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Direct and indirect regulation of *derrière*, a *Xenopus* mesoderm-inducing factor, by VegT

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SUMMARY

One candidate for an endogenous mesoderm-inducing factor in *Xenopus* is derrière, a member of the TGFβ family closely related to Vg1. In this paper we first show that derrière is able to exert long-range effects in the early *Xenopus* embryo, reinforcing the view that it functions as a secreted factor required for proper formation of posterior structures. Analysis of the *derrière* promoter shows that expression of the gene is controlled through a complex inductive network involving VegT and TGFβ-related molecules and also, perhaps, FGF family members. The work confirms that *derrière* plays an important role in mesoderm formation and it illustrates the complex regulation to which inducing factors are subject.

Key words: *Xenopus*, Mesoderm induction, derrière, TGFβ family, T-box family, VegT, Smad, Fast

INTRODUCTION

The mesoderm of the amphibian embryo is formed through an inductive interaction in which cells of the vegetal hemisphere of the late blastula act on overlying equatorial cells (Sive, 1993; Heasman, 1997). The strongest candidates for natural mesoderm-inducing signals include members of the transforming growth factor β (TGFβ) family, and particularly the *nodal-related* genes *Xnr1* and *–2* and *–4* to *–6* and *derrière* (Jones et al., 1995; Joseph and Melton, 1997; Sun et al., 1999; Takahashi et al., 2000). These genes are expressed at the right times and in the right places in the embryo to act as endogenous inducing factors, and inhibition of their effects, by various means, causes defects in mesodermal and endodermal differentiation (Osada and Wright, 1999; Sun et al., 1999; Agius et al., 2000; Onuma et al., 2002).

Although these factors play important roles in the early embryo, rather little is known about the transcriptional regulation of the *nodal-related* genes or of *derrière*. One of the most significant observations in this respect concerns the requirement for VegT in their activation. VegT is a maternally expressed member of the T-box family whose transcripts are restricted to the vegetal hemisphere of the *Xenopus* egg and early embryo (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). Ablation of maternal VegT transcripts causes loss of endodermal and mesodermal structures, and expression of the *nodal-related* genes and of *derrière* does not occur (Zhang et al., 1998; Kofron et al., 1999; Takahashi et al., 2000). VegT is a transcriptional activator, and it is possible that the *nodal-related* genes and *derrière* number among its direct targets. However, only *Xnr1* has been shown to contain T-box binding sites in its promoter, and these sites appear not to be required for the vegetal expression of a reporter gene driven by the *Xnr1* promoter (Hyde and Old, 2000).

We have investigated the transcriptional regulation of *derrière*. This gene has been little-studied compared with the *nodal-related* genes, yet is a strong candidate for an endogenous inducing agent: it is expressed in the right cells, inhibition of its function impairs mesoderm development (Sun et al., 1999), and in this paper we show that derrière, unlike, for example, Xnr2, is able to exert long-range effects in the developing embryo. Our results show that *derrière* is subject to complex regulation involving not only VegT but also members of the TGFβ family and perhaps FGF family members. The combined effects of these different signals, acting in a series of autoregulative loops, may help ensure the rapid activation of mesoderm-inducing agents at the mid-blastula transition.

MATERIALS AND METHODS

Xenopus embryos and microinjection

Xenopus embryos were obtained by artificial fertilisation and maintained in 10% normal amphibian medium (NAM) (Slack, 1984). They were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Embryos at the one- or two-cell stage were injected

with RNA dissolved in 10 nl water as described by Smith (Smith, 1993). For animal cap assays, embryos were dissected and cultured in 75% NAM. Dexamethasone was dissolved in ethanol to a concentration of 2 mM and then diluted to a final concentration of 1 μM in 75% NAM containing 0.1% bovine serum albumin. Protein synthesis inhibition experiments were performed as described by Smith et al. (Smith et al., 1991), except that cycloheximide was applied continuously at a concentration of 10 μM. This treatment was sufficient to reduce incorporation of [^{35}S]methionine into isolated animal pole regions by over 90%.

Animal cap recombinants were performed as described previously (Jones et al., 1996). Conjugates were fixed when sibling embryos reached stage 10.5 and expression of Xbra (Smith et al., 1991) was analysed by in situ hybridisation.

Constructs

pSP64T.*VegT-GR* was produced by fusing the ligand-binding domain of the human glucocorticoid receptor (hGR) to the C terminus of VegT, thereby creating a construct similar to the previously published Xbra-GR (Tada et al., 1997). Details are available on request. A mouse activin A cDNA (Albano et al., 1993) was cloned into pSP64T to create pSP64T.*mactivinA*. pCS2-*derrière* (Sun et al., 1999), pSP64T.*Xnr2* (Jones et al., 1995) pΔXAR (Hemmati-Brivanlou and Melton, 1992), pXFD/Xss (Amaya et al., 1991), pd50 (Amaya et al., 1991) and p*lacZ* (Kolm et al., 1997) were as described. Capped RNAs were synthesised using SP6 RNA polymerase.

To obtain *derrière* promoter sequence, a *Xenopus* genomic library, prepared in the vector λFIXII (Stratagene), was screened using a probe corresponding to the first 356 base pairs of the *derrière* open reading frame. Restriction digestion and Southern blotting identified a 2 kb *Xba*I fragment, which was sub-cloned into pBluescriptII (SK) and sequenced. This fragment (d1.2.1) consists of the 247 base pair exon 1 of *derrière* preceded by 850 base pairs of upstream sequence and 844 base pairs of intron (see Fig. 2A). The transcription start site was mapped by RNAase protection and is located approximately 30 base pairs downstream of a TATA box.

For luciferase assays, the 5′ 850 base pairs of d1.2.1 were amplified by PCR and sub-cloned into the *Mlu*I and *Bgl*II sites of the vector pGL3-basic (Promega). For transgenesis, the same fragment was sub-cloned into pGL3-GFP, a modified version of pGL3-basic in which the luciferase coding region is replaced by the GFP coding region (Casey et al., 1999). Deletion constructs were produced by PCR. In vitro mutagenesis was carried out by a PCR-based approach. Details are available on request.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as described by Trindade and colleagues (Trindade et al., 1999). Proteins used in EMSAs were produced by in vitro translation of synthetic RNA. pSP64TBX.VegT-HA (Conlon et al., 2001) was linearised with *Sal*I and transcribed with SP6 RNA polymerase and pFTX9-XlFast1 (Howell et al., 1999) was linearised with *Xba*I and transcribed with T7 RNA polymerase.

