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The homeobox gene *Siamois* is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm

Gilles Carnac[1], Laurent Kodjabachian[2], J. B. Gurdon[1] and Patrick Lemaire[1,2,*]

[1]Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR, United Kingdom and Department of Zoology, Cambridge, UK
[2]Laboratoire de Génétique et de Physiologie du Développement, IBDM CNRS-INSERM-Université de la Méditerranée, Campus de luminy, Case 907, F-13288 Marseille cédex 09, France

*Author for correspondence at address 2

SUMMARY

Siamois*, a *Xenopus* zygotic homeobox gene with strong dorsalising activity, is expressed in the dorsal-vegetal organiser known as the Nieuwkoop centre. We show that, in contrast to Spemann organiser genes such as *goosecoid*, *chordin* and *noggin*, *Siamois* gene expression is not induced following overexpression of mesoderm inducers in ectodermal (animal cap) cells. However, *Siamois* is induced by overexpressing a dorsalising Wnt molecule. Furthermore, like Wnt, *Siamois* can dorsalise ventral mesoderm and cooperate with *Xbrachyury* to generate dorsal mesoderm. These results suggest that *Siamois* is a mediator of the Wnt-signalling pathway and that the synergy between the Wnt and mesoderm induction pathways occurs downstream of the early target genes of these two pathways. Overexpression of *Siamois* in animal cap cells reveals that this gene can act in a non vegetal or mesodermal context. We show the following. (1) Animal cap cells overexpressing *Siamois* secrete a factor able to dorsalise ventral gastrula mesoderm in tissue combination experiments. (2) The Spemann organiser-specific genes *goosecoid*, *Xnr-3* and *chordin*, but not *Xlim.1*, are activated in these caps while the ventralising gene *Bmp-4* is repressed. However, the dorsalising activity of *Siamois*-expressing animal caps is significantly different from that of *noggin*- or *chordin*-expressing animal caps, suggesting the existence of other dorsalising signals in the embryo. (3) Ectodermal cells overexpressing *Siamois* secrete a neuralising signal and can differentiate into cement gland and, to a lesser extent, into neural tissue. Hence, in the absence of mesoderm induction, overexpression of *Siamois* is sufficient to confer organiser properties on embryonic cells.

Key words: *Siamois*, *noggin*, *goosecoid*, *chordin*, *brachyury*, *Xwnt-8*, *Xnr-3*, *Xenopus*, dorsalisation, embryonic axis, Spemann organiser, Nieuwkoop centre, late blastula organiser, Wnt signalling, neural induction

INTRODUCTION

In amphibians, patterning of mesoderm is thought to be set up as a result of sequential inductive interactions during early embryogenesis. Several lines of evidence indicate that the formation of dorsal mesoderm in *Xenopus laevis* is controlled by signalling molecules produced by a small number of cells localised in the so-called 'dorsalising centres'. Two dorsalising centres have been identified, acting in a temporal hierarchy: the Nieuwkoop centre and Spemann's organiser (Boterembrood and Nieuwkoop, 1973; Gimlich and Gerhart, 1984; Gimlich, 1986). Both centres were defined embryologically by heterotopic transplantation of dorsal cells into ventral territories (see Hamburger, 1988 and references therein). According to these data, the Nieuwkoop or dorsal-vegetal organising centre, fated to become pharyngeal endoderm, acts during the blastula stages by inducing, in the overlaying equatorial region of the embryo, the Spemann organiser, fated to become axial and head mesoderm. In turn, this centre recruits more lateral mesoderm into the axial structures during gastrulation. Understanding the ontogeny of the Spemann organiser therefore requires a better understanding of the events responsible for the creation of the Nieuwkoop centre and of its mode of action.

Several pieces of evidence suggest that creation of the Nieuwkoop centre results from the activation of a Wnt-signalling pathway before the midblastula transition (referred in what follows as the Pre-MBT Wnt-signalling pathway). First, ventral injection of mRNA for several Wnt family members (see Moon, 1993 and references therein) or for molecules acting along the Wnt pathway (as defined from work in *Drosophila*) such as a *Xenopus* homologue of the *Drosophila Dishevelled* protein (Sokol et al., 1995), a mutant version of the GSK-3 kinase or β-catenin (see Gumbiner, 1995 and references therein), leads to the creation of an ectopic Nieuwkoop centre. Second, depleting the embryos of β-catenin, thereby blocking the Pre-MBT Wnt pathway, prevents the formation of the endogenous Nieuwkoop centre (Heasman et al., 1994). These experiments provide convincing evidence that activation of the pre-MBT Wnt pathway is an important step in the creation of the Nieuwkoop centre. However, this does not necessarily imply the involvement of a dorsalising Wnt molecule in axis determination, as several other molecules can directly activate more downstream components of the pathway (Heasman et al., 1994).

