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Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser

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SUMMARY

The maternal dorsal determinants required for the specification of the dorsal territories of *Xenopus* early gastrulae are located at the vegetal pole of unfertilised eggs and are moved towards the prospective dorsal region of the fertilised egg during cortical rotation. While the molecular identity of the determinants is unknown, there are dorsal factors in the vegetal cortical cytoplasm (VCC). Here, we show that the VCC factors, when injected into animal cells activate the zygotic genes *Siamois* and *Xnr3*, suggesting that they act along the Wnt/β-catenin pathway.

In addition, *Siamois* and *Xnr3* are activated at the vegetal pole of UV-irradiated embryos, indicating that these two genes are targets of the VCC factors in all embryonic cells. However, the consequences of their activation in cells that occupy different positions along the animal-vegetal axis differ. Dorsal vegetal cells of normal embryos or VCC-treated injected animal cells are able to dorsalise ventral mesoderm in conjugate experiments but UV-treated vegetal caps do not have this property. This difference is unlikely to reflect different levels of activation of FGF or activin-like signal transduction pathways but may reflect the activation of different targets of *Siamois*. *Chordin*, a marker of the head and axial mesoderm, is activated by the VCC/*Siamois* pathway in animal cells but not in vegetal cells whereas *cerberus*, a marker of the anterior mesendoderm which lacks dorsalising activity, can only be activated by the VCC/*Siamois* pathway in vegetal cells. We propose that the regionalisation of the organiser during gastrulation proceeds from the differential interpretation along the animal-vegetal axis of the activation of the VCC/β-catenin/*Siamois* pathway.

Key words: *Siamois*, *Xnr3*, *chordin*, *noggin*, *cerberus*, *Xenopus*, regionalisation, Organiser, maternal dorsal determinants, Wnt, cortex

INTRODUCTION

Establishment of a correct vertebrate body plan relies on the formation of a functional Spemann's organiser by the early gastrula stage (reviewed by Lemaire and Kodjabachian, 1996). Therefore, understanding the molecular mechanisms underlying the formation of this structure has been a major goal of modern embryology. At the early gastrula stage, Spemann's organiser is located in the dorsal marginal zone of amphibian embryos, in a territory fated to form dorsoanterior mesoderm. Recent evidence has indicated that two types of information are involved in the formation of the organiser: mesoderm induction and a dorsal pathway triggered by cortical rotation.

Mesoderm induction is mediated by polypeptide growth factors which probably belong to the TGF-β or FGF families. These factors are required for the induction of the correct amount of mesoderm (Hemmati-Brivanlou and Melton, 1992; Amaya et al., 1991) and cooperate with an independent dorsal pathway to give rise to dorsal mesoderm (Kimelman et al., 1992).

The dorsal pathway is likely to make use of components of the Wnt signalling pathway (reviewed by Kuhl and Wedlich, 1997). Ventral overexpression of several Wnt family members or of downstream components of their transduction pathway such as Xdsh and β-catenin, leads to the formation of an ectopic organiser. Conversely, inactivation of β-catenin or of the maternal transcription factor Xtcf-3, homologous to the *Drosophila* Wg transducer pangolin (Brunner et al., 1997), leads to the ventralisation of *Xenopus* embryos. Consistent with these findings, β-catenin is translocated to the nucleus in a broad dorsal domain during the blastula stages (Schneider et al, 1996; Larabell et al., 1997). Several zygotic targets of this pathway have been identified (Carnac et al., 1996; Yang-Snyder et al., 1996; Brannon and Kimelman, 1996; Ryan et al., 1996; Fagotto et al., 1997). Among these, *Siamois* encodes a homeodomain transcription factor with strong dorsalising activity (Lemaire et al., 1995), *Xnr-3* codes for a TGF-β family member (Smith et al., 1995) and *chordin* and *noggin* code for inhibitors of Bmp4 protein (Smith and Harland, 1992; Sasai et al., 1994; Piccolo et al., 1996; Zimmermann et al., 1996).

Siamois and *Xnr3* are activated very early and might be direct targets of the Wnt pathway. In addition, Fan and Sokol (1997) showed that a fusion protein between the N-terminal repressor domain of *Drosophila* engrailed and the DNA-binding domain of *Siamois* acted as a dominant repressor mutant of *Siamois* and suppressed axis formation, thus suggesting that *Siamois* activity is required for the formation of the organiser.

In contrast, activation of *chordin* may be indirect as this gene is activated later during development (Sasai et al., 1994; Ryan et al., 1996) and is a target of *Siamois* (Carnac et al., 1996; Fan and Sokol, 1997).

These results have greatly improved our understanding of the early events during axis formation in *Xenopus* but have also left several issues unresolved. The first one concerns the identity of the endogenous maternal determinants that initiate the cascade of events leading to the stabilisation and translocation of β-catenin. Overexpression of a dominant negative form of Xdsh, which acts upstream of β-catenin in the Wnt pathway, does not prevent the formation of the organiser (Sokol, 1996), suggesting that the endogenous maternal determinants that regulate β-catenin may not belong to the Wnt family. These determinants are located at the vegetal pole before cortical rotation and subsequently relocated to the future dorsal side of the embryo as cortical rotation proceeds (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993; Kikkawa et al., 1996; Sakai, 1996). A more precise analysis of the subcellular localisation of the determinants has revealed that they are associated with the cortex (Kageura, 1997). Furthermore, transplantation of vegetal cortical cytoplasm (VCC) from fertilised eggs into ventral blastomeres is sufficient to induce formation of a secondary set of axial structures (Holowacz and Elinson, 1993; Fujisue et al., 1993). In these experiments, the VCC, like Wnts or noggin, has no mesoderm inducing activity but can synergise with FGF to give rise to more dorsoanterior tissue (Holowacz and Elinson, 1995). These results suggest that the dorsal factors present in the VCC are the maternal dorsal determinants. However, a direct link between the VCC factors and the Wnt/β-catenin pathway has not been reported.

A second unresolved issue concerns the regionalisation of the dorsal territories. Following cortical rotation, maternal dorsal determinants are translocated to a large domain on the prospective dorsal side of the embryo (reviewed by Elinson and Holowacz, 1995), a process that can be witnessed by the translocation of β-catenin to the nucleus at the mid-blastula stage. By the mid-gastrula stage, the dorsal territories have been regionalised in at least three functional domains: anterior endomesoderm (Bouwmeester et al., 1996), head organiser, and trunk/tail organiser (reviewed by Lemaire and Kodjabachian, 1996). How the early broad dorsal domain is regionalised is not known.

In this study, we have addressed these two issues. We first show that the VCC factors activate the Wnt/β-catenin pathway as demonstrated by the activation of *Siamois* and *Xnr3*. We then present data indicating that the activation of the Wnt/β-catenin pathway is interpreted differently along the animal-vegetal axis. In response to the activation of this pathway, vegetal cells acquire an anterior endomesodermal fate, marked by the expression of *cerberus* (Bouwmeester et al., 1996), while more animal cells adopt a head/trunk organiser fate, marked by the expression of *chordin*.

MATERIALS AND METHODS

Embryo manipulations and injections

Embryos were in vitro fertilized, dejellied, UV treated and cultured in 0.1× MBS as previously described (Lemaire et al., 1995). For cell lineage studies, 4.6 nl of fluorescein lysinated dextran or rhodamine lysinated dextran (FLDx, 10 mg/ml in water; RLDx, 5 mg/ml in water; Molecular Probes, Inc., Eugene, OR) were injected into both blastomeres at the two-cell stage or into the two ventral blastomeres at the four-cell stage. Cytoplasmic transplantations were carried out essentially as described in Holowacz and Elinson (1993). Briefly, 30-40 nl of animal or vegetal cortical cytoplasm was taken from UV-treated fertilized eggs and injected into two animal blastomeres of eight-cell embryos, which were used as donors of the animal caps. To overexpress *noggin*, *chordin* or *Siamois* in tissue explants, 4.6 nl of synthetic mRNA diluted in water were injected into two animal or vegetal blastomeres at the two to four-cell stage. Synthetic mRNAs for *Siamois*, *noggin* and *chordin*, were prepared as described by Carnac et al. (1996). Synthetic mRNAs for *v-ras* and *tAR* were prepared according to the methods of Whitman and Melton (1992) and Hemmati-Brivanlou and Melton (1992).