Luciferase assays

Luciferase assays were performed using the Promega Dual-Luciferase assay kit. Embryos were injected with 20 pg pGL3-basic containing the appropriate promoter fragment, 20 pg pRL-SV40/TK, and an appropriate amount of RNA encoding either β-galactosidase or the inducing agent being tested. Animals caps were dissected at stage 8.5 and cultured in 75% NAM for the desired period. They were then suspended in 10 μl of 1× Passive Lysis Buffer per cap and after centrifugation 5 μl was taken for assay. Oocytes were suspended in 20 μl 1× Passive Lysis Buffer per oocyte and 20 μl was taken for assay. All values are expressed as Relative Luciferase Units (Firefly luciferase activity/Renilla luciferase activity).

Transgenesis

Transgenic *Xenopus* embryos were created as described by Sparrow and colleagues (Sparrow et al., 2000), itself a modified version of the original protocol of Kroll and Amaya (Kroll and Amaya, 1996).

Whole-mount in situ hybridisation

In situ hybridisations were carried out essentially as described previously (Harland, 1994), except that BM purple was used as a substrate. *derrière* (Sun et al., 1999) and *VegT* (Zhang and King, 1996) probes were as described. A GFP construct (mgfp5) (Zernicka-Goetz et al., 1996) in pBluescriptII(SK) was linearised with *Nco*I and a *Xenopus Brachyury* construct (pXT1) (Smith et al., 1991) was linearised with *Bgl*II. Both were transcribed with T7 RNA polymerase in the presence of digoxigenin-11-UTP (Roche). β-galactosidase staining was performed as described previously (Kolm and Sive, 1995).

RNA isolation and RNAase protection assays

RNA was prepared from pooled animal caps using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNAase protection analysis was carried out essentially as described by Jones and colleagues (Jones et al., 1992), using RNAase T1 alone for all probes. A *derrière* probe was made by cloning a PCR fragment of *derrière* (nucleotides 775-939) into pBluescriptII, linearising with *Not*I and transcribing with T7 RNA polymerase. Probes for *Bix4* (Casey et al., 1999) and *ornithine decarboxylase* (Isaacs et al., 1992) were as described.

RESULTS

derrière, like activin but unlike Xnr2, can exert long-range effects in the *Xenopus* embryo

derrière is a member of the TGFβ superfamily that is expressed in the vegetal hemisphere of the *Xenopus* embryo from the late blastula stage. Mis-expression of *derrière* can induce isolated animal pole regions to form mesoderm, and the factor is therefore a strong candidate for an endogenous mesoderm inducing agent (Sun et al., 1999). Consistent with this idea, *derrière* is expressed in the same domain as *VegT* (Fig. 1), a maternal transcript that is necessary both for mesoderm induction and for zygotic activation of *derrière*.

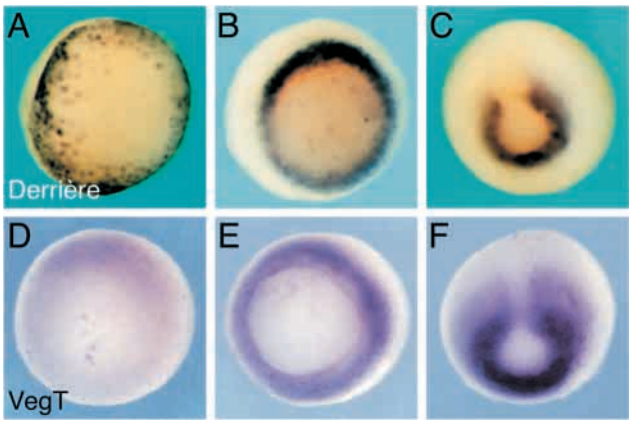

Fig. 1. Comparison of the expression patterns of *derrière* (A-C) and *VegT* (D-F) at stages 9 (A,D), 10.5 (B,E) and 12 (C,F). Embryos were fixed at the indicated stages and processed for in situ hybridisation. Note the similarities between the expression patterns of the two genes.

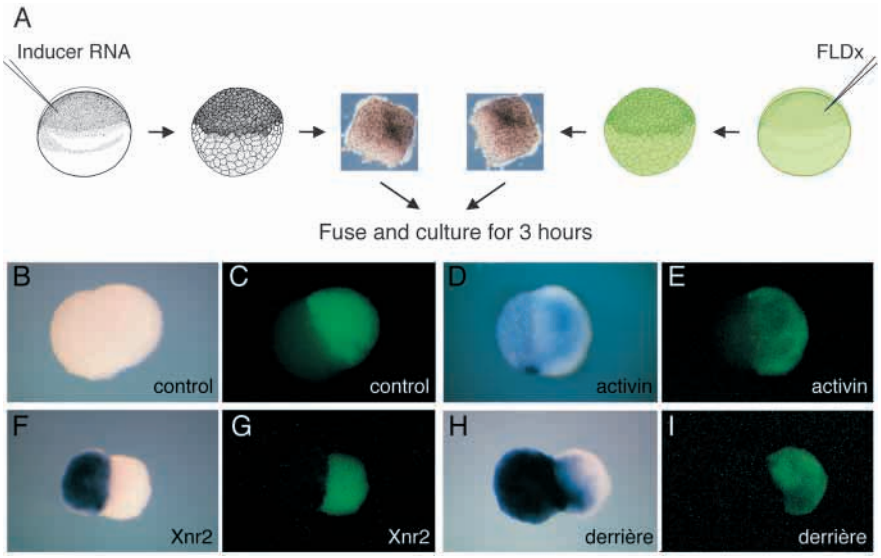

Fig. 2. Derrière, like activin, but unlike Xnr-2, can exert long-range effects in *Xenopus* tissue. (A) The experimental design. Animal pole regions dissected from embryos uniformly labelled with the cell lineage marker fluorescein-lysine-dextran (FLDx) were juxtaposed with animal pole regions derived from embryos injected with RNA encoding activin (5 pg), derrière (500 pg), or Xnr-2 (500 pg). Caps were cultured for 3 hours and then examined by whole-mount in situ hybridisation for expression of *Xbra* (B,D,F,H) or by fluorescence microscopy for detection of FLDx (C,E,G,I). (B,C) A control conjugate in which an FLDx-labelled animal cap was juxtaposed with an uninjected cap. *Xbra* is not activated. (D,E) A conjugate in which an FLDx-labelled animal cap was juxtaposed with an cap derived from an embryo injected with RNA encoding activin. Note induction of *Xbra* in FLDx-labelled tissue, as previously described (Gurdon et al., 1994; Jones et al., 1996). (F,G) A conjugate in which an FLDx-labelled animal cap was juxtaposed with an cap derived from an embryo injected with RNA encoding Xnr-2. Induction of *Xbra* is restricted to unlabelled tissue (see Jones et al., 1996). (H,I) A conjugate in which an FLDx-labelled animal cap was juxtaposed with a cap derived from an embryo injected with RNA encoding derrière. Note induction of *Xbra* in both unlabelled and FLDx-labelled tissue. In this respect the pattern of *Xbra* expression differs from that induced by activin (see text).

