation and to regulate the promoter of

Fig. 4. (A) Experimental design to test the effect of blocking Xnr1, Xnr2, Xnr4 and Derrière signaling activity in relation to *VegT*. Oocytes were first injected with 7 ng VegT antisense oligo to deplete VegT mRNA and then injected with 200 pg cmXnr2 mRNA and/or 200 pg cmDerrière mRNA. Oocytes were then injected with 100 pg VegT mRNA. (All injections were at the vegetal pole.) Oocytes were fertilized following transfer to a host female. (B) cmXnr2 and cmDerrière block rescued gastrulation movements, as determined by the presence or lack of the blastopore (arrows). From left to right: brown, uninjected; red, VegT antisense oligo injected only; blue, VegT antisense oligo and VegT mRNA injected; purple, VegT antisense oligo, VegT mRNA and cmXnr2 mRNA injected; green, VegT oligo, cmDerrière mRNA and VegT mRNA injected; orange, VegT antisense oligo, cmXnr2 mRNA, cmDerrière mRNA and VegT mRNA injected.

cerberus and *Xlim1* expression in VegT-depleted embryos rescued with *VegT* mRNA.

The results from Fig. 5 are summarized in Table 2. The ability of the dominant negative signaling molecules to block *VegT* mRNA rescue was categorized by severity of reduced gene expression. Gene expression values of embryos expressing the dominant negatives together with VegT mRNA [cmXnr2 (blue bar); cmDerrière (red bar); both (green bar)] were compared with VegT mRNA alone (black bar) and expressed as a percentage. The categories include severely reduced, reduced, unaffected or overexpressed (Table 2). Gene groupings of different categories were apparent. Most gene expression was severely repressed at both early and late gastrula stages, when both cmXnr2 and cmDerrière were

Fig. 5. Dominant negative *Xnr2* and *derrière* blocks the ability of VegT mRNA to rescue VegT-depleted embryos. VegT-depleted embryos were rescued with VegT mRNA (black bars) and co-injected with repressors of TGFβ signaling (VegT mRNA + cmXnr2 mRNA (blue bar); VegT mRNA + cmDerrière (red bar); VegT mRNA + cmXnr2 + cmDerrière (green bar)). For easier comparison, relative gene expression is normalized to ODC levels. Histograms depict a severely reduced rescue for homeobox gene expression by both cmXnr2 and cmDerrière in early (stage 10.5) gastrula embryos and at least a reduced rescue by both dominant negatives in late gastrula (stage 12) embryos. cmXnr2 and cmDerrière similarly reduce *Xsox17α*, *Gata4/5/6* and *endodermin* rescued expression. *Xhex*, *cerberus* and *Xlim1* rescues are also severely reduced by these dominant negatives. All genes were rescued by VegT mRNA injection in the oocyte (this figure) and in the embryo (data not shown).

Table 2. Summary of gene expression rescue blocked by cleavage mutants for Xnr2 and Derrière

(A) Stage 10.5

Reduced by cmXnr2	Reduced by cmDerrière	Reduced by both	Severely reduced by cmXnr2	Severely reduced by cmDerrière	Severely reduced by both	Unaffected by cmXnr2	Unaffected by cmDerrière	Unaffected by both	Overexpressed by cmXnr2	Overexpressed by cmDerrière
Bix1				Bix1	Bix1					
Bix3	Bix3	Bix3								
	Bix4		Bix4		Bix4					
Milk	Milk				Milk					
Mix.1	Mix.1				Mix.1					
Mix.2	Mix.2				Mix.2					
	Mixer		Mixer		Mixer					
	Xsox17α		Xsox17α		Xsox17α					
	Gata4		Gata4		Gata4					
	Gata5		Gata5		Gata5					
	Gata6		Gata6		Gata6					
				endodermin		endodermin		endodermin		
	Xhex		Xhex		Xhex					
			cerberus	cerberus	cerberus					
Xlim1	Xlim1				Xlim1					