Construction of pBSRN3 enR-Sia

pBSRN3 enR-Sia was constructed as follows: the region encoding the N-terminal engrailed repressor domain (aa 1-298) was PCR-amplified with the oligonucleotides en-F (5′- CGG AAT TCA ACT TTG GCC ATG GCC CTG GAG GAT CGC TGC -3′; the underlined sequence contains a cloning *Eco*RI site placed in front of the initiator methionine of engrailed) and en-R (5′- GGA TCC CAG AGC AGA TTT CTC -3′). The region encoding amino acids 135-208 from Siamois, corresponding to the homeodomain and a few flanking amino acids, was PCR-amplified with the two oligonucleotides SiaEV-F (5′- CCA GAG AAA TCT GCT CTG GGA TCC TCT CCA GCC ACC AGT A -3′) and SiaEV-R (5′- ATA AGA ATG CGG CCG CTA CTG GGG AGA GTG GAA AGT GG -3′, the underlined sequence introduces a cloning *Not*I site and an in frame stop codon). The two amplified fragments were mixed and reamplified with the two oligos en-F and SiaEV-R. Because of the partially complementary sequences of en-R and SiaEV-F, a fragment of 1141 bp was amplified, corresponding to a fusion protein between the N-terminal repressor domain of engrailed and the homeodomain of Siamois. This fragment was cloned into the *Eco*RI/*Not*I sites of pBluescriptRN3 (Lemaire et al., 1995) to generate pBSRN3 enR-Sia. To prepare mRNA for enR-Sia, pBSRN3 enR-Sia was linearised with Sfi1 and synthetic capped mRNA was synthesised from the T3 promoter.

Tissue explants combinations

All explants were cut in 1× MBS with no. 5 Dumont forceps. Marginal zone explants were cut at stage 10-10.25. Vegetal explants were cut out at stage 8.5 and comprised cells located within a 30° arc of the vegetal pole. Animal caps were excised at stage 8.5. Recombination between ventral marginal zone explants and animal or vegetal caps were carried out according to Carnac et al. (1996). Conjugates were cultured in 1× MBS with 0.2% BSA until control sibling embryos reached the appropriate stage.

Immunohistochemistry and in situ hybridisation

Detection of muscle with the monoclonal antibody 12/101 was performed as described by Carnac et al. (1996) for Figs 5 and 6. 12/101 was detected (see Fig. 4) using lissamine rhodamine-conjugated donkey anti-mouse IgG (1:100 dilution, BIO/CAN Sci. Inc., Mississauga, Ontario). In situ hybridisation on 10 μm embryo sections were performed as previously described (Lemaire et al., 1995) except that the posthybridisation RNase treatment was omitted. The *Xnr3* and *chordin* probes were synthesized using T7 polymerase from *Eco*RI-

linearised pKSfug (Ecochard et al., 1995) and pSKChd (Sasai et al., 1994) plasmids respectively.

RT-PCR analysis

For the experiments shown in Figs 1 and 6B, RNA was extracted by the proteinase K/LiCl method according to Sambrook et al. (1989). In all other experiments, embryos or tissue pieces were homogenised in NETS buffer (0.3 M NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% SDS). Following two rounds of phenol/chloroform extractions, total nucleic acids were ethanol precipited and resuspended in water. Oligo(dT)-primed first strand cDNA was prepared from the equivalent of 4-12 caps or 1 embryo using Superscript II reverse transcriptase (Gibco-BRL). A negative control excluding enzyme was included for each sample. 1/3 to 1/20 of the cDNA obtained was used for each PCR reaction which was carried out using 0.5 unit of Taq DNA polymerase (Promega), 10 pmol of each primer, 100 μM dNTPs, 3 mM $MgCl_2$, 0.5 μCi of [α-^{32}P] dATP in a 25 μl volume of 1× PCR buffer. During amplification the temperature profile of each cycle was: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. For all genes studied except *cerberus*, 29 amplification cycles were performed. For *cerberus*, 25 cycles were performed. In these conditions, the signal obtained was a linear function of the imput cDNA (data not shown). As an internal loading control, PCR primers for the ubiquitously expressed FGF-R1 gene were included in all PCR reactions (Lemaire and Gurdon, 1994). 1/8 to 1/3 of the PCR reaction was loaded on a 6% sequencing gel which was subsequently dried and autoradiographed.

To avoid confusion between amplification of genomic DNA and cDNA, we chose, for each gene, PCR primers that flanked an intron. The sequences of the *Xbra* and FGF-R1 primers were described previously (Lemaire and Gurdon, 1994). The others primers were as follows: *Siamois* forward 5′- AAA CCA CTG ATT CAG GCA GAG G -3′, reverse 5′- GTA GGG CTG TGT ATT TGA AGG G -3′; *Xnr-3* forward 5′- GTG AAT CCA CTT GTG CAG TT-3′, reverse 5′-ACA GAG CCA ATC TCA TGT GC -3′; *chordin* forward 5′- CTG TAC CAA CCC AAT CCG TGC C -3′, reverse 5′- CTT GGT GCA ACA TCT GTC CCG C -3′; *cerberus* forward 5′- GCT TGC AAA ACC TTG CCC TT -3′, reverse 5′- CTG ATG GAA CAG AGA TCT TG -3′.

RESULTS

Vegetal cortical cytoplasm induces *Siamois* and *Xnr3* expression in animal cap cells

The dorsal factor in vegetal cortical cytoplasm (VCC) behaves as a competence modifier like Xwnt-8 or noggin rather than as a dorsal mesoderm inducer (Holowacz and Elinson, 1995), suggesting that the dorsal factor in VCC may function to activate a Wnt signalling cascade. If this is the case, injection of the VCC dorsal factor in animal caps should induce the two Wnt targets genes *Siamois* and *Xnr3* but not the early trunk mesodermal marker *Xbra* (Smith et al., 1991).

To test this idea, 8-cell embryos were injected with VCC in the animal blastomeres and cultured until stage 10. Animal caps were excised and analysed for *Xnr3*, *Siamois* and *Xbra* expression at stage 10.5 by semi-quantitative RT-PCR (see Materials and Methods). As maternal mRNA for *Siamois* and *Xnr3* are present at very low levels (Lemaire et al., 1995; Smith et al., 1995) the presence of *Siamois* or *Xnr3* transcripts in injected animal caps reflects the activation of the genes rather than a possible carry-over of maternal mRNAs in the injected cortical cytoplasm. As shown in Fig. 1, *Xnr3* and *Siamois* mRNAs were present in VCC-injected caps but not in caps injected with animal cortical cytoplasm (ACC). In contrast, *Xbra* was activated neither by the VCC nor by the ACC, confirming that the VCC lacks mesoderm-inducing activity (Holowacz and Elinson, 1995). Comparison of the levels of expression of *Siamois* and *Xnr3* in VCC-injected caps and whole embryos showed that *Siamois* and *Xnr3* were activated at similar levels in both situations.

These experiments demonstrate that injection of VCC into animal cap cells is sufficient to activate two targets of the Wnt/β-catenin pathway, *Siamois* and *Xnr3* in the absence of detectable trunk mesoderm.

Xnr3 and *Siamois* are activated in cortical cells at the vegetal pole of UV irradiated embryos

We next wanted to ask whether the inheritance of the VCC factors by vegetal cells would lead to the activation of the same dorsal genetic programme at the early gastrula stage. To test this, we UV-irradiated fertilised eggs, thus preventing cortical rotation and causing the dorsal determinants to remain at the vegetal pole of these embryos.