The phenomenon of mesoderm induction was discovered in an assay in which animal pole tissue was induced to form mesoderm following juxtaposition with vegetal blastomeres (Nieuwkoop, 1969). According to this assay, a mesoderm inducing factor should be capable of acting in a non cell-autonomous fashion. That is, the signal should be able to activate mesoderm-specific gene expression some cell diameters away from its site of production.

The signalling range of derrière was tested by the technique employed by Jones and colleagues (Jones et al., 1996). Animal pole regions derived from embryos injected with RNA encoding derrière were juxtaposed with animal caps dissected from embryos injected with the lineage label fluorescein-lysine-dextran (FLDx). As controls, caps were derived from embryos injected with RNA encoding activin, which is known to exert long-range effects, or with RNA encoding Xnr2, which in this assay acts essentially cell-autonomously (Jones et al., 1996). The conjugates were cultured for 3 hours and then fixed and analysed for expression of *Xbra* by in situ hybridisation. Fig. 2 shows that derrière, like activin and unlike Xnr2, can activate expression of *Xbra* in FLDx-labelled cells, indicating that it can exert long-range effects.

Interestingly, the pattern of *Xbra* activation in *derrière*-expressing conjugates differs from that in *activin*-expressing conjugates. In the former, *Xbra* is activated throughout the *derrière*-expressing animal cap and appears to spread from there into the FLDx-labelled tissue. In the latter, and as described by others, *Xbra* is most strongly induced in the FLDx-labelled animal cap, in a domain that presumably corresponds to a particular concentration of activin. We are now investigating whether this difference is due to different diffusion properties of the two factors (see Ohkawara et al., 2002) or to their different concentration-dependent effects. Whatever the explanation, the results show clearly, at least in this over-expression system, that *derrière* is capable of exerting long-range effects in the *Xenopus* embryo.

derrière is an immediate-early target of VegT

Ablation of mRNA encoding the vegetally localised transcription factor VegT prevents zygotic expression of *derrière* in the *Xenopus* embryo, indicating that VegT is essential for expression of *derrière* during late gastrula stages of development (Kofron et al., 1999). Is *derrière* a direct target of VegT? To answer this question we constructed a hormone-inducible version of VegT termed VegT-GR, in which the ligand-binding domain of the glucocorticoid receptor is fused to the C terminus of VegT. This construct is inactive unless the steroid hormone dexamethasone (DEX) is added to the embryo culture medium (data not shown). In combination with the protein synthesis

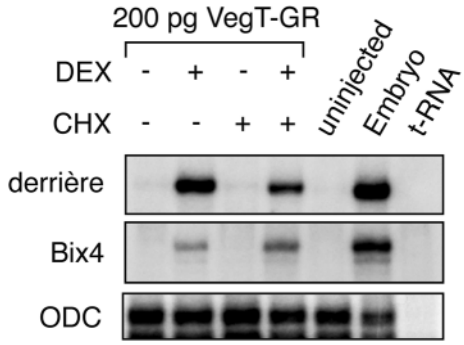

Fig. 3. *Derrière* is a direct target of VegT. VegT-GR, a hormone-inducible form of VegT, was injected into *Xenopus* embryos at the one-cell stage and the embryos were allowed to develop to the late blastula stage. Animal caps were dissected from these embryos and they were incubated in dexamethasone (DEX), cycloheximide (CHX) or both. After 3 hours of culture they were analysed for expression of *derrière* or *Bix4* by RNAase protection. *Ornithine decarboxylase* (*ODC*) was used as a loading control. Note that both *derrière* and *Bix4* are induced by DEX in the presence of CHX, but that CHX causes a decrease in levels of activation of *derrière*.

Fig. 4. T-box and Fast binding sites in the *derrière* promoter. (A) Schematic diagram of the *derrière* genomic fragment obtained in this work. It comprises 851 base pairs 5′ of the first exon, the whole of exon 1 (247 base pairs) and 844 base pairs of the first intron. F1 and F2 represent the putative Fast sites and Tbs1 and Tbs2 the putative T box sites. (B) Partial sequence of the *derrière* promoter region. The transcription start site is indicated by an arrow and the beginning of the protein coding region is shown in lower case. T box sites Tbs1 and Tbs2 are underlined, as are two matches to the consensus Fast binding site (F1 and F2). (C) Comparison of Tbs1 and Tbs2 with the T box binding site deduced from binding site selection experiments (Kispert and Herrmann, 1993; Conlon et al., 2001).

inhibitor cycloheximide (CHX), this construct allows one to examine the ability of VegT to activate *derrière* directly.

RNA encoding VegT-GR was injected into *Xenopus* embryos at the 1-cell stage. Animal pole regions were then dissected at the late blastula stage, and groups of 15-20 animal caps were incubated in the absence of factors, in DEX or CHX alone, or in both reagents. *Derrière* proved to be activated by dexamethasone even in the presence of cycloheximide (Fig. 3), indicating that the effects of VegT do not require intervening protein synthesis. This observation is consistent with the suggestion that VegT acts directly to induce expression of *derrière*. We note, however, that the level of activation of *derrière* by VegT-GR is reduced by cycloheximide, and this does not occur with the induction of *Bix4* (Fig. 3). As we discuss below, one explanation of this observation is that optimal activation of *derrière* by VegT involves some indirect effects.