(B) Stage 12

Reduced by cmXnr2	Reduced by cmDerrière	Reduced by both	Severely reduced by cmXnr2	Severely reduced by cmDerrière	Severely reduced by both	Unaffected by cmXnr2	Unaffected by cmDerrière	Unaffected by both	Overexpressed by cmXnr2	Overexpressed by cmDerrière
	Bix1				Bix1				Bix1	
		Bix3					Bix3		Bix3	
	Bix4				Bix4	Bix4				
Milk	Milk	Milk								
	Mix.1	Mix.1							Mix.1	
	Mix.2	Mix.2							Mix.2	
Mixer				Mixer	Mixer					
Xsox17α				Xsox17α	Xsox17α					
	Gata4		Gata4		Gata4					
			Gata5	Gata5	Gata5					
Gata6	Gata6				Gata6					
	endodermin				endodermin	endodermin				
Xhex	Xhex	Xhex								
			cerberus	cerberus	cerberus					
		Xlim1				Xlim1	Xlim1			

Relative expression was determined as a percentage of the rescue (VegT-depleted + VegT mRNA). ≤30%, severely reduced rescue; >30% ≤80%, reduced rescue; >80% ≤120%, unaffected rescue; >120%, overexpressed (see Fig. 4).

intestinal fatty acid binding protein (IFABP) (Gao et al., 1998). GATA4/5/6 may function much earlier than these later stages to specify endoderm, and their role for endoderm formation during *Xenopus* gastrulation is just beginning to be examined. Overexpression of the *C. elegans* GATA-like factor, END-1, in the *Xenopus* animal cap causes ectopic endodermal gene expression and suggests a conserved role for GATA factors in early endoderm formation (Shoichet et al., 2000). Additionally, when injected into *Xenopus* animal caps, GATA4 and GATA5 induce endodermal gene expression, and GATA5 can respecify a mesodermal fate to an endodermal fate when overexpressed (Weber et al., 2000).

As a more rigorous test of the role of *Mixer* and *Gata5* in endoderm formation, we asked to what extent they rescue the phenotype of VegT-depleted embryos. A total of 600 pg Mixer mRNA and/or 600 pg GATA5 mRNA was injected into four vegetal D-tier cells of 16-32-cell embryos. Phenotypically, Mixer mRNA injection did not rescue the VegT-depleted embryo (Fig. 7B). We next analyzed whether gene expression was rescued in these embryos. Mixer mRNA injected into VegT-depleted embryos rescued expression of *Xsox17α* in the early gastrula (stage 10.5) to 35% of control levels (Fig. 7A). Similarly in the late gastrula (stage 12), Mixer rescued

expression of *Gata6*. In contrast, the other homeobox genes *Bix1*, *Bix3*, *Bix4*, *Milk*, *Mix.1* and *Mix.2* (anterior endodermal genes), and *Gata5* were not rescued by Mixer mRNA injection. We confirmed that Mixer does not cause the expression of mesodermal genes such as *Xbra* (Fig. 7A) and does not itself switch on the TGFβ growth factors Xnr1, Xnr2, Xnr4 and Derrière (data not shown).

Like Mixer, GATA5 mRNA injection alone did not rescue the phenotype resulting from VegT mRNA depletion. However, GATA5 mRNA injection had a different rescue profile than Mixer mRNA injection alone, only rescuing *Xlim1* expression (35% stage 10.5, 50% stage 12) and *Xhex* (20% stage 12) during mid and late gastrula stages (Fig. 7A). Interestingly, when *Mixer* and *Gata5* were co-expressed, the level of *Xsox17α*, *Xhex*, *Gata*4 and *Gata6* expression were synergistically enhanced in the gastrula. Mixer mRNA injection did not rescue *Gata5* expression and GATA5 mRNA injection did not rescue *Mixer* expression (data not shown). Lastly, Xsox17α mRNA injection into VegT-depleted embryos did not rescue either early or late endodermal markers (data not shown).