We first examined if *Siamois* and *Xnr3* were expressed in whole irradiated embryos at the early gastrula stage (Stage 10.5) (Figure 2A). Like *Siamois*, which was previously reported to be expressed in UV-treated embryos (Brannon and Kimelman, 1996; Cui et al., 1996), *Xnr3* was also activated in these embryos when assayed by semi-quantitative RT-PCR (Fig. 2). Activation of these two genes in UV-irradiated embryos did not reflect a general activation of all organiser

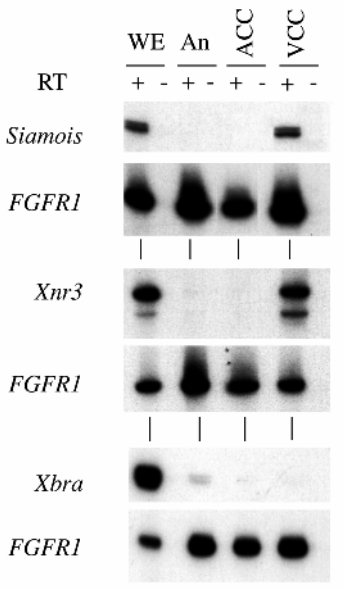

Fig. 1. Animal caps injected with vegetal cortical cytoplasm express *Siamois* and *Xnr3* but not *Xbra*. Animal caps from embryos injected with vegetal cortical cytoplasm (VCC) or animal cortical cytoplasm (ACC) were analysed by RT-PCR at stage 10.5 for the expression of *Siamois*, *Xnr3* and *Xbra*. Endogenous *FGFR1* transcripts were used here as an internal standard. Analysis of the same samples by RNase protection gave similar results, as did independent northern analysis of *Xnr3*. + and – refers in this and the following figures to the presence or absence of reverse transcriptase, as a control for DNA contamination. WE, whole embryo; An, uninjected animal caps.

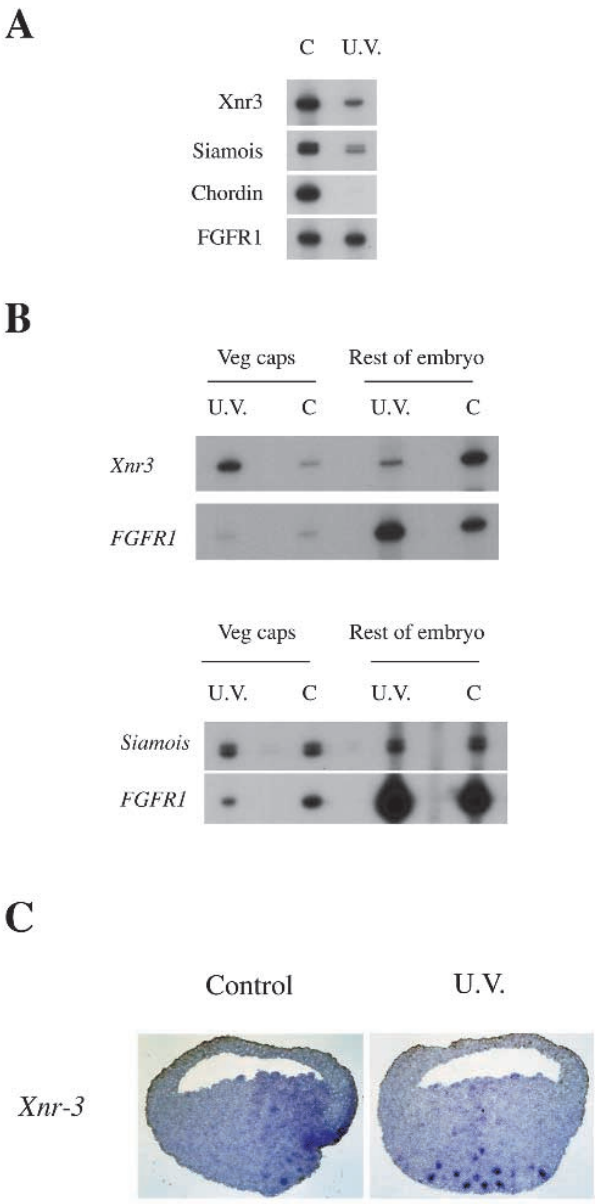

Fig. 2. Expression of *Xnr3* and *Siamois* in UV-irradiated gastrulae. (A) Expression in whole embryos. Fertilised eggs were irradiated and the expression of *Xnr3*, *Siamois* and the *FGFR1* genes was assayed by RT-PCR at stage 10.25. In the experiment shown, the average dorsoanterior index (DAI) of the embryos was 0.27 at the tadpole stage. C, control embryo; U.V., irradiated embryo. (B) Distribution of the transcripts in control (C) or UV-irradiated (U.V.) early gastrulae (DAI=0.26). Vegetal pole explants were dissected at stage 10, and RNA from both these explants and the rest of the embryos was prepared at stage 10.25. Analysis of transcript abundance was carried out by RT-PCR. *Siamois* and *Xnr3* transcripts are enriched at the vegetal pole of UV-irradiated embryos. (C) In situ hybridisation with an *Xnr3* antisense RNA probe on sectioned control or UV-irradiated early gastrulae. Due to a better penetration of the probe on sectioned embryos, it can be observed that the distribution of *Xnr3* transcripts in normal early gastrulae is not restricted to the epithelial outer cell layer, but extends into the deep marginal and vegetal cells. In UV treated embryos, *Xnr3* transcripts are detected in the perinuclear space of cortical cells. A similar result was obtained with a *Siamois* probe (not shown). Note that in normal embryos, vegetal expression of *Xnr3* extends nearly down to the vegetal pole.

genes as *chordin* was not activated in the UV-irradiated embryos. To determine the localisation of the transcripts for *Xnr3* and *Siamois*, vegetal poles of normal or UV-irradiated embryos were explanted at stage 10 and analysed by RT-PCR at stage 10.25 (Fig. 2B). *Xnr3* transcripts, like those of *Siamois* (Brannon and Kimelman, 1996; Cui et al., 1996), were mainly present at the vegetal pole of UV-irradiated embryos (Fig. 2B). Furthermore, analysis by *in situ* hybridisation of the precise distribution *Siamois* and *Xnr3* transcripts confirmed that they were radially distributed at the vegetal pole and showed that *Xnr3*, like *Siamois*, is most strongly activated in the cells of the surface layer of the embryo (Fig. 2C and not shown). As these cells have probably inherited the vegetal cortical cytoplasm, this distribution strengthens the idea that expression of *Xnr3* and *Siamois* is a consequence of the inheritance of factors present in the VCC. Since vegetal pole cells are larger than dorsal marginal zone cells, fewer cells inherit the dorsal determinants in UV-irradiated embryos than in normal embryos, thus providing an explanation for the reduced expression of *Siamois* and *Xnr3* in irradiated embryos (Fig. 2A). Taken together, these results indicate that *Xnr3* and *Siamois* are targets of the VCC in cells of both animal and vegetal origin.

The activation of organiser genes by VCC factors in animal and vegetal pole cells prompted us to test the dorsalising properties conferred by dorsal determinants to animal and vegetal pole cells.

VCC-injected animal caps have the ability to dorsalise ventral marginal zone explants but UV-irradiated vegetal caps do not

Carnac et al. (1996) showed that animal cap cells injected with either *Siamois* RNA or *Xwnt-8* RNA secrete a dorsalising signal. As the vegetal cortical factor behaves similarly to *Xwnt-8* RNA, we expected that the cortical dorsal factor would also induce animal cap cells to secrete a dorsalising signal.

To test this possibility, we injected VCC into animal blastomeres of eight-cell embryos and conjugated stage 8.5 animal caps from these embryos to pieces of ventral marginal zone (VMZ) from early gastrulae which had been labelled with the fluorescent lineage tracer, fluorescein lysinated dextran (FLDx). The conjugates were cultured until the equivalent of stage 32 and analysed for the presence of muscle by immunostaining with the 12/101 antibody (Kintner and Brockes, 1984). Animal caps from embryos injected with ACC or uninjected embryos were used as controls. A schematic representation of this protocol is presented in Fig. 3A.