The mode of action of the Nieuwkoop centre has also been analysed in some detail (reviewed by Christian and Moon, 1993; Holowacz and Elinson, 1995; see also Dale and Slack, 1987). From these experiments, dorsal vegetal cells appear to emit a combination of factors, some of which have general mesoderm-inducing activity, while others, referred to as modifiers or dorsalisers, lack this activity but are able to cooperate with mesoderm inducers to generate the dorsal-most cell types. This cooperation could take place at different levels. For example, the binding of dorsalisers and inducers to the cell surface could: (1) alter the properties of receptors for mesoderm inducers; (2) activate a transduction pathway that converges with that activated by mesoderm inducers before reaching the nucleus; (3) directly activate or repress specific target genes that will in turn cooperate with target genes downstream of mesoderm inducers. The distinction between these models has so far been hampered by our ignorance of the identity of the endogenous Nieuwkoop factors.

Recently, using an expression cloning strategy, Lemaire et al. (1995) have cloned a zygotic homeobox gene named *Siamois*, which induced a secondary axis when over-expressed in ventral-vegetal blastomeres of *Xenopus* embryos. The progeny of the injected blastomeres do not contribute to the ectopic axis, indicating that overexpression of *Siamois* in ventral-vegetal cells is sufficient to confer a Nieuwkoop centre identity. Consistent with this idea, the expression of *Siamois* is localised during normal development in the dorsal vegetal cells of *Xenopus* blastulae and young gastrulae. As *Siamois* codes for a transcription factor, it was proposed that it acts by regulating the expression of components of the Nieuwkoop centre signal. In the work reported in this paper, we have analysed the regulation and mode of action of *Siamois*. We show that *Siamois* is activated by the Pre-MBT Wnt pathway but not by mesoderm inducers. Furthermore, we demonstrate that overexpression of *Siamois* in animal caps confers two organiser properties to these cells in the absence of detectable mesoderm differentiation: they can dorsalise ventral mesoderm, and secrete a neural inducer. Our data also suggest that *Siamois*, in addition to antagonising the BMP-4 pathway, is activating a novel dorsalising pathway.

MATERIALS AND METHODS

Embryo injections

Embryos were in vitro fertilised, dejellied, cultivated in 10% MBS, and injected with mRNA as previously described (Lemaire and Gurdon, 1994). 4.6 nl rhodamine lysinated dextran (RLDx; 5 mg/ml in water; M_r 10×10^3, Molecular Probe) was injected into both blastomeres of two-cell embryos.

RNA expression constructs

Synthetic capped mRNA was prepared as previously described (Lemaire et al., 1995). *noggin* and *goosecoid* mRNAs were synthesised as described by Lemaire et al. (1995). Synthetic *Siamois* mRNA was prepared from a plasmid, pBSRN3 XSia-ORF, containing the *Siamois* open reading frame but lacking the 5′ and 3′ UTR. Synthetic *chordin* and *Xbra* mRNAs were synthesised as in Sasai et al. (1994) and Cunliffe and Smith (1992) respectively. *Bmp-4* mRNA was prepared as described by Dale et al. (1992). *Activin*βB mRNA was prepared from pSP64TActivin βB (a gift from Doug Melton). *bFGF* mRNA was made from plasmid containing bFGF cDNA in pSP64TbFGF (a gift from Betsy Pownall). *Xnr-2* mRNA was prepared as described by Jones et al. (1995). *Xnr-3* mRNA was synthesized from pCS2/Xnr-3 (a gift from Chris Wright). *bVg1* mRNA was prepared as described by Thomsen and Melton (1993). *Xwnt-8* mRNA was prepared as described by Lemaire et al. (1995).