The effect of rescuing the VegT-depleted embryo with Mixer and GATA5 mRNAs was also examined at the tailbud stage
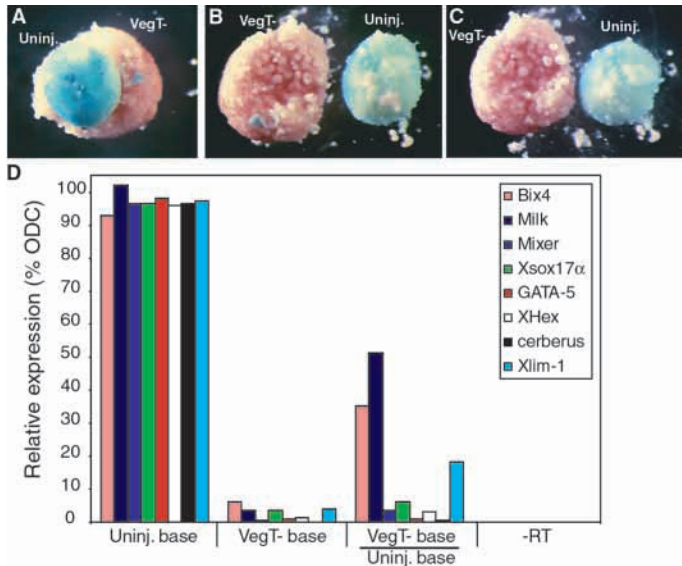
Fig. 6. VegT-depleted vegetal explants can receive a signal to restore endodermal gene expression. (A) VegT-depleted vegetal explants (red) were cultured with uninjected vegetal explants (blue) for 1 hour. Following culture, VegT-depleted explants were separated (B) and cleaned (C) to remove any contaminating cells. (D) Histograms show that *Bix4*, *Milk* and *Xlim1* gene expression is restored after culture. (Relative gene expressed was normalized to ODC).

(stage 32). Injected singly or together, they rescued the expression of *Gata4*, *Gata6*, *endodermin*, *Xlhbox8* and *Xsox17α* to different extents (Fig. 7C). Thus, *Gata5* rescued the expression of *Xlim1*, while Mixer mRNA alone did not (Fig. 7C). Interestingly, rescued endodermal gene expression at this stage was many fold greater than in wild-type sibling embryos for some genes. For example, Mixer mRNA rescued *endodermin* expression to make it over fourfold higher than wild-type levels at the tailbud stage, although *endodermin* was not rescued at all at the gastrula stage. When GATA5 mRNA was injected, both *endodermin* (>twofold) and *Xsox17α* (>twofold) expression was rescued, although this expression was rescued in the gastrula. Co-injection of Mixer and GATA5 mRNAs rescued *Xlhbox8*, *endodermin*, *Gata4*, *Gata6* and *Xsox17α* to above normal levels. Thus *Mixer* and *Gata5* are capable of rescuing specific endodermal genes in both gastrula and tailbud stages.

DISCUSSION

The vegetal area of the early *Xenopus* blastula has a deceptively homogeneous appearance. It has long been known to play essential roles in patterning the embryo, specifically in mesoderm induction (Nieuwkoop, 1969), axis formation (Vincent et al., 1986) and in germ-cell lineage establishment (Holwill et al., 1987). These functions have been shown to be controlled by maternal determinants, synthesized during oogenesis and inherited by vegetal cells derived from the vegetal hemisphere of the oocyte (Heasman et al., 2000; Houston and King, 2000; Kofron et al., 1999; Zhang et al., 1998). Recently, the mechanism by which this vegetal area self-differentiates into endodermal tissue has also been

examined. In an earlier study we showed that the maternal transcription factor VegT was essential for late endodermal gene expression (Zhang et al., 1998). Here, we have examined the role of maternal VegT in both the initiation and maintenance of the expression of a large array of zygotic genes whose expression is localized to the vegetal mass of the blastula and gastrula stage embryo. Many of these genes had been implicated in endoderm differentiation by overexpression and dominant negative expression studies (Henry and Melton, 1998; Hudson et al., 1997; Lemaire et al., 1998). We found that the early endodermal genes, *Bix1*, *Bix3*, *Bix4*, *Milk (Bix2)*, *Mix.1*, *Mix.2*, *Mixer*, *Gata4/5/6*, *endodermin*, *Xhex*, *cerberus* and the organizer gene *Xlim1*, are directly or indirectly downstream of *VegT*. This proves that maternal *VegT* establishes the endodermal germ layer, and indicates that there are no parallel maternal or zygotic pathways that can compensate for the loss of maternal VegT activity. In comparison, neural and epidermal differentiation pathways are intact in VegT-depleted embryos, while mesodermal tissue is also lost (Kofron et al., 1999; Zhang et al., 1998). Of all the endodermal genes examined, only expression of *Xsox17α* was initiated in VegT-depleted embryos, and then at a much reduced level. It will be important to determine if another maternal transcription factor is responsible for the onset of *Xsox17α* transcription.