As shown in Table 1 and Fig. 3B, 58.5% of the conjugates with VCC-injected animal caps contained muscle in the VMZ explant. However, muscle was rarely seen in the conjugates with ACC-injected or noninjected animal caps, and notochordal tissue was not morphologically detectable in either case. These results indicate that VCC-injected animal cap cells, like animal cap cells in which the Wnt/β-catenin pathway has been activated, secrete a dorsalising signal which respecifies VMZ into muscle cells.

Like VCC-injected animal caps, vegetal poles from UV-irradiated embryos and dorsal vegetal cells from normal embryos express *Siamois* and *Xnr3* in response to the inheritance of maternal dorsal determinants. To test their ability to dorsalise

ventral marginal zone explants, we conjugated stage 10 VMZ explants with (i) stage 8.5-9 vegetal poles from UV or control embryos and (ii) stage 8.5-9 dorsal vegetal explants (see schematic representation in Fig. 4A). As a positive control, the dorsalising properties of stage 10 dorsal marginal zone (DMZ) explants was also analysed.

Unlike VCC-injected animal caps, we found that vegetal poles from UV-irradiated or control embryos were unable to dorsalise the VMZ explants (Fig. 4B and Table 2). In contrast, stage 8.5-9 dorsal vegetal explants secreted a factor able to cause muscle differentiation in 52% of the VMZ explants (Fig. 4B and Table 2). In the latter case, dorsal mesoderm differentiation was limited to the cells derived from the VMZ explants, demonstrating that the dorsal vegetal explants were not contaminated with mesoderm. Thus, we find that, like DMZ cells, dorsal vegetal cells secrete a signal able to dorsalise ventral mesoderm whereas UV-vegetal pole cells do not.

Cui and colleagues (1996) also assessed the inducing properties of UV-irradiated vegetal cells by analysing their ability to induce dorsal mesoderm in conjugated animal caps. Under their experimental conditions, UV-vegetal cells could induce muscle and, in a minority of cases notochord, in the ectoderm. This contrasts with our results. This discrepancy could result from differences in the assay system. In our assay, we measure the secretion of a dorsalising signal able to dorsalise mesoderm after its induction, a property which the mesoderm inducer activin lacks (Smith et al., 1993). Cui and colleagues measure the ability of vegetal cells to secrete factors that will induce dorsal mesoderm in ectoderm. In their assay, induction of muscle and notochord by the vegetal caps might arise from the secretion by the vegetal caps of mesoderm inducers rather than of dorsalising molecules. Consistent with this proposition, we find that our UV-irradiated vegetal pole explants, which lack dorsalising activity, can induce notochord and muscle in a similar proportion of ectodermal explants to that reported by Cui and colleagues (not shown).

From these experiments, we conclude that the interpretation of the inheritance of maternal dorsal determinants depends on the cellular context: vegetal pole cells do not secrete dorsalisers while cells located higher up along the animal-vegetal axis do.

Vegetal pole cells can secrete dorsalisers but fail to do so in response to *Siamois*

Several explanations could account for the failure of vegetal

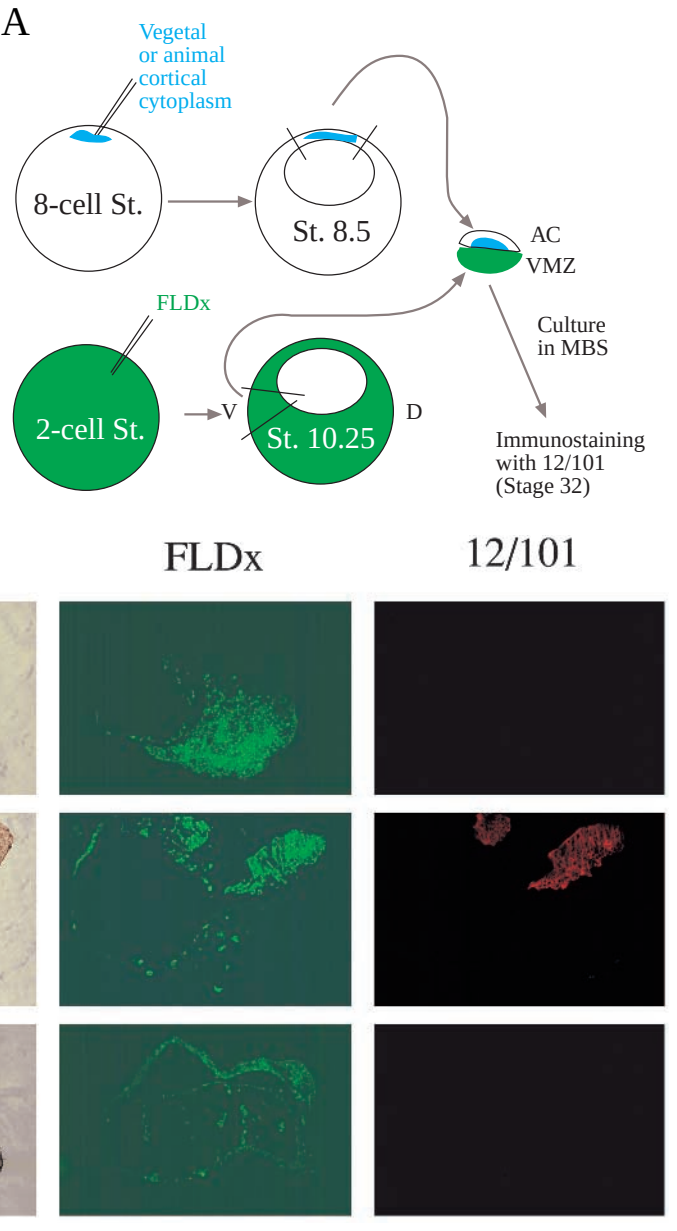

Fig. 3. Animal caps injected with vegetal cortical cytoplasm secrete a dorsalising signal. (A) Diagrammatic representation of the experiment. Ventral marginal zone explants (composed mostly of ventral mesodermal cells) were taken from embryos (stage 10.25) previously injected with the lineage tracer FLDx. The explants were immediately combined with blastula animal caps (stage 8.5) derived from embryos previously injected with animal or vegetal cortical cytoplasm (ACC, VCC). The conjugates were cultured until their mesodermal component reached the equivalent of stage 32 and they were then immunostained with the muscle antibody 12/101. (B) Photographs of sections through conjugates composed of a ventral marginal zone explant labelled with fluoresceine lysinated dextran (FLDx) combined with an animal cap derived either from uninjected embryos (none), from embryos injected with vegetal cortical cytoplasm (VCC), or from embryos injected with animal cortical cytoplasm (ACC). Left panels, bright-field images; middle panels, position of FLDx-labelled cells; right panels, 12/101 staining. Muscle-specific staining is seen only in the progeny of the FLDx-labelled marginal zone cells which were conjugated to the VCC-injected animal cap.

Table 1. Respecification of ventral mesoderm (VMZ) into muscle cells by a dorsalising signal from VCC-injected animal caps

Injected samples	Number of conjugates examined	Number of conjugates with muscle cells in			% of conjugates with muscle cells in VMZ§
		VMZ*	AC†	VMZ+AC‡	
None	10	1	0	0	10.0
Vegetal cortical cytoplasm	41	24	1	2	58.5
Animal cortical cytoplasm	29	3	1	0	10.3

Stage 8.5 animal caps, uninjected or injected with vegetal or animal cortical cytoplasm (VCC or ACC), were conjugated to stage 10.25 ventral marginal zone (VMZ) explants. The conjugates were cultured until stage 32 before sectioning. Secretion of a dorsalising signal was detected by the presence of 12/101-positive muscle cells in the VMZ explant.

*Muscle cells were derived only from the ventral marginal zone explants (VMZ).

†Muscle cells were derived only from the animal cap (AC).

‡ Muscle cells were derived from VMZ and AC.