Tissue explant combinations

All explants were cultured in 1× MBS. To determine the competence of ventral mesoderm to be dorsalised by *noggin*-, *Siamois*- or *chordin*-expressing caps, ventral marginal zone explants, composed mostly of ventral mesoderm cells, were cultured until stage 12 or 13 as sandwiches (two combined explants) in 1× MBS containing 0.1% BSA. At the appropriate stage, the two ventral marginal zone pieces were separated and combined with stage 9 animal caps (see also Fig. 6A). All sandwiches were cultured in 1× MBS with 0.1% BSA until sibling control embryos reached the indicated stage.

Immunostaining

Tissue explants were fixed in MEMFA (Hemmati-Brivanlou and Harland, 1989) for 3 hours and kept (overnight or longer) in methanol at −20°C. 10 μM sections were cut from tissue explants or whole embryos embedded in Histoplast:beeswax (98:2). Immunostainings were performed using four monoclonal antibodies: 12/101 (muscle specific; Kintner and Brockes, 1984), MZ15 (notochord specific; Smith and Watt, 1985), D7F2 (recognising the muscle-specific protein MyoD; Hopwood et al., 1992) and 4d (recognising the pan-neural molecule N-CAM; Watanabe et al., 1986). Incubations, washes and colour reactions (using NBT-BCIP (Boehringer MA) or the alkaline phosphatase substrate I (Vector labs) were performed according to the method of Hopwood et al. (1992). When double staining was performed, the first colour reaction was stopped by incubating the sections in 1× MBS with 5 mM EDTA overnight at 4°C before incubation with the second mAb. Where indicated, the nuclei were stained with Hoechst 33258 (Boehringer, 2 μg/ml during 40 minutes).

RNase protection assays

RNase protection assays were performed as previously described (Lemaire and Gurdon, 1994) using 10-15 animal cap sandwiches per tube. Quantitations were performed using a Molecular Graphics phosphorimager running the ImageQuant software. Antisense RNA probes for the FGF receptor, *Xbra*, *gsc* and *Xlim-1* were prepared as described by Lemaire et al. (1995). The *Bmp-4* probe was prepared as described by Dale et al. (1992). The *Siamois* probe was generated using T7 polymerase from a plasmid named pXSia BglII 350 that contained the 5′ 312 bp *Bgl*II fragment of the *Siamois* cDNA (Lemaire et al., 1995) cloned in pBluescript SK(−). The *chordin* antisense probe was derived from the *Xenopus chordin* full-length clone (a gift from Dr E. De Robertis; Sasai et al., 1994) as described by Ryan, K., Garret, N., Mitchell, A. and Gurdon, J. B. (unpublished data). The *noggin* probe was prepared using T7 RNA polymerase from pnogginΔAS, a derivative of the plasmid pnoggin Δ5′(a gift from Dr R. Harland; Smith and Harland, 1992) lacking an *Avr*II-*Sph*I fragment in the 5′ end of the *noggin* cDNA. The *Xnr-1* and *Xnr-2* probes were prepared as described by Jones et al. (1995). The *Xnr-3* probe was prepared from the plasmid pdor3 (a gift from Dr W. C. Smith; Smith et al., 1995). The plasmid pdor3 was linearised with *Pvu*II and the antisense probe was synthesised using T7 polymerase.

RESULTS

Siamois is activated by Wnt signalling but not by mesoderm inducers in animal caps

Organiser genes such as *goosecoid*, *noggin* or *chordin* have been shown to be activated in ectodermal (animal cap) cells by high but not by low doses of the mesoderm inducer *activin*

(reviewed by Dawid, 1994; Kessler and Melton, 1994). To test if *Siamois* could also be activated by *activin*, we injected several concentrations of *activin* mRNA into animal blastomeres of two-cell stage embryos, excised the animal tissue at stage 9 and analysed for the presence of transcripts for *Siamois* and several mesoderm or organiser genes at stage 10. As described previously, *Xbrachyury* (*Xbra*) induction was strongest at low *activin* concentration while induction of dorsal genes such as *goosecoid* (*gsc*), *noggin* or *chordin* was highest at high concentrations (Fig. 1A and data not shown). In contrast, *Siamois* was not induced in animal caps injected with low or high amounts of *activin* mRNA (Fig. 1A). In addition, injection into animal blastomeres of high or low doses of mRNA for the mesoderm inducers, *Xnr-2*, *Bvg1*, encoding a processed form of *Vg1*, *Bmp-4* or *bFGF* (Dawid, 1994; Kessler and Melton, 1994; Jones et al., 1995) led to the activation of *Xbra* but also failed to activate *Siamois* (Fig. 1B). Therefore *Siamois*, in contrast to *gsc*, *noggin* and *chordin*, is not a target of mesoderm inducers in animal cap cells.