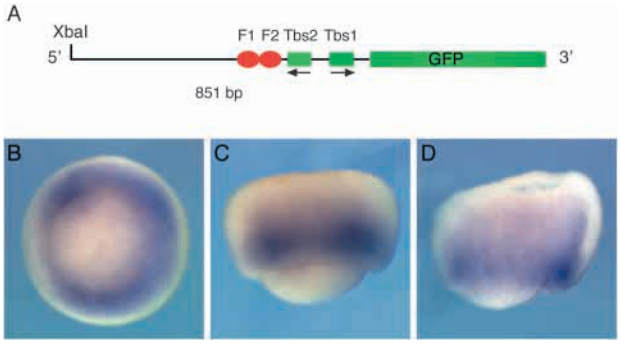

Fig. 5. The *derrière* 5′ regulatory region drives mesendodermal expression of a GFP reporter gene. (A) The reporter construct. (B-D) Views of transgenic *Xenopus* embryos showing expression of GFP by in situ hybridisation. (B) Stage 10.5 embryo, vegetal view. Expression of GFP is detectable in the mesoderm and (weakly) in the endoderm. (C) Dorsal view of the embryo shown in B. (D) Lateral view of a stage 10.5 embryo with dorsal blastopore lip to the right. This embryo was bisected before the staining procedure. Note that expression of the reporter construct differs from that of the endogenous gene in that transcripts persist in the involuted mesoderm.

The *derrière* promoter contains two potential T-box binding sites

As a first step towards confirming the idea that VegT acts directly on the *derrière* promoter, we isolated a genomic fragment comprising the whole of the first exon of *derrière* flanked by 851 nucleotides of 5′ sequence and 844 nucleotides of intron 1 (Fig. 4A; GenBank accession number AF527059). Consistent with the idea that VegT activates expression of *derrière* directly, we noticed two sites resembling a T-box ‘half site’ (Kispert and Herrmann, 1993; Conlon et al., 2001) positioned 90 (Tbs1) and 169 (Tbs2) nucleotides 5′ of the transcription start site (Fig. 3B,C). We also noted the presence of two Fast binding sites at 194 and 209 nucleotides 5′ of the transcription start site.

To determine whether sequence 5′ of the *derrière* transcription start site can drive correct expression of a reporter gene, we placed this region upstream of green fluorescent protein (GFP) and made transgenic *Xenopus* embryos (Fig. 5A). Expression of the reporter was not strong enough to be observed by GFP fluorescence, but it was readily detectable by in situ hybridisation using a GFP probe. As is observed with the endogenous gene (Fig. 1A-C), expression of the reporter construct is strongest in the marginal zone, but it is also weakly detectable in the vegetal hemisphere (Table 1; Fig. 5B,C and

Table 1. Expression patterns of wild-type *derrière* reporter constructs and reporter constructs in which one or both T box sites are mutated

Reporter construct	Cases (number of experiments)	Embryos with no reporter gene expression	Embryos with expression in mesendoderm (%)
pGFP-d851	211 (6)	90	121 (57.3)
pGFP-d851ΔTbs1	118 (3)	55	63 (53.4)
pGFP-d851ΔTbs2	97 (3)	43	54 (55.7)
pGFP-d851ΔTbs1,2	153 (3)	66	87 (56.9)

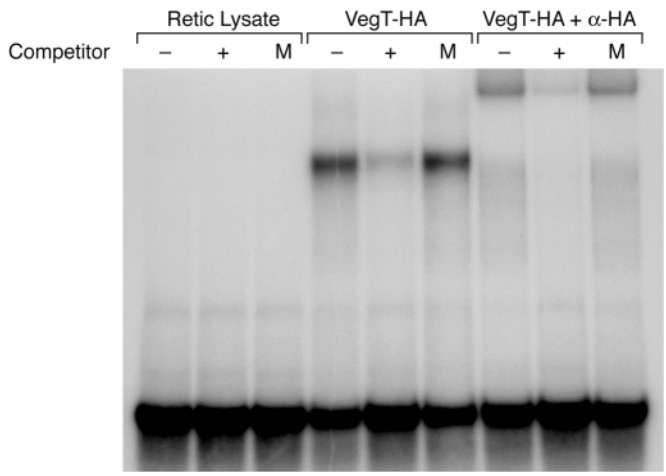

Fig. 6. Electrophoretic mobility shift assay demonstrating that VegT binds Tbs1. A ^{32}P-labelled 36 base pair probe including Tbs1 was incubated with uncharged reticulocyte lysate (lanes 1-3) or with in vitro translated HA-tagged VegT (lanes 4-9). Assays in lanes 2, 5 and 8 included an excess of unlabelled probe as competitor (+) and assays in lanes 3, 6 and 9 included an excess of unlabelled mutated probe (M). Supershift assays in lanes 7-9 included a rat anti-HA monoclonal antibody, with incubation carried out for 10 minutes at 4°C. A specific shift is visible in lanes 4 and 6, and these are 'supershifted' in lanes 7 and 9.

data not shown). One difference from the endogenous expression pattern, however, is that expression of the transgene persists in involuted mesoderm (Fig. 5D). This might reflect perdurance of GFP RNA; alternatively, our construct may lack an element that is responsible for down-regulation of *derrière* following involution.

VegT interacts with the *derrière* promoter

Electrophoretic mobility shift assays were used to test the possibility that VegT regulates expression of *derrière* through direct interaction with Tbs1 or Tbs2. VegT proved to interact with the Tbs1 site (Fig. 6), but poorly, if at all, with Tbs2 (data not shown). These observations are consistent with the fact that Tbs1 is a better match to the consensus T box binding sequence derived from binding site selection experiments (Kispert and Herrmann, 1993; Conlon et al., 2001) than is Tbs2 (Fig. 4C). Results described below, however, suggest that Tbs2 may nevertheless play a role, albeit minor, in the regulation of *derrière*.

Tbs1 and 2 are not necessary for VegT mediated induction in animal caps

The results described above suggest that VegT regulates expression of *derrière* through direct interaction with the its 5′ regulatory region. Consistent with this idea, we observe that VegT can drive expression of a luciferase reporter gene placed downstream of the *derrière* promoter. Constructs were injected into *Xenopus* embryos at the 1- to 2-cell stage in the presence or absence of VegT RNA. Animal caps then were dissected at the mid blastula stage and cultured to the early gastrula stage when they were assayed for luciferase activity. VegT caused a 25- to 30-fold induction of luciferase expression (Fig. 7A,B). Xbra and Eomes are also able to activate this construct (data not shown) and therefore may also contribute to *derrière* regulation, although this has yet to be investigated.