§The percentage of the conjugates which had muscle cells from VMZ.

pole cells to secrete dorsalising factors in response to maternal dorsal determinants. One of them is that the secretory machinery of vegetal cells may not support secretion of dorsalising polypeptides. To test this, we overexpressed *chordin* or *noggin*, two genes coding for dorsalising polypeptides (Carnac et al., 1996) in vegetal pole cells, and analysed the dorsalising properties of the resulting explants. Vegetal poles of embryos injected vegetally with chordin (900 pg) or noggin (100 pg) mRNA were explanted at stage 8.5-9, conjugated with stage 10 VMZ explants and cultured until tailbud stages (Fig. 5A). Here, we chose to use vegetal pole cells from normal embryos, which also lack dorsalising activity (Table 2) and withstand the mRNA injection better than vegetal pole cells from UV-irradiated embryos. As a positive control, animal caps from embryos injected in their animal pole with the same amount of mRNA were conjugated with stage 10 VMZ explants. Table 3 and Fig. 5B show that injection of both *chordin* and *noggin* mRNA resulted in the secretion of a dorsalising factor by both animal and vegetal caps. Moreover, the proportion of conjugates containing muscle cells in the VMZ part was similar in animal cap/VMZ conjugates and in vegetal cap/VMZ conjugates. This indicates that the ability of animal and vegetal cells to secrete a dorsalising polypeptide was not significantly different and could not account for the observed difference in dorsalising properties.

As the level of expression of *Siamois* is decreased in UV-irradiated embryos compared to controls, the lack of dorsalising activity of UV-vegetal pole cells could also be due to an insufficient level of expression of this gene. To test this hypothesis, we conjugated stage 10 VMZ explants with stage 8.5-9 *Siamois*-injected vegetal poles from UV-irradiated embryos. As a positive control, stage 8.5-9 *Siamois*-injected animal caps were also conjugated with stage 10 VMZ explants (Fig. 5). As previously reported (Carnac et al., 1996), injection of 100 pg of *Siamois* mRNA into animal pole cells conferred dorsalising activity to injected cells (Fig. 5B; Table 3). However, vegetal pole cells from UV-irradiated embryos injected with 100 pg of *Siamois* mRNA lacked dorsalising activity in our conjugate assay (Fig. 5B; Table 3). To rule out that the inability of UV-irradiated vegetal pole cells to secrete a dorsaliser in response to *Siamois* was due to the damaging effects of the ultraviolet irradiation, we also conjugated *Siamois*-injected vegetal poles from control embryos to VMZs. In this case, injection of up to 250 pg of *Siamois* mRNA did not lead to the secretion of a dorsalising signal (Table 3). Hence, the lack of dorsalising activity of vegetal pole cells is unlikely to result from the insufficient level of activation of *Siamois*. Rather it appears that the interpretation of the cue provided by Siamois depends on the cellular context.

Cornell et al. (1995) have suggested that the level of activation of the FGF pathway may be lower in vegetal pole cells than in other embryonic cells, while work by Henry et al. (1996) suggests that vegetal cells differ from other embryonic cells due to their exposure to higher concentrations of an activin-like mesoderm inducing factor. We therefore asked whether artificial activation of the FGF pathway or impairment of an activin-like pathway would confer dorsalising activity to *Siamois*-injected vegetal pole cells. In these experiments, the FGF/MAP kinase pathway was activated by overexpressing *v-ras*, the constitutively active oncogenic form of the protooncogene *c-ras* (Cornell et al., 1995), while interference with an activin-like pathway was obtained by overexpressing a truncated form of the type II activin receptor, tAR (Hemmati-Brivanlou and Melton, 1992). 100 pg of *Siamois* mRNA was injected into the vegetal pole of early 4-cell embryos either singly or with mRNA encoding either *v-ras* (100 pg) or *tAR* (1 ng or 4.2 ng). The amounts of *v-ras*

Table 2. Dorsalising activity of dorsal marginal, dorsal vegetal and vegetal pole cells

Type of explant conjugated to VMZ tissue pieces	Number of conjugates analysed	Number of conjugates with muscle cells in VMZ	% of conjugates with muscle cells in VMZ
none	47	2	4.2
DMZ cells	19	16*	84.2
Dorsal vegetal Cells	25	13*	52
Veg. pole	22	3	13.6
Veg. pole from UV emb.	34	0	0

Stage 8.5-9 animal cap, dorsal vegetal or vegetal pole explants from control or UV-irradiated embryos were conjugated to stage 10 ventral marginal zone (VMZ) explants. The conjugates were cultured until stage 30-35 before sectioning. Secretion of a dorsalising signal was detected by the presence of 12/101-positive muscle cells in the VMZ explant. No muscle was ever detected in the other part of the conjugates. Notochord was present in DMZ explants (see Fig. 2B) but was never detected by morphological criteria in the VMZ part of the conjugates. *Large patches of muscle were present.

and *tAR* mRNA injected were similar to those used by Cornell et al. (1995) and Henry et al. (1996) respectively. Vegetal poles from injected embryos were dissected at stage 8.5-9, immediately conjugated to stage 10 VMZ and cultured until the tailbud stages. Analysis of muscle formation in the injected vegetal pole/VMZ conjugates demonstated that injection of either *v-ras* or *tAR* mRNA failed to enable vegetal pole cells to secrete dorsalisers in response to *Siamois* (Table 3). Therefore, differences in the levels of activation of the activin-like or FGF pathways in vegetal pole and animal cap cells are unlikely to explain the differential response of these cells to the VCC/β-catenin/*Siamois* pathway.

Activation of different *Siamois* targets in animal and vegetal cells

The strong contrast in dorsalising activity conferred by *Siamois* to vegetal caps and to animal caps suggests that this gene activates different targets in these two cell types. *Chordin*, coding for a Bmp4 antagonist (Piccolo et al., 1996) is one of the targets of *Siamois*: it is activated in animal caps overexpressing *Siamois* (Carnac et al., 1996) and is repressed in embryos in which *Siamois* function has been disrupted by the dorsal overexpression of SE, a fusion protein between the N-terminal repressor domain of *Drosophila* engrailed and the DNA binding domain of Siamois (Fan and Sokol, 1997). Another organiser gene, *cerberus*, is expressed in a broader dorsal domain (Boumeester et al., 1996). To determine if this gene is also a target of *Siamois*, we overexpressed *Siamois* in the vegetal ventral blastomeres of 4-cell embryos, explanted the ventral vegetal domain of stage 10 embryos and cultured the explants until stage 11. Analysis of the abundance of *cerberus* transcripts by RT-PCR indicated that this gene could be activated by *Siamois* (Fig. 6A). To test whether the presence of *Siamois* was also required for *cerberus* expression, we used *enR-Sia*, encoding an engrailed repressor domain/Siamois homeodomain fusion protein similar to SE (Fan and Sokol, 1997), and which also antagonises *Siamois* function (not shown). Dorsal injection of *enR-Sia* mRNA in 4-cell embryos led to the repression of *cerberus* at stage 11 (Fig. 6A), confirming that this gene is a target of *Siamois*.

We then compared the expression of *chordin* and *cerberus* in VCC-injected animal caps and UV vegetal caps. Like *Xnr3* and *Siamois*, *chordin* was activated in VCC-injected animal caps. However, the level of accumulation of *chordin* transcripts was lower in VCC-injected animal caps than in control embryos (Fig. 6B). This result may reflect the fact that *chordin*, unlike *Xnr3* or *Siamois*, may require the presence of mesoderm inducers to be fully activated. In contrast to *chordin*, *cerberus* was not activated in animal caps injected with either VCC or *Siamois* mRNA (Fig. 6B and data not shown).

The behaviour of *chordin* and *cerberus* also differed in UV-irradiated embryos. Analysis of their expression pattern in UV-irradiated or control embryos revealed that while *chordin* was expressed at less than 2% of control levels in irradiated embryos (Fig. 2A), the total amount of *cerberus* mRNA present in an embryo was barely affected by the UV treatment (Fig. 6C). RT-PCR analysis of the distribution of *cerberus* transcripts in UV-treated embryos showed that the major site of expression was at the vegetal pole (Fig. 6C). Furthermore, this vegetal expression was dependent on Siamois as overexpression of *enR-Sia* at the vegetal pole of UV-treated embryos led to the complete loss of *cerberus* expression (Fig. 6C).