Gene hierarchy initiated by maternal VegT

Our results lead us to propose a gene hierarchy for endoderm formation. Previously, we showed that maternal VegT directly activated *Xnr1* expression (Kofron et al., 1999). Our results suggest that Nodal signaling then initiates the expression of the early endodermal genes such as *Mixer* and *Gata5* in gastrulae. It is also possible that VegT directly activates expression of these genes, although the severe reduction of expression observed with cmXnr2 in this study indicates that TGFβ signaling plays an important role in their expression. Although *Mixer* and *Gata5* are both considered to be general endodermal markers, our results suggest they act in separable pathways, *Mixer* maintaining *Xsox17α* expression and *Gata5* initiating *Xlim1* expression in the early gastrula (stage 10.5) (Fig. 8A). Furthermore, the rescue experiments suggest that *Mixer* and *Gata5* cooperate to initiate *Xhex* and *Gata4* expression in the early gastrula. We also examined later endodermal markers in *Mixer* and *Gata5* mRNA-injected, VegT-depleted embryos at the taibud stage. We found that several genes including *endodermin*, *Gata6* and *Xsox17α* were not only rescued but even overexpressed at this stage. It is difficult to interpret these results since *Mixer* is not normally expressed in wild-type embryos at this time. However, it is likely that endodermal genes may be overexpressed because VegT-depleted embryos lack mesoderm, which may normally act as an inhibitory influence on endoderm formation.