To investigate the roles of Tbs1 and Tbs2 in the activation of *derrière* reporter constructs, the sequences were mutated so as to disrupt T box binding (Fig. 7A). Single mutations, or mutation of both binding sites, proved to have no effect on the ability of VegT to activate the construct (Fig. 7B). Furthermore, when such constructs were introduced into *Xenopus* embryos by transgenesis, expression still occurred in endoderm and mesoderm (Table 1; Fig. 7C-F). Thus, although VegT might be capable of activating expression of *derrière*

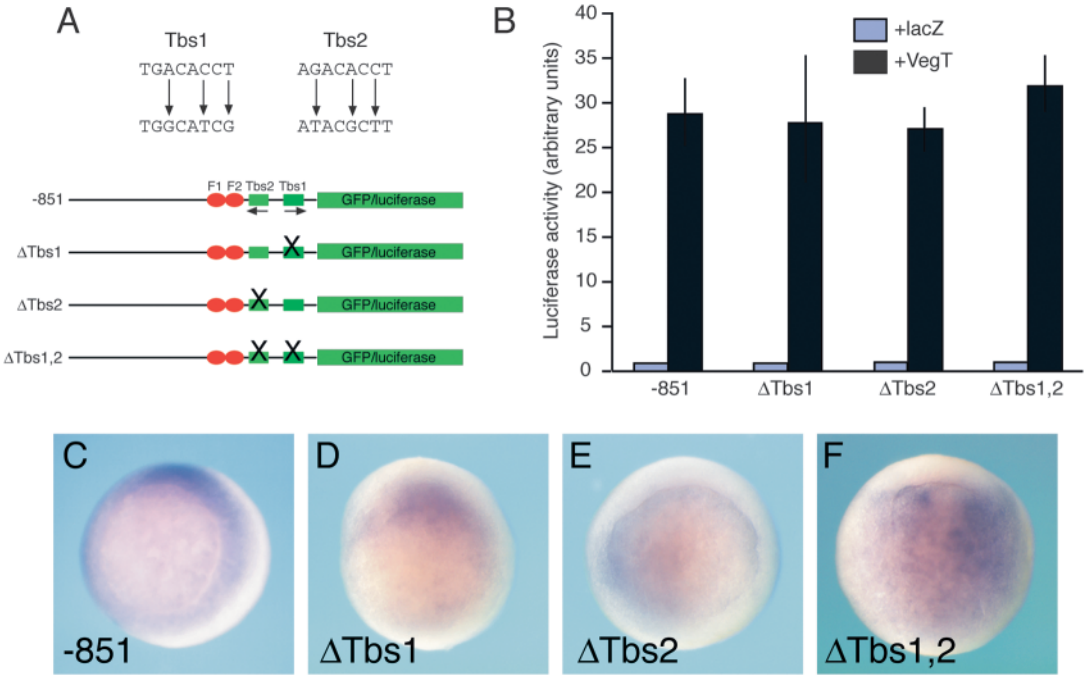

Fig. 7. Mutational analysis of the putative T-box binding sites in the *derrière* promoter. (A) Diagrams showing the constructs used and the mutations made in the T-box binding sites. (B) Luciferase assay in animal caps showing that induction of the firefly luciferase reporter gene is independent of the T-box sites. (C-F) Vegetal views of stage 10.5 transgenic *Xenopus* embryos expressing the indicated transgenes. Note that all four constructs are expressed in the mesendoderm. Expression of the ΔTbs1,2 construct in the case illustrated in F is higher in the endoderm than the mesoderm, but this is not a consistent observation.

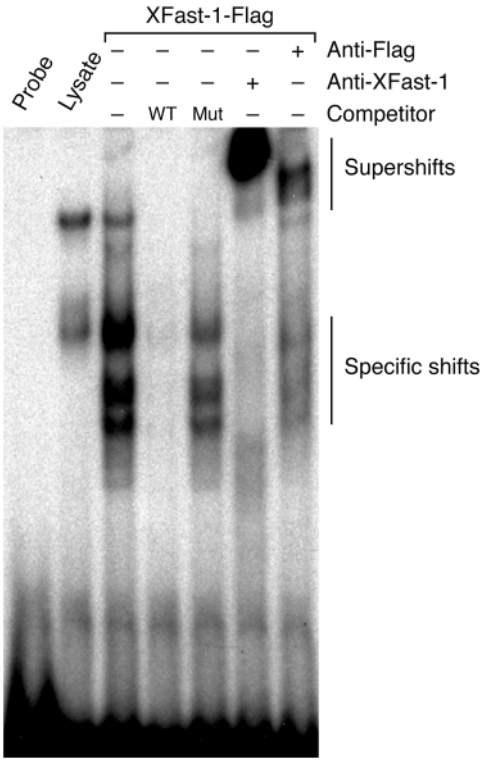

Fig. 8. Electrophoretic mobility shift assay demonstrating that Fast-1 binds the Fast sites identified in Fig. 4. A ^{32}P-labelled 36 base pair probe including both Fast sites was incubated with uncharged reticulocyte lysate (lane 2) or with in vitro translated Flag-tagged Xfast-1 (lanes 4-7). The assay in lane 4 included an excess of unlabelled probe as competitor (WT) and the assay in lane 5 included an excess of unlabelled mutated probe (Mut). Supershift assays included an anti XFast-1 antibody (lane 6) and an anti Flag antibody (lane 7).

directly, it also appears to be able to induce transcription indirectly.

The *derrière* promoter responds to TGFβ signals through Fast sites

VegT activates the expression of the *nodal-related* genes *Xnr1-4* as well as that of *derrière* itself (Clements et al., 1999; Kofron et al., 1999; Sun et al., 1999; Yasuo and Lemaire, 1999). One possibility, therefore, is that the indirect induction of *derrière* reporter constructs by VegT occurs through activation of TGFβ family members such as these. Consistent with this idea we have identified two potential Fast sites in the *derrière* 5′ regulatory region (Fig. 4), and electrophoretic mobility shift assays confirm that these do indeed bind Fast-1 (Fig. 8).