From these data, we conclude that whereas early genes like *Siamois* and *Xnr3* are activated by the VCC/β-catenin pathway in both animal and vegetal cells, these two cell types differ in their ability to express *cerberus* and *chordin*, two targets of *Siamois*. *Chordin*, which is activated in animal but not in vegetal cells, is a potent dorsalising molecule. In contrast, *cerberus* can only be activated in vegetal cells and Bouwmeester et al. (1996) have shown that cerberus is not a dorsaliser, as *cerberus* mRNA-injected VMZs do not form dorsal mesoderm (lack of dorsalising activity was also confirmed in our mRNA-injected animal caps/VMZ conjugate assay; data not shown). The differential activation of *cerberus* or *chordin* in response to *Siamois* could therefore account for

Table 3. Dorsalising activity of animal and vegetal cells injected with *Siamois*, *chordin*, *noggin*, *v-ras* or *tAR* mRNA

Type of explant conjugated to VMZ tissue pieces	mRNA injected	Number of conjugates analysed	Number of conjugates with muscle cells in VMZ	% of explants with muscle cells in the VMZ
None	–	56	4	7.1
Veg. pole from UV emb.	Sia 100 pg	32	4	12.5
Veg. pole from control embryos	Sia 100 pg	12	2	16.6
	Sia 250 pg	8	0	0
An Caps	Sia 100 pg	30	27*	90
Veg. pole from control embryos	chd 900 pg	15	9*	60
An caps	chd 900 pg	16	10*	62.5
Veg. pole from control embryos	nog 100 pg	11	8*	72.7
An. caps	nog 100 pg	19	15*	78.9
Veg. pole from control embryos	Sia 100 pg + v-ras 100 pg	18	0	0
Veg. pole from control embryos	Sia 100 pg + tAR 1 ng	11	0	0
	Sia 100 pg + tAR 4,2 ng	22	0	0

Stage 8.5-9 animal cap or vegetal pole explants derived from control or UV-irradiated embryos injected as indicated with mRNA for *Siamois*, *chordin noggin*, *v-ras* or *tAR* were conjugated to stage 10 ventral marginal zone (VMZ) explants. The levels of *tAR* mRNA used were sufficient to block mesoderm induction during normal development as assayed by the expression of Xbra, while injection of 100 pg of *v-ras* mRNA into animal caps led to ventral mesoderm differentiation (determined morphologically; not shown). The conjugates were cultured until stage 30-35 before sectioning and staining with the muscle-specific 12/101 antibody. When present, the muscle cells were always in the VMZ part of the conjugate. Notochord was never detected by morphological criteria in the VMZ part of the conjugates. Sia, Siamois; Chd, chordin; nog, noggin; tAR, truncated activin type II receptor. *Large patches of muscle were present.

the different dorsalising properties of VCC-injected animal caps and UV-vegetal caps.

DISCUSSION

In this study, we show that the VCC dorsal factor is able to activate the Wnt-responsive genes *Siamois* and *Xnr3* in animal caps as well as at the vegetal pole of embryos in which cortical rotation has been blocked. This suggests that all cells that inherit maternal determinants express *Siamois*. However, the consequences of this activation differ along the animal-vegetal axis. VCC-injected animal caps acquire two properties of the head organiser: they express *chordin* and are able to dorsalise ventral mesoderm. In contrast, vegetal caps from UV-irradiated embryos adopt an anterior mesendoderm fate, as demonstrated by the expression of *cerberus*.

The VCC dorsal factors act along the Wnt/β-catenin pathway

Formation of the dorsal axis of amphibian embryos is initiated by cortical rotation, which moves dorsal factors from the vegetal cortical region to a more equatorial position. Microinjection of vegetal cortical cytoplasm (VCC) into the ventral equatorial region of a recipient embryo produces an ectopic dorsal axis, demonstrating that the dorsal factors can be experimentally transferred (Yuge et al. 1990; Fujisue et al. 1993; Holowacz and Elinson 1993). The axis-inducing property of the cortex was further demonstrated by Kageura (1997), who showed that transplantation of the dorsal vegetal cortex to ventral positions led to the induction of a secondary axis. A clue as to the nature of the dorsal factors comes from microinjection of VCC into animal pole cells. In this location, the dorsal factors enhance dorsoanterior development, without inducing mesoderm, suggesting that they act as competence modifiers (Holowacz and Elinson 1995).

Both noggin and members of the Wnt/β-catenin pathway have the capacity to produce a dorsal axis by acting as competence modifiers, rather than as mesoderm inducers. Recent experiments using UV-irradiated embryos suggest a connection between the dorsal activity of the VCC and the Wnt/β-catenin pathway. Cortical rotation is inhibited by UV, and two Wnt/β-catenin dependent events, namely nuclear translocation of β-catenin and expression of *Siamois*, now occur in cells at the vegetal pole rather than at the equatorial region (Schneider et al., 1996; Brannon and Kimelman 1996; Cui et al., 1996). Dorsal activity of the VCC also remains at the vegetal pole in UV irradiated eggs (Fujisue et al. 1993; Holowacz and Elinson 1993).

Our present results greatly strengthen the hypothesis that the VCC dorsal factors act in the Wnt/β-catenin pathway. Microinjection of VCC into animal pole cells leads to the expression of *Siamois* and *Xnr3*, both of which are downstream targets of the Wnt/β-catenin pathway and are not expressed in response to either noggin or mesoderm inducers (Carnac et al., 1996; Smith et al., 1995).