Fig. 8B represents a more complete model of the interaction of these genes in early development, combining our current data with previously reported results. Because activin remains expressed in VegT-depleted embryos and endoderm does not form, it is unlikely to be important in the initiation of endoderm formation (Kofron et al, 1999). Maternal VegT directly initiates transcription of *Bix1*, *Bix4* and possibly other homeobox genes (Casey et al., 1999; Tada et al., 1998), as well as the nodals (represented as 'Xnr1' in Fig. 7B) (Chang and Hemmati-
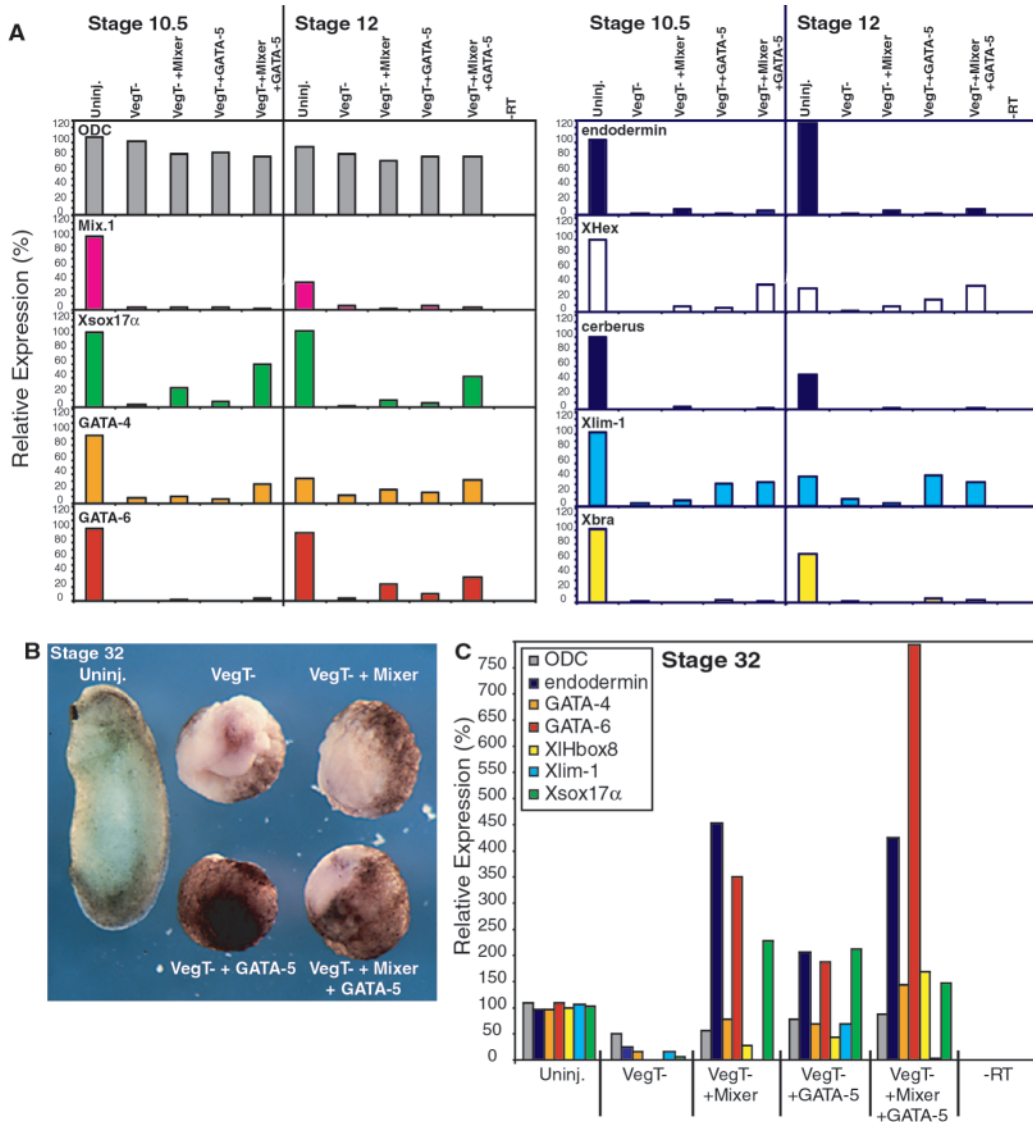
Fig. 7. Mixer and GATA5 mRNA injection rescue specific endodermal gene expression in VegT-depleted embryos. (A) VegT-depleted embryos injected with 600 pg Mixer and/or 600 pg GATA5 show rescued endodermal gene expression in early (stage 10.5) and late (stage 12) gastrula embryos. Mixer mRNA injected alone rescues expression of *Xsox17α* at the early gastrula (stage 10.5), and rescues *Gata4* and *Gata6* at the late gastrula (stage 12). GATA5 mRNA injection alone rescues *Xlim1* expression and shows a delayed rescue for *Xhex* (stage 12). Co-injecting Mixer (600 pg) and GATA5 (600 pg) mRNAs rescues expression 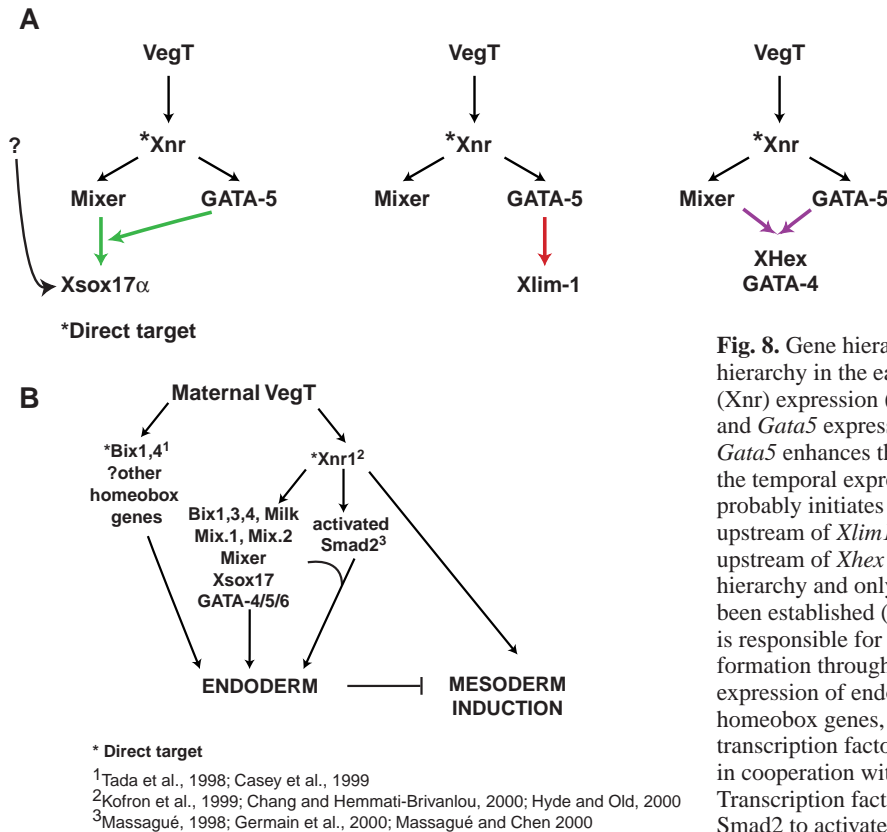of *Xsox17α*, *Gata4*, *Gata6*, *Xhex* and *Xlim1*. (B) Mixer and GATA5 mRNA injections do not rescue the phenotype of VegT-depleted embryos. VegT-depleted embryos rescued with Mixer mRNA and/or GATA5 mRNA resemble VegT-depleted embryos. (C) Rescued expression in later development (stage 32) differs from early development. Mixer mRNA injection rescues *endodermin*, *Gata4*, *Gata6* and *Xsox17α* expression. GATA5 mRNA injection rescues expression of these same genes as well as *Xlim1*. Injecting both Mixer and GATA5 mRNAs rescues *Xsox17α*, *Gata4*, *Gata6* and *Xlhbox8* expression.