Further experiments demonstrated that the TGFβ family member activin can activate a *derrière* reporter construct in isolated animal pole regions, and results obtained with a deletion series of the *derrière* 5′ regulatory region are consistent with the idea that this occurs through the Fast sites (Fig. 9A). We also note, however, that mutation of the Fast sites does not abolish activin responsiveness completely (Fig. 9B), and one possibility is that activation of the reporter construct by TGFβ family members can also occur indirectly, through the induction of T box family members such as *Xbra* (Smith et al., 1991), *eomesodermin* (Ryan et al., 1996) and *Antipodean* (Stennard et al., 1996). Indeed, mutation of the T box sites in addition to the Fast sites prevents activin-induced activation of luciferase activity (Fig. 9B).

Autoregulation of the *derrière* promoter

The above results suggest that activation of the *derrière* promoter by VegT can occur indirectly, through the induction of TGFβ family members, which in turn act through the Fast

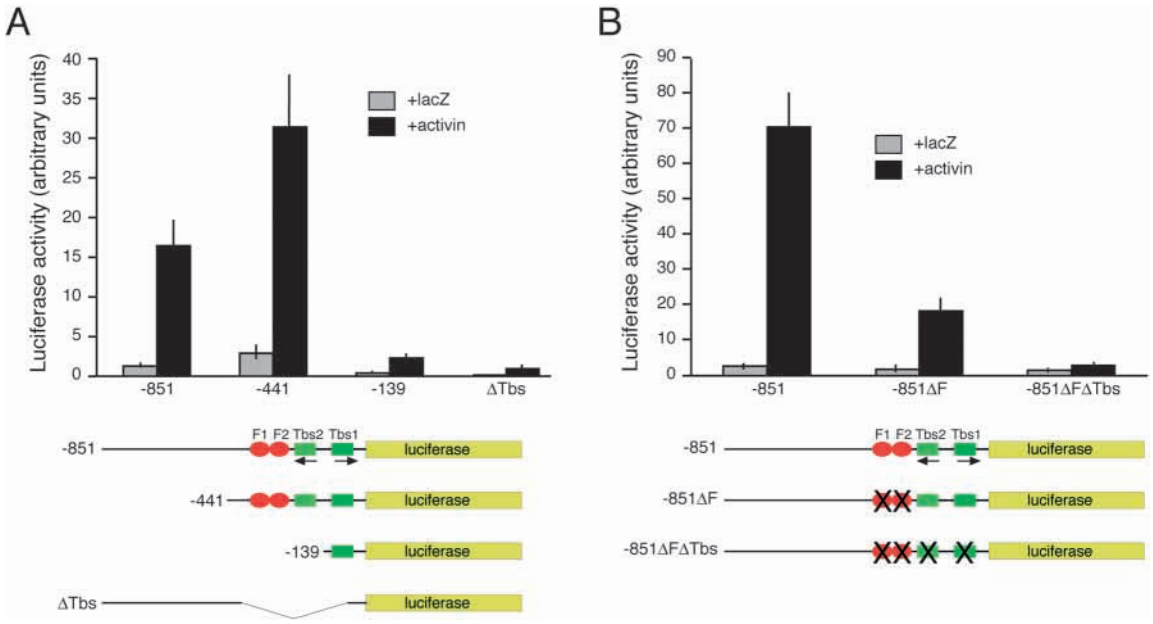

Fig. 9. The *derrière* 5′ regulatory region responds to activin. (A) Deletion analysis shows that an activin-responsive element lies between –441 and –139 nucleotides in a region that includes the two Fast sites. (B) Targeted mutation of the T box and Fast sites suggests that activin induction can also occur in an indirect fashion, through the T box sites.

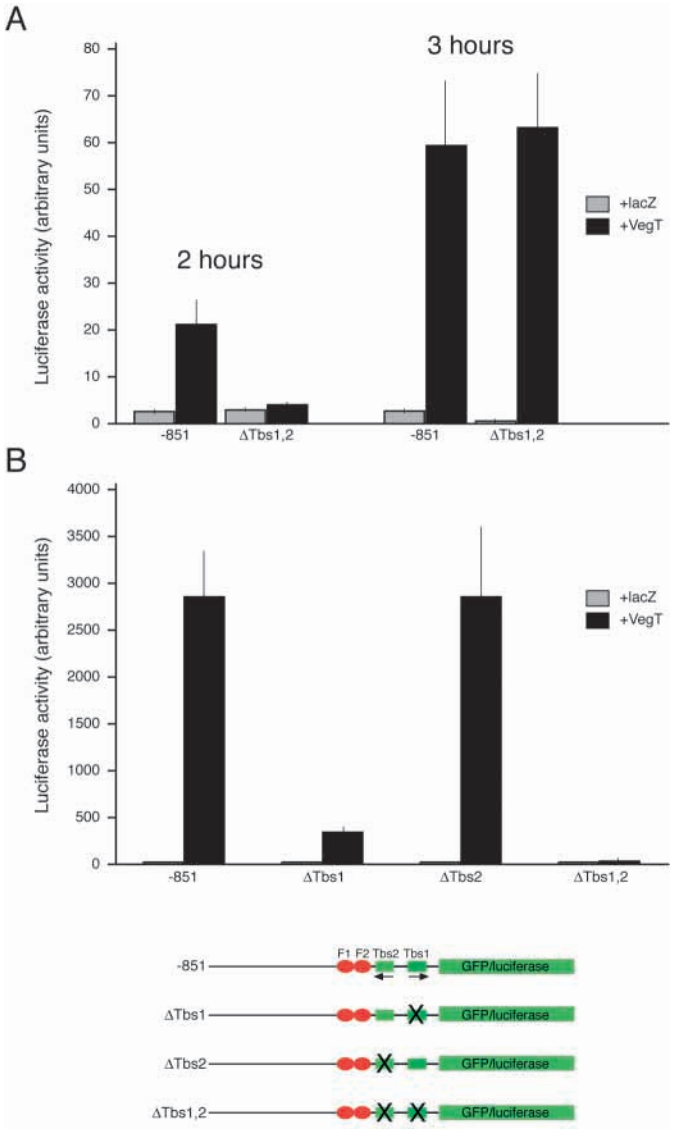

Fig. 10. (A) The initial phase of reporter gene activation in response to VegT requires intact T box sites. The indicated reporter constructs were injected into *Xenopus* eggs at the one-cell stage along with the VegT RNA or, as a control, lacZ RNA. Animal caps were dissected at stage 9 and cultured for 2 or 3 hours, as indicated. Analysis of luciferase activity shows that reporter gene activity was abolished by mutation of the T box sites in the 2-hour time-point, but not in the 3-hour time-point. (B) Activation of the indicated reporter constructs in *Xenopus* oocytes. Mutation of Tbs1 causes a dramatic reduction in luciferase activity and mutation of both sites abolishes the ability to respond to VegT.