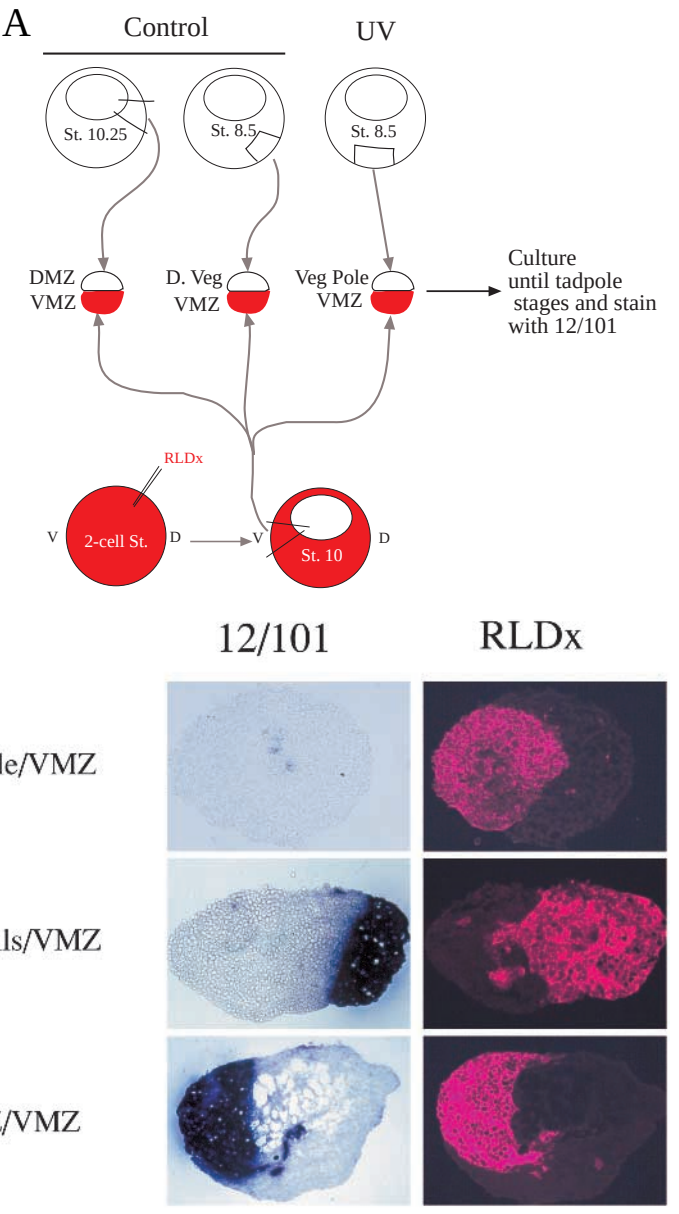

Fig. 4. Dorsalising activity of dorsal marginal zone cells, dorsal vegetal cells or vegetal pole cells from UV-irradiated embryos. (A) Experimental strategy. Explants of ventral marginal zone (VMZ) were taken at stage 10 from embryos injected with the lineage tracer rhodamine lysinated dextran (RLDx). They were combined with either vegetal pole from UV-treated embryos (UV Veg Pole), dorsal vegetal cells from stage 8.5-9 embryos (D. Veg) or stage 10 dorsal marginal zone (DMZ). The conjugates were cultured until stage 30-35 and immunostained with the muscle antibody 12/101. (B) Photographs of sections through conjugates of ventral marginal zone explants (VMZ) with UV vegetal pole (top), dorsal vegetal cells (middle) or dorsal marginal zone explants (bottom). While DMZ and dorsal vegetal cells have a dorsalising activity, UV vegetal poles fail to dorsalise VMZ explants. Notochordal differentiation, monitored by the presence of highly vacuolated cells, is found in the DMZ explants but never observed in the dorsal vegetal explants. Left panels, 12/101 staining; right panels, position of RLDx labelled cells.

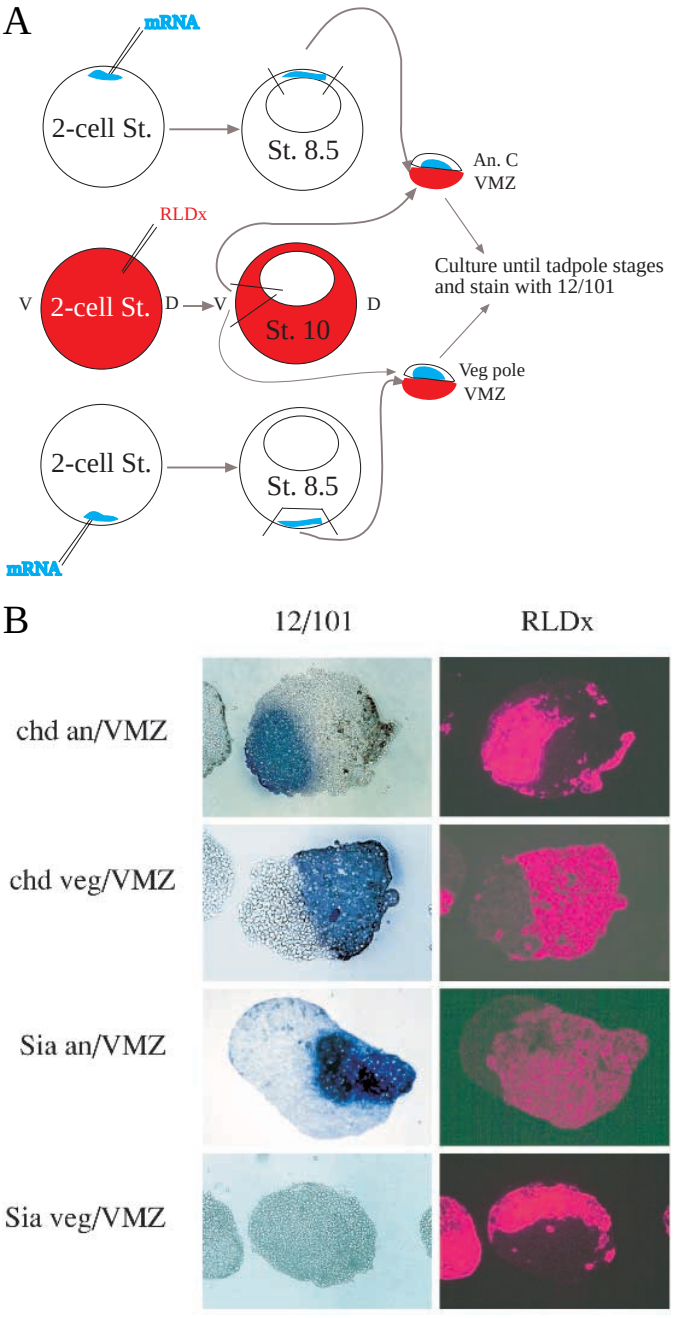

Fig. 5. Dorsalising activity of animal or vegetal pole cells injected with *Siamois* or *chordin* mRNA. (A) Experimental strategy. Stage 10 ventral marginal cells (derived from embryos injected with RLDx) were combined either with animal cap (stage 8.5-9) derived from embryos previously injected in the animal pole with 900 pg of *chordin* mRNA or 100 pg *Siamois* mRNA or with vegetal cap derived from embryos injected similarly in the vegetal pole. The conjugates were cultured until stage 30-35 and immunostained with the muscle antibody 12/101. (B) Photographs of sections through conjugates of VMZ explants with *chordin* mRNA injected animal caps (chd an) or vegetal poles (chd veg), or with *Siamois* mRNA-injected animal caps (Sia An) or vegetal poles (Sia veg). While injection of *chordin* mRNA confers dorsalising activity to both animal and vegetal pole cells, injection of *Siamois* mRNA leads to the secretion of dorsalising signals in animal caps cells only. chd, chordin; Sia, Siamois. Left panels: 12/101 staining; right panels: RLDx labelled cells.

Furthermore, our results show that the amount of dorsal activity in the VCC of one egg is sufficient to activate *Siamois* and *Xnr3* ectopically. This observation indicates that overexpression of Wnt/β-catenin pathway members is likely to be mimicking the natural activity found in the VCC. Consistent with this idea, analysis by *in situ* hybridisation of the precise location of *Xnr3* and *Siamois* transcripts in UV-irradiated embryos demonstrate that expression of these genes is strongest in the cells of the surface layer that have inherited the VCC.

Siamois and *Xnr3* are activated in all embryonic cells that inherit dorsal maternal determinants

In his elegant cortical transplantation experiments, Kageura (1997) found that transplantation of dorsal vegetal cortex to the ventral marginal zone caused the development of an ectopic axis, while transplantation to more animal or vegetal positions failed to do so. Kageura's interpretation of these results was that the dorsal factors are only active when they come in contact with a core factor localised in the equatorial region of the egg. Our results do not support this interpretation. We find that activation of *Siamois* and *Xnr3* by vegetal cortex cytoplasm is not restricted to the marginal zone. This activation can occur in both animal and vegetal pole cells and is therefore not regulated by cellular context. Also, the strong activation of *Siamois* and *Xnr3* at the vegetal pole of UV-irradiated embryos, which do not undergo cortical rotation, indicates that this process is not needed for the activation of the maternal dorsal determinants.

This constitutive relationship between the activation of the VCC/β-catenin pathway and the expression of *Xnr3*/*Siamois* also appears to hold true during normal development: during the extensive pre-gastrula cell movement (Bauer et al., 1994), the broad domain of nuclear localisation of β-catenin at the mid-blastula stage (Fig. 1C from Schneider et al., 1996) is shifted to a more vegetal position which corresponds to the early gastrula domain of expression of *Xnr3*/*Siamois*.