Brivanlou, 2000; Hyde and Old, 2000; Kofron et al., 1999). As we have shown, zygotic TGFβs indirectly or directly activate expression of early endodermal transcription factors, which is consistent with previous work showing one of these genes, *Mix.2*, to be downstream of TGFβ signaling (Howell et al., 1999). The zygotic TGFβs also cause Smad activation (Massagué, 1998; Massagué and Chen, 2000; Faure et al., 2000), which then acts in concert with other vegetally expressed transcription factors such as Mixer to activate gene expression (Germain et al., 2000). We have found here that Xsox17α mRNA does not rescue endodermal gene expression, and Mixer mRNA only rescues *Xsox17α*, suggesting that they may require partners such as the Smads or β-catenin. Recently, it was shown that Xsox17α/β and Xsox3 physically interact with β-catenin (Zorn et al., 1999a), and that Mixer interacts with Smad2 (Germain et al., 2000). In contrast, ectopic expression of *Xsox17α* or *Mixer* in animal caps induces an array of endodermal markers (Henry and Melton, 1998; Hudson et al., 1997). This could be explained by inherent differences in the embryo's animal and vegetal regions.

Intercellular signaling and endoderm formation

Maternal VegT mRNA is localized to the vegetal hemisphere of the oocyte (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) and the protein is equally expressed in nuclei throughout the vegetal mass of the blastula stage embryo (Stennard et al., 1999). To what extent then is endoderm formation a cell-autonomous event since each cell inherits VegT? To study this, we first identified the major growth factors downstream of *VegT*, the TGFβs Xnr1, Xnr2, Xnr4 and Derrière, and tested their ability to rescue endodermal genes in VegT-depleted embryos. We found that they rescued all endodermal genes. Next we blocked signaling via these growth factors using nonsecreted mutant forms of Xnr2 and Derrière, and showed that this severely inhibited the expression of most endodermal genes, particularly when both Derrière and Xnr signaling was blocked. We conclude that only a basal level of activity of the early endodermal genes is initiated and maintained by cell-autonomous direct action of VegT. These results are in general agreement with cell dissociation studies (Chang and Hemmati-

A

*Direct target

B

* Direct target

[1]Tada et al., 1998; Casey et al., 1999
[2]Kofron et al., 1999; Chang and Hemmati-Brivanlou, 2000; Hyde and Old, 2000
[3]Massagué, 1998; Germain et al., 2000; Massagué and Chen 2000

Fig. 8. Gene hierarchy and network downstream of *VegT*. (A) Gene hierarchy in the early gastrula (stage 10.5). *VegT* initiates Nodal (Xnr) expression (Kofron et al., 1999) which contributes to *Mixer* and *Gata5* expression. *Mixer* regulates *Xsox17α* expression, and *Gata5* enhances the expression of *Xsox17α* (green arrows). Based on the temporal expression of *Xsox17α*, a different upstream activator probably initiates the expression of this gene. Alone, *Gata5* acts upstream of *Xlim1* (red arrow) and, in cooperation with *Mixer*, acts upstream of *Xhex* and *Gata4* (purple arrows). All arrows represent a hierarchy and only the direct interaction between VegT and Xnr has been established (asterisk) (Kofron et al., 1999). (B) Maternal VegT is responsible for initiating mesoderm induction as well as endoderm formation through zygotic TGFβs. These TGFβs activate the expression of endodermal transcription factors including the homeobox genes, GATA factors and *Xsox17*. Zygotic endodermal transcription factors such as Mixer and GATA5 can act alone and/or in cooperation with Smad2 to activate endodermal gene expression. Transcription factors such as Xsox17 may require a partner such as Smad2 to activate gene expression.