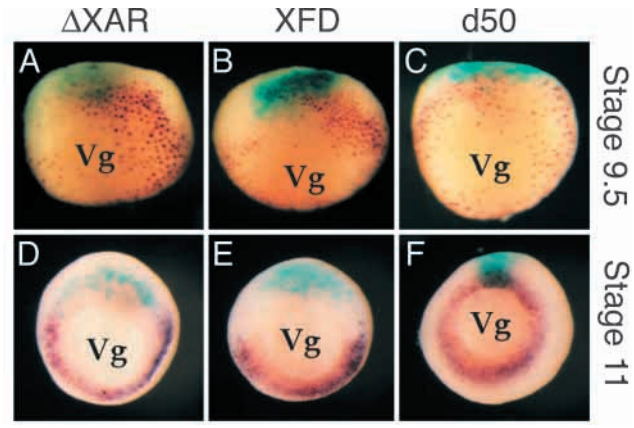

Fig. 11. Inhibition of activin-like or FGF signalling does not interfere with activation of *derrière* but does prevent its maintenance. Embryos were injected at the two-cell stage with 800 pg RNA encoding the truncated activin receptor ΔXAR (A,D), the truncated FGF receptor XFD (B,E), or the control construct d50 (C,F). RNA encoding β-galactosidase was co-injected as a cell lineage label in all cases. Embryos were allowed to develop to stage 9.5 (A-C) or 11 (D-F) and expression of *derrière* was analysed by in situ hybridisation. The initial activation of *derrière* is not affected by inhibition of activin-like (A) or FGF (B) signalling; note the overlap of β-galactosidase staining (blue) with the in situ hybridisation reaction product (purple), which at this early stage is largely nuclear. Continued expression of *derrière*, however, does require activin-like and FGF signalling; note down-regulation of expression in D and E. Vg, vegetal pole.

sites we identified (Fig. 4). One prediction of this model is that if one were to analyse reporter gene activation at an earlier stage, before the indirect effects had occurred, mutation of the T box sites should abolish the response to VegT. Another prediction is that if one were to analyse reporter gene activation in oocytes rather than in animal caps, thereby preventing TGFβ-mediated indirect effects, mutation of the T box sites should again abolish the response to VegT. Fig. 10 shows that these predictions are realised. Analysis of reporter gene expression 2 hours after dissection of animal pole regions reveals that mutation of the two T box sites reduces significantly the ability of VegT to induce luciferase activity, while dissection at 3 hours reveals no difference between the wild-type and mutated constructs (Fig. 10A). Similarly, mutation of Tbs1 and Tbs2 prevents the response of a *derrière* reporter construct to VegT in oocytes (Fig. 10B). Mutation of Tbs1 in this assay has a more dramatic effect than does mutation of Tbs2, suggesting that much of the induction is mediated by Tbs1 (Fig. 10B).

This scheme suggests that the initial activation of *derrière* occurs in response to VegT alone, and that amplification and maintenance of expression (including activation in the marginal zone) involves signalling by members of the TGFβ family and perhaps other factors (see Clements et al., 1999; Yasuo and Lemaire, 1999). To investigate the importance of such factors in the maintenance of *derrière* expression during normal development, RNA encoding the dominant-negative activin receptor ΔXAR (Hemmati-Brivanlou and Melton, 1992), or the truncated FGF receptor XFD (Amaya et al., 1991), or the control construct d50 (Amaya et al., 1991) was injected into one blastomere of embryos at the two-cell stage along with RNA encoding β-galactosidase as a lineage marker (Fig. 11). Embryos were fixed at late blastula stage 9.5 or mid-gastrula stage 11 and examined for expression of *derrière* by in situ hybridisation. The dominant-negative receptors only weakly inhibited activation of *derrière* at stage 9.5, with expression occurring in 75% of XFD-expressing embryos (n=32) and in 81% of ΔXAR-expressing embryos (n=37; Fig. 11A-C). However, they completely prevented maintenance of expression to stage 11 (n=26 for XFD-

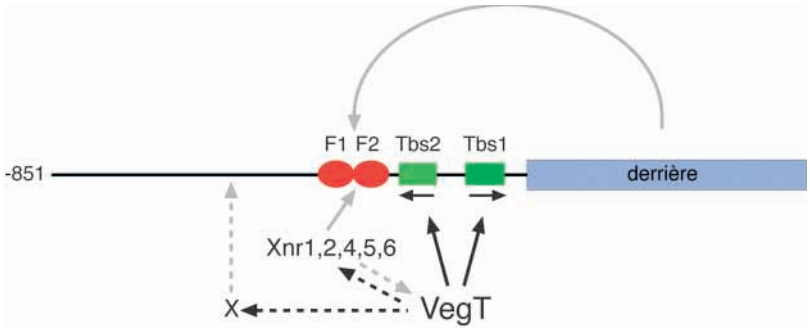

Fig. 12. A network of interactions in the regulation of *derrière*. Solid black arrows indicate direct actions of VegT and dotted black arrows indicate effects of VegT that may be indirect. Solid grey arrows indicate probable direct interactions of derrière and nodal-related gene products on the *derrière* promoter, which occur after the initial effects of VegT. Dotted grey arrows indicate the effects of gene X on the *derrière* promoter and of the nodal-related proteins on the *VegT* 5′ regulatory region, causing the activation of *Antipodean*. Gene X is yet to be identified, but may represent a member of the FGF family. See text for details.

expressing embryos and n=19 for ΔXAR-expressing embryos; Fig. 11D-F).

DISCUSSION

derrière is a candidate for an endogenous mesoderm-inducing factor in *Xenopus*

Although it is clear that members of the TGFβ family play important roles in mesoderm induction in the *Xenopus* embryo, little is known about the different functions of the many TGFβs that are expressed in the early embryo, or about the regulation of their expression. In this paper we turn our attention to *derrière*. Previous work using a dominant-negative *derrière* construct has demonstrated that the function of this gene is required for posterior mesoderm formation (Sun et al., 1999), and our own preliminary experiments using an antisense *derrière* morpholino oligonucleotide are consistent with this suggestion, although the phenotype is milder than is observed with the dominant-negative version of the protein (R. J. W. and J. C. S., data not shown). It is possible that there are additional copies of *derrière* in the *Xenopus* genome that are inhibited by the dominant-negative construct but not by the antisense oligonucleotide. Alternatively, the dominant-negative derrière construct may not be completely specific, perhaps inhibiting to some extent the effects of members of the *nodal-related* family (Sun et al., 1999).