To study the effect of the activation of the Pre-MBT Wnt pathway on the expression of *Siamois* in animal cap cells, we injected 1-100 pg of *Xwnt-8* mRNA into the animal pole of 2-cell embryos and analysed the expression of *Siamois* in stage 10 explanted animal caps. Injection of as little as 1 pg of *Xwnt-8* mRNA into animal blastomeres was sufficient to induce *Siamois* but none of the *Xwnt-8* mRNA concentrations tested resulted in the induction of *gsc* or *Xbra* gene expressions (Fig. 1C). Therefore, *Siamois*, like *Xnr-3* (Smith et al., 1995), is activated by the pre-MBT Wnt-signalling pathway but not by mesoderm inducers.

Siamois respecifies mesoderm differentiation from ventral to dorsal fates

While injection of *Xbra* mRNA into ectodermal (animal cap) cells only gives rise to ventral mesodermal cell types (Cunliffe and Smith, 1992), *Xbra* mRNA can cooperate with *Xwnt-8* mRNA to give rise to dorsal mesodermal cell types (Cunliffe and Smith, 1993). As the results described in the previous section indicated that *Siamois* acts downstream of the Pre-MBT Wnt pathway, we tested if *Siamois* can also cooperate with *Xbra*. *Xbra* mRNA, *Siamois* mRNA or a combination of both were injected into the animal pole of two-cell embryos, and animal caps obtained from these injected embryos were excised at stage 9.5 (Fig. 2A). At stage 17, animal caps injected with *Xbra* mRNA or *Siamois* mRNA alone showed no elongation, suggesting the absence of dorsal mesoderm (Fig. 2B-D). In contrast, substantial elongation was observed in all animal caps co-injected with both messages (n=6/6; Fig. 2E). To characterise the type of mesoderm present in the elongated caps, they were cultured until stage 32 and immunostained with the monoclonal antibodies 12/101 (Kintner and Brockes, 1984) and MZ15 (Smith and Watt, 1985) which recognise muscle and notochord cells, respectively. Muscle or notochord differentiation was not observed in *Siamois*-injected animal caps (n>20) (data not shown) and few or no muscle cells were present in animal caps injected with *Xbra* alone (Fig. 2F). However, injection of a combination of *Xbra* and *Siamois* mRNA led to the formation of large blocks of muscle (n=6/6) and notochord (n=2/6) (Fig. 2G). Therefore, *Siamois*, like *Xwnt-8*, can cooperate with *Xbra* to give rise to dorsal mesoderm.

To test if *Siamois* could also dorsalise normal ventral mesoderm, different amounts of *Siamois* mRNA were injected into the two ventral-vegetal blastomeres of 4-cell embryos. *Siamois*-expressing ventral mesoderm tissue were explanted at the early gastrula stage, cultured until stage 26, and then analysed for the presence of muscle or notochord (Fig. 3A). Injection of as little as 1 pg of *Siamois* mRNA was sufficient to lead to massive muscle differentiation. Injection of 10 pg of