Brivanlou, 2000; Clements et al., 1999; Yasuo and Lemaire, 1999). However, we find that *Xsox17α* and *Mix.1* are blocked in their expression as severely as the other endodermal markers, while others found them to be enhanced (Clements et al., 1999) or less affected (Yasuo and Lemaire, 1999). A problem with disaggregation studies is that the manipulation itself may affect gene expression.

TGFβs may be involved in regulating gene expression in several ways. They may act as inducers, transmitting signals to adjacent cells. Alternatively, they could be involved in auto-regulatory loops, enhancing the expression of downstream genes within the same cell (Hyde and Old, 2000). To test the importance of cell/cell interactions in endodermal gene regulation we co-cultured explants of uninjected and VegT-depleted embryos and found that only a subset of endodermal genes were rescued, and then only to 20-50% of control levels. This indicates that while some induction happens between cells of the vegetal mass, it is not as robust as the mesodermal induction shown in previous experiments between equatorial and vegetal explants (Kofron et al., 1999), even when the co-culture period is extended. This difference may be explained in several ways. It is possible that only one part of the wild-type vegetal mass may have inducing activity, and this may not be sufficiently exposed in these experiments. It has been shown, for example, that the dorsal, and not the ventral, vegetal mass is able to induce cement gland formation in animal cap tissue (Jones et al., 1999). Alternatively, the lack of induction in the vegetal co-culture experiments may indicate that cell interaction plays only a minor role in switching on endodermal genes.

If the latter explanation is correct, why is there such an effective inhibition of endoderm formation by blocking TGFβ signaling? One explanation may be that the signals act over a very short range, and bind mainly to the secreting cell's own receptors to produce autoregulatory loops. The Smads downstream of these TGFβs would then transactivate endodermal genes directly or via VegT. Xnr1 and VegT have been shown to act in autoregulatory loops in other studies (Hyde and Old, 2000; Lustig et al., 1996; Zhang and King, 1996). Thus, the total expression level of genes such as *Bix4* is the combination of expression levels produced by three means: direct initiation due to VegT binding, indirect regulation via autoregulatory loops within one cell and by inductive interactions between neighboring cells.

Heterogeneity of gene expression within the endodermal germ layer

Although *VegT* is expressed apparently equally throughout the vegetal mass, several of its target genes are asymmetric in their expression patterns (Bouwmeester et al., 1996; Taira et al., 1992; Wright et al., 1988; Zorn et al., 1999b). Several factors may contribute to this. Firstly, zygotic transcription may begin at different times across the blastula, leading to waves of zygotic gene expression. Secondly, other localized factors may interact with VegT or its target genes. It has been suggested, for example, that interactions between β-catenin/Xtcf3 and VegT may regulate the expression of *Xhex* in the anterior endoderm (Zorn et al., 1999b). Certainly, *Xhex* expression is inhibited in both VegT-depleted and β-catenin-depleted embryos (Heasman et al., 2000). Also there is evidence of binding of both Smads and homeodomain transcription factors to the promoter of *goosecoid* (Germain et al., 2000). Thirdly,

VegT targets may interact with each other to alter the level of expression of downstream genes. Rescue experiments carried out here with genes downstream of VegT, *Mixer* and *Gata5*, illustrate this phenomenon. *Mixer* expression in VegT-depleted embryos rescued *Xsox17α* expression, but this was significantly enhanced when *Gata5* was co-expressed. The anterior endodermal gene, *Xhex*, is not rescued well by either *Gata5* or *Mixer* individually, but requires both for enhanced rescued expression. A final source of heterogeneity in the vegetal mass arises from the fact that although endodermal genes such as *Xsox17α*, *Gata5* and *Mixer* are expressed in the same general area, our rescue experiments show they have different functions. For example, *Gata5* alone of the three regulates *Xlim1* expression. Interestingly, even though many of the genes downstream of VegT including *Xlim1*, *cerberus*, *Gata5* and *Xhex* have been implicated in head formation, none of them rescue heads in VegT-depleted embryos (Fig. 7B and data not shown). It seems likely that gastrulation movements and mesoderm formation are pre-requisites for head formation. This work illustrates that endoderm formation proceeds from a very simple beginning, and rapidly becomes complex, as a heterogeneous network of zygotic transcription factors and signaling molecules are activated downstream of *VegT* at mid-blastula transition.

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