Regionalisation of the anterior territories of the organiser

By the mid-gastrula stage, the domain of expression of *Xnr3*/*Siamois* can be subdivided along the anteroposterior axis into 3 territories, defined by different patterns of expression of the *Siamois* target genes *cerberus* and *chordin*. The anteriormost domain, located at the leading edge of migrating cells, is the anterior endomesoderm (AEM) which expresses *cerberus* but not *chordin*. Cells in the intermediate domain, which corresponds roughly to the presumptive head mesoderm, express both genes, while more posterior organiser cells express *chordin* but not *cerberus* (Bouwmeester et al., 1996). These three domains differ in their inducing properties. The AEM lacks dorsalising activity, as demonstrated by the fact that Einsteck grafts of AEM do not induce ectopic axial or head structures (Bouwmeester et al., 1996). Although the role of this domain in *Xenopus* pattern formation has not been thoroughly investigated, in the mouse the anterior primitive endoderm is required for the formation of rostral neurectoderm (Thomas and Beddington, 1996). In *Xenopus*, Einsteck experiments with head mesoderm lead to ectopic head formation. Similar experiments with the posterior domain lead to axis duplications, indicating that this territory has dorsalising activity (Lemaire

and Kodjabachian, 1996). Fate maps from 32-cell embryos indicate that late gastrula AEM is mainly derived from the vegetal-most dorsal blastomeres, while the more posterior domains are derived from blastomeres located in a progressively higher position along the animal-vegetal axis (Bauer et al., 1994; Vodicka and Gerhart, 1995).

Our results suggest that the position along the animal-vegetal axis of the cleavage stage blastomeres from which the AEM and head mesoderm are derived could play a major role in the distinction between these two territories. We find that inheritance of dorsal determinants by vegetal pole cells leads to the activation of *cerberus* but not of *chordin*, suggesting that these cells have acquired an anterior mesendodermal fate. Consistent with this proposition, these cells lack dorsalising activity. In contrast, activation of the VCC/*Siamois* pathway in animal cells leads to the activation of *chordin* but not *cerberus* and to the acquisition of dorsalising activity. Although this has not been tested here, it is likely that marginal zone cells, which also have dorsalising activity, activate both cerberus and chordin in response to the activation of the VCC/β-catenin/*Siamois* pathway.

Taken together, our results suggest that the regionalisation of the anterior domain of the organiser into AEM and presumptive head mesoderm results from the differential interpretation of the activation of the VCC/β-catenin/*Siamois* pathway along the animal-vegetal axis. In the light of these findings, an alternative interpretation of the results of the cortex transplantation studies (Kageura, 1997) can be proposed. Transplantation of dorsal vegetal cortex to both equatorial and vegetal positions leads to *Siamois*/*Xnr3* expression. However, while transplantation to an equatorial position leads to the secretion of a dorsalising factor and to ectopic axis induction, transplantation to a vegetal position results instead in the formation of AEM-like tissue, which lacks axis-inducing properties.

Molecular basis for the differential response to the VCC/β-catenin/*Siamois* pathway

In an attempt to understand why vegetal pole cells do not secrete dorsalising factors in response to *Siamois*, we have tested three possibilities. Firstly, we found that the observed difference is not due to a general inability of these cells to secrete dorsalising peptides. Secondly, it has been proposed that the FGF pathway may be activated at a lower level in vegetal cells than in marginal zone cells (Cornell et al., 1995). We therefore examined the effect of artificially increasing the level of activation of the FGF pathway in vegetal cells. Under the experimental conditions tested, this did not restore the ability of vegetal cells to secrete dorsalising factors in response to *Siamois*. Finally, Henry and colleagues (1996) also suggested that decreasing the level of activity of an activin-like pathway could convert vegetal cells into more animal cells. In our hands however, injection of *tAR* mRNA did not lead to the secretion of dorsalising polypeptides by *Siamois* mRNA-injected vegetal pole cells. These experiments do not exclude the possibility that a very precise level of activation of

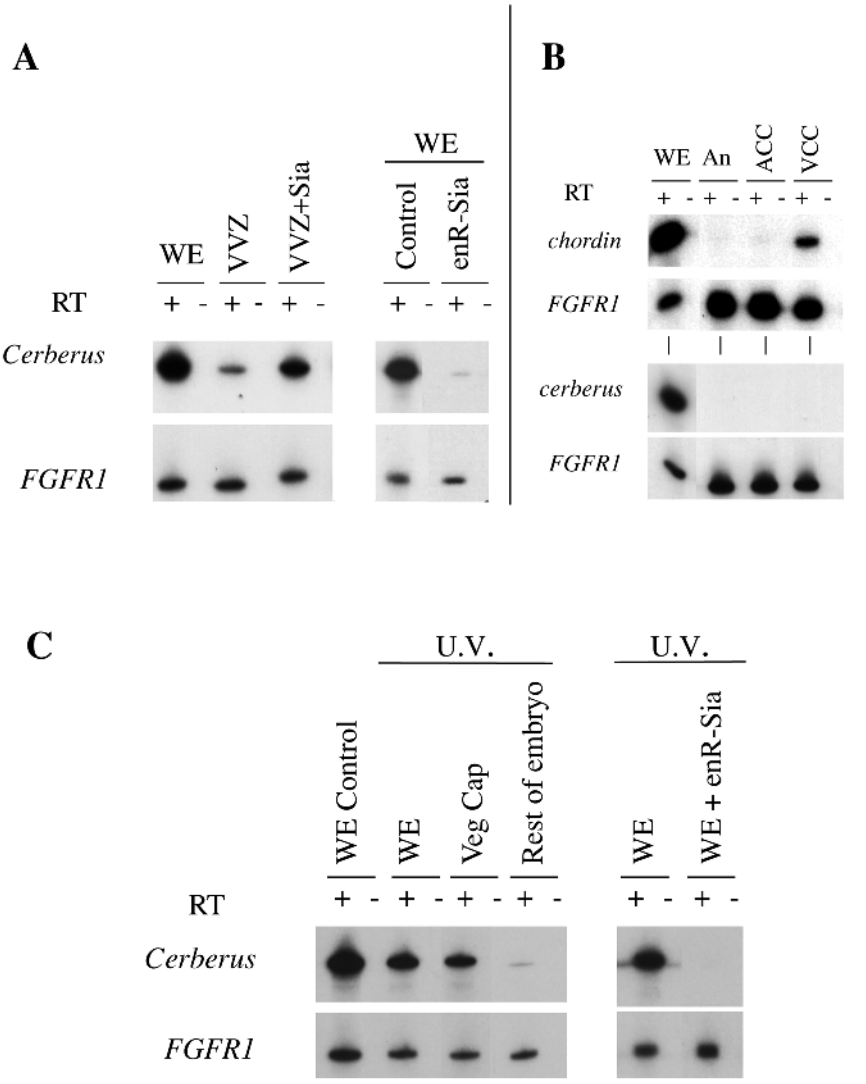

Fig. 6. Activation of *chordin* and *cerberus* by Siamois is context dependent. (A) *Cerberus* is a target of Siamois. 4-cell embryos were injected with 100 pg of *Siamois* mRNA in a ventral vegetal location or with 100 pg of *enR-Sia* mRNA in a dorsal vegetal position. Control and injected embryos were reared until stage 10 before excising ventral vegetal cells when needed. Whole embryos (WE) and ventral vegetal explants from uninjected (VVZ) or *Siamois*-injected embryos (VVZ+Sia) were analysed at stage 11 for *cerberus* expression. (B) *Chordin*, but not *cerberus*, is activated by the VCC in animal caps. The same cDNA samples as in Fig. 1 were subjected to RT-PCR analysis for the expression of *chordin* and *cerberus*. Abbreviations are as in Fig. 1. (C) *cerberus* is expressed at the vegetal pole of UV-irradiated embryos. Control or UV-irradiated (U.V.) whole embryos (WE) as well as explants were analysed at stage 11 by RT-PCR. In the experiment shown on the left panel, *chordin* expression was undetectable in the UV-irradiated whole embryos (not shown). enR-Sia: embryos injected at the vegetal pole with 100 pg of *enR-Sia* mRNA.

the FGF and/or activin-like pathways is needed for embryonic cells to secrete dorsalisers in response to *Siamois*. However they suggest that the differential response to *Siamois* does not solely rest on the difference in the level of activation of these two pathways. Several maternal T-box transcription factors expressed in the vegetal cells have recently been isolated (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). In the future, it will be of interest to investigate whether these factors play a role in the differential response of vegetal and sub-equatorial cells to *Siamois*.

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