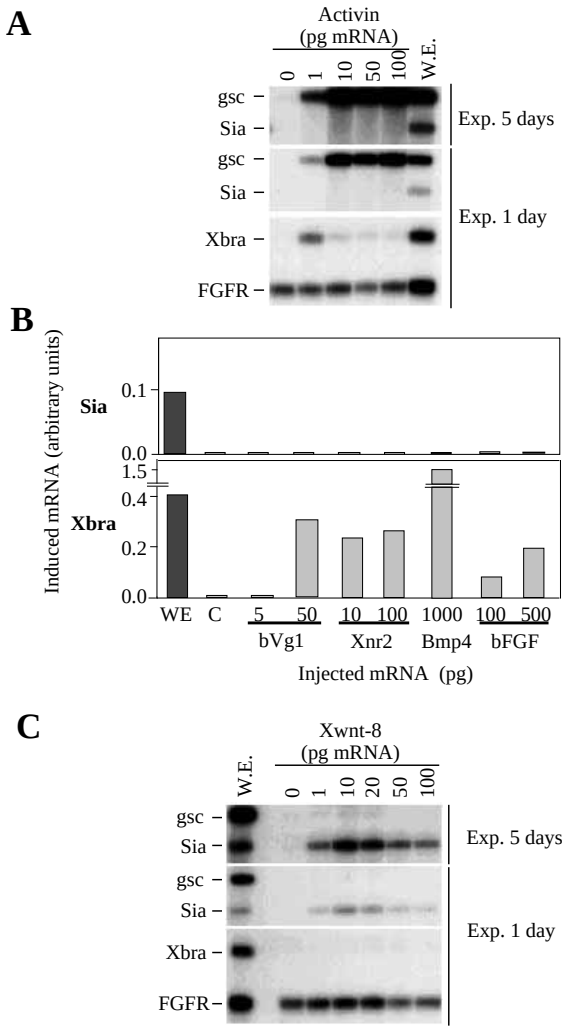


Fig. 1. Activation of *Siamois* by the Wnt-signalling pathway but not by mesoderm inducers in animal caps. mRNA of *activin βB*, *bVg1*, *Xnr2*, *Bmp-4* and *bFGF* was injected in the animal pole of 2-cell embryos. The animal caps were excised at stage 9 and gene expression was analysed by RNase protection assay at stage 10. (A,B) Injection of mRNA coding for mesoderm inducers in animal pole blastomere leads to the activation of *Xbra* and *goosecoid* (*gsc*) but not of *Siamois* (*Sia*). (A) Both a short and a long exposure of the autoradiograph are presented to demonstrate the lack of activation of *Siamois*. (B) The *Xbra* and *Siamois* signals have been normalised using the signal for the constitutively active *FGFR*. (C) *Siamois* but not *gsc* or *Xbra* is activated by the Wnt-signalling pathway. Exposure times were 5 days (top panel) and 24 hours (bottom panel). The apparent decrease in the activation of *Siamois* at high concentrations of *Xwnt-8* mRNA is due to a weaker loading of the corresponding lanes (see the FGFR signal). WE, whole embryo; C, control uninjected animal caps.

Siamois mRNA induced mostly notochord differentiation (Fig. 3B). Injection of 100 pg of *Siamois* mRNA suppressed all muscle differentiation and led to the presence of limited amounts of notochord (Fig. 3B), which may reflect the conversion of *Siamois*-expressing mesoderm cells into the most dorsoanterior mesoderm: the prechordal plate.

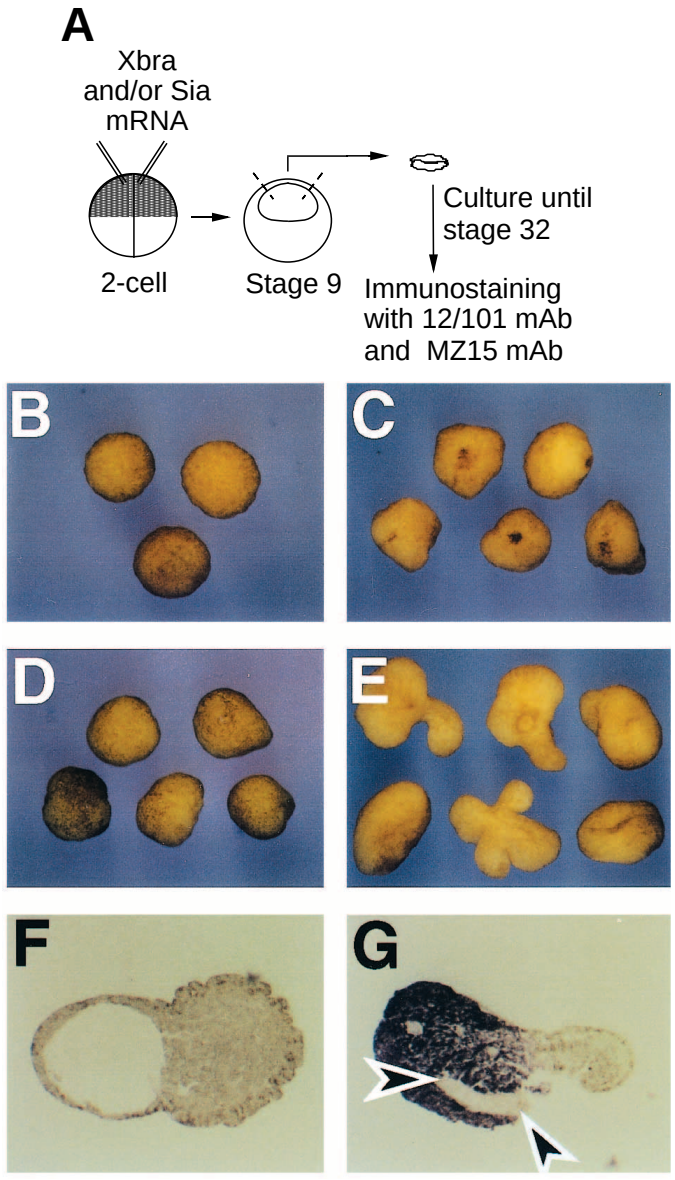


Fig. 2. *Siamois* cooperates with *Xbra* to induce dorsal mesoderm in animal caps. (A) Diagrammatic representation of the experiment: embryos were injected into their animal poles at the two cell stage with *Xbra* mRNA (2.5 ng), *Siamois* mRNA (50 pg), or with a mixture of both. Animal caps were explanted at stage 9 and cultured as doublets. (B-E) Stage 17 animal caps: (B) uninjected, (C) injected with *Xbra* mRNA, (D) with *Siamois* mRNA or (E) with a mixture of both. (F,G) Representative photographs of sections through animal caps overexpressing *Xbra* (F) or *Xbra* and *Siamois* (G), cultured until stage 32 and stained with the 12/101 muscle-specific mAb (black). Strongly vacuolated notochord cells can be seen in the animal caps overexpressing both *Siamois* and *Xbra* (arrows). Animal caps from embryos injected with *Siamois* mRNA alone showed neither morphological signs of mesodermal differentiation nor staining with the 12/101 antibody (not shown).

Thus, like *Xwnt-8* (Sokol et al., 1991; Cunliffe and Smith, 1993), *Siamois* can respecify, to dorsal fates, both normal ventral mesoderm tissue and that obtained by overexpression of *Xbra* in ectoderm. However, *Siamois* can dorsalise embryonic ventral mesoderm tissue more strongly than it can dorsalise *Xbra*-induced ventral mesoderm. This suggests that these two types of ventral mesoderm are qualitatively different and that, in addition to *Xbra*, *Siamois* cooperates with other mesodermalising factors present in the marginal zone.

Animal caps injected with *Siamois*, *Xwnt-8* and *noggin*, but not *goosecoid* mRNA can dorsalise early ventral mesoderm in sandwich experiments

The results above establish that *Siamois* can dorsalise ventral mesoderm of normal or 'artificial' nature, but does not discriminate between a cell-autonomous or non cell-autonomous

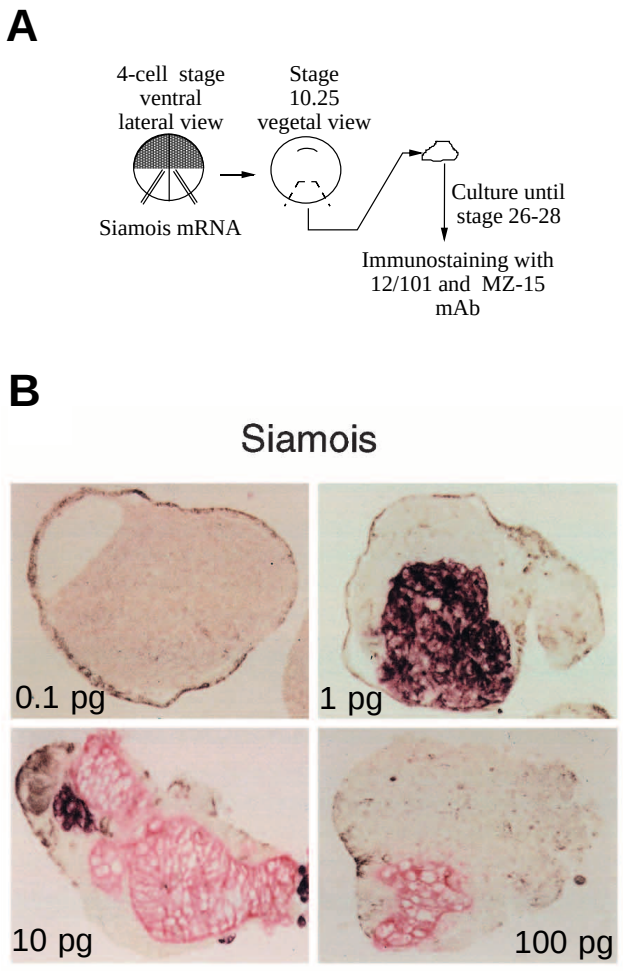
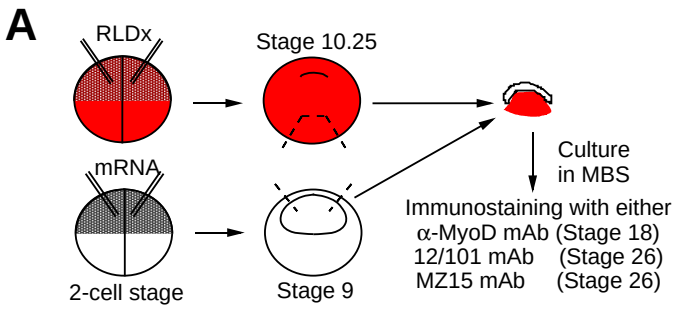
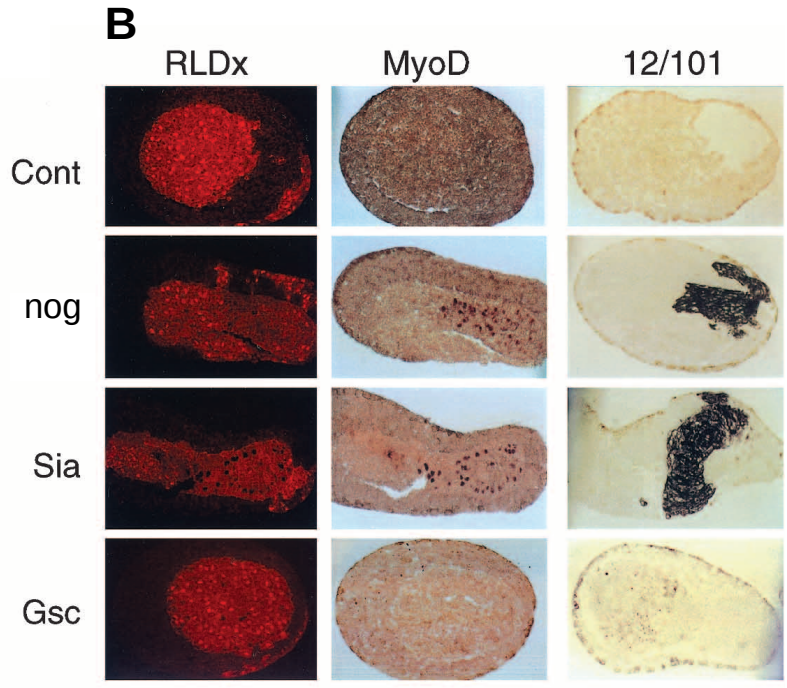


Fig. 3. Effects of the overexpression of *Siamois* in embryonic ventral mesoderm tissue. (A) Diagrammatic representation of the experiment. Pieces of ventral marginal zone (composed mostly of ventral mesoderm cells) were explanted at stage 10.25 from embryos injected in their two ventral vegetal blastomeres at the 4-cell stage with different amounts of *Siamois* mRNA. The marginal explants were fixed when sibling embryos reached stage 26-28, then double-stained with the muscle-specific antibody 12/101 and the notochord specific antibody MZ15. (B) Double-stained ventral marginal zone explants from embryos injected with dilutions (0.1-100 pg) of *Siamois* mRNA. Cells labelled with the MZ15 and 12/101 antibodies are stained red and brown, respectively.

mode of action. As during embryogenesis *Siamois* is mainly expressed in dorsal-vegetal cells underlying the mesoderm (Lemaire et al., 1995), *Siamois* probably respecifies mesoderm from ventral to dorsal fates in the embryo by activating a secreted dorsaliser(s). To confirm this hypothesis, we injected *Siamois* mRNA (25, 50 or 100 pg) into animal poles of 2-cell embryos and conjugated stage 9 animal caps derived from these embryos to pieces of early gastrula ventral mesoderm labelled with the fluorescent lineage tracer rhodamine-lysinated dextran (RLDx). The conjugates were cultured until the equivalent of stage 18 and analysed for the presence of the skeletal muscle determining protein MyoD using the D7F2 monoclonal antibody (Hopwood et al., 1992), or cultured until the equivalent of stage 26 and analysed for the presence of differentiated dorsal tissues such as mature skeletal muscle and notochord by immunostaining (Fig. 4A). Animal caps from embryos injected with 25, 50 or 100 pg of *noggin* mRNA were used as a positive control for dorsalisation in conjugate experiments (Smith et al., 1993).

When the ventral mesoderm tissue was cultured alone (data not shown) or conjugated with an uninjected animal cap, no 12/101 or MyoD staining was detected (Fig. 4B; Table 1). In contrast, both *Siamois*- and *noggin*-expressing animal caps can dorsalise early gastrula ventral mesoderm as demonstrated by the presence of muscle cells (MyoD+ and 12/101+) only in the mesodermal part of the conjugate (Fig. 4B; Table 1). MyoD or 12/101 staining was never detected in animal cap cells. As the injection of *Siamois* or *noggin* mRNA into the ventral marginal zone of early embryos results in the conversion of ventral mesoderm into axial mesoderm including notochord (Fig. 3 and data not shown), we were surprised to find no sign of notochord differentiation in conjugated ventral mesoderm explants (Table 1). A possible explanation for this difference is that, in our conjugate experiments, the ventral mesoderm is exposed to factors secreted by the animal caps from a later stage than when mRNAs are directly injected into the ventral marginal zone of





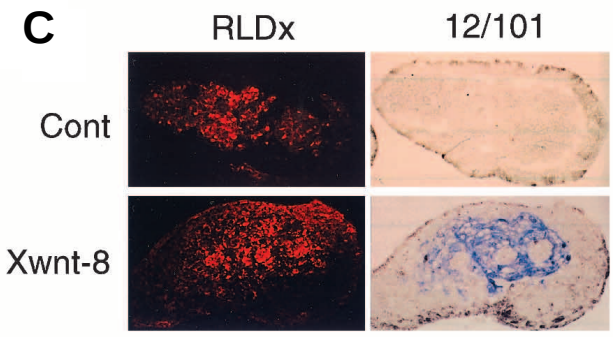


Fig. 4. *Siamois*-, *noggin*-, and *Xwnt-8* but not *goosecoid*-expressing animal caps secrete a dorsalising signal. (A) Diagramatic representation of the experiment. Pieces of ventral equatorial tissue (composed mostly of ventral mesoderm cells) were dissected from embryos (stage 10.25) previously injected with the lineage tracer RLDx (red). The explants were immediately combined with blastula animal caps (stage 9) derived from embryos previously injected with different amounts of *Siamois* (25 pg, 50 pg, 100 pg), *noggin* (25 pg, 50 pg, 100 pg), or *gsc* (100 pg, 500 pg) mRNA or with 10 pg of*Xwnt-8* mRNA. The 'sandwiches' were cultured until their mesoderm component reached the equivalent of stage 18 for analysis of the presence of the muscle marker MyoD (B) or stage 26-28 for double immunostaining with the muscle and notochord antibodies 12/101 and MZ15 (B,C, and not shown). (B,C) Photographs of sections through sandwiches composed of a piece of ventral mesoderm tissue labelled with fluorescent RLDx combined with an animal cap derived either from control uninjected embryos (cont) or from embryos injected with 50 pg of *noggin* mRNA (nog), 50 pg of *Siamois* mRNA (Sia), 500 pg of *gsc* mRNA (gsc) or 10 pg of Xwnt-8. B) Left panels, RLDx-labelled cells at stage 18; middle panels, MyoD-positive cells in the same sections as the left panels; right panels, 