One noteworthy feature of derrière is that it is able to exert long-range effects in our animal cap assay, whereas Xnr2, for example, is not. In this regard, we note that although the N-terminal region of derrière contains a group of four basic amino acids (KKRR), this is not as extensive a domain as the basic region in BMP4 that interacts with heparan sulphate proteoglycans and thereby restricts its signalling range (Ohkawara et al., 2002). The ability of derrière to exert long-range effects in the *Xenopus* embryo, together with results obtained following inhibition of its activity (see above) mark this protein out as a strong candidate for an endogenous mesoderm-inducing factor, and makes analysis of its regulation all the more significant.

derrière is a direct target of VegT

Experiments using a hormone-inducible version of VegT and cycloheximide suggest that *derrière* is a direct target of VegT (Fig. 3). Our results indicate, however, that cycloheximide does cause some reduction in the level of activation of *derrière* by VegT-GR, a phenomenon we do not observe with induction of *Bix4* (Fig. 3). This reduction is consistent with the conclusion of this paper that the activation of *derrière* by VegT also involves indirect effects, mediated in part by the induction of TGFβ family members.

The direct action of VegT on the 5′ regulatory region of *derrière* is likely to occur through the T box sites highlighted in Fig. 4. These sites are required for the early activation of a *derrière* reporter construct in isolated animal pole regions (Fig. 10A) and for the activation of such constructs in *Xenopus* oocytes, in which indirect activation does not occur (Fig. 10B). Consistent with binding site selection experiments (Kispert and Herrmann, 1993; Conlon et al., 2001), the site designated Tbs1 (TGACACCT) proved to interact more strongly with VegT in electrophoretic mobility shift assays than did Tbs2 (AGACACCT) (Fig. 6). We note that the nucleotide that differs between the two sites (the first ‘T’ in Tbs1 becomes an ‘A’ in Tbs2) is directly contacted by Xbra in the crystal structure of that protein (Muller and Herrmann, 1997).

Mutation of Tbs1 did not, however, completely prevent reporter gene activation in response to VegT in *Xenopus* oocytes (Fig. 10B), indicating that Tbs2 may also play a role in the activation of *derrière* by VegT. It is possible that the two sites cooperate to ensure the normal regulation of the gene, in a manner resembling that suggested to occur in the regulation of *Ci-trop* in *Ciona intestinalis* (Di Gregorio and Levine, 1999). This is under investigation.

The *derrière* promoter is also subject to indirect regulation

Although the T box sites in the *derrière* promoter are required for the early response to VegT and for activation in a system such as the *Xenopus* oocyte where indirect effects do not occur, they are not required for later responses. Thus, mutation of the T box sites does not prevent the vegetal activation of *derrière* reporter constructs in transgenic *Xenopus* embryos (Fig. 7C-F), and nor does it prevent induction by VegT of similar constructs in animal pole regions following culture for 3, rather than 2 hours (Fig. 7B). Our results suggest that the activation of *derrière* reporter constructs under these conditions occurs indirectly, due, at least in part, to the activation of members of the TGFβ family such as the *nodal-related* genes and perhaps even *derrière* itself. Thus activin, a member of the TGFβ family, can induce expression of endogenous *derrière* and can activate the expression of *derrière* reporter constructs. This inducing

activity is likely to occur through the Fast sites, as shown in Fig. 4; deletion analysis demonstrates that loss of the Fast sites reduces significantly the ability of activin to induce reporter gene expression (Fig. 9A), although there may be indirect effects at work here too, because there remains some residual activity that is substantially abolished by deletion of the T box sites (Fig. 9B). It is possible that while VegT can exert indirect effects through the activation of TGFβ family members, TGFβ family members can exert indirect effects through the activation of T box genes. These might include *Xbra* (Smith et al., 1991) and *eomesodermin* (Ryan et al., 1996), as well as *Antipodean* (Stennard et al., 1996), itself an alternatively spliced isoform of VegT (Stennard et al., 1999).

Investigation of this indirect pathway will require carefully timed experiments that make use of specific inhibitors of particular members of the TGFβ family. Our initial experiments along these lines indicate that a truncated activin receptor does not prevent the initial activation of *derrière* but does inhibit its maintenance (Fig. 11). Interestingly, a recent report making using of dominant-negative versions of *Xnr5* and *Xnr6* found no evidence of down-regulation of *derrière* at any stage, even though the *Xnr5* construct also inhibited the functions of *Xnr2*, *Xnr4*, *Xnr6*, *derrière* itself and *BVg1* (Onuma et al., 2002). This suggests that maintenance of *derrière* expression can occur through Xnr1 or activin signalling.

Other regulatory elements may also play a role in *derrière* regulation

The data described so far suggest that VegT and members of the TGFβ family participate in a network of autoregulatory loops (see Fig. 12). But VegT and members of TGFβ family are unlikely to be the only members of this network, and we may not have identified all the regulatory elements in the *derrière* promoter. For example, expression of our reporter construct persists in involuted mesoderm (Fig. 5D); as discussed above, this might reflect perdurance of GFP RNA, but it may be that our constructs lack an element that is responsible for the down regulation of *derrière* following involution. Furthermore, we find that the late response to VegT, and the expression of reporter constructs in transgenic embryos, is not abolished (and if anything is slightly elevated) even if both the Fast sites and the T-box sites are mutated (data not shown). This suggests that there may be another gene 'X' that is regulated by VegT and which acts on the *derrière* promoter to enhance and maintain its expression (Fig. 12). Candidates for such a gene include members of the FGF family; inhibition of FGF function does not inhibit the initial expression of *derrière* but does prevent its continued expression (Fig. 11).

Why should *derrière* be subject to such complicated regulation? One possibility is that a network of autoregulatory interactions of this sort might facilitate the rapid changes in gene expression that occur during early *Xenopus* development. Another idea is that the network might act to restrict later expression of *derrière* to the mesoderm rather than to the mesendoderm (Sun et al., 1999). If this were the case, gene X may be represented by *eFGF* (Isaacs et al., 1992), whose function might be to maintain expression of *derrière* in the marginal zone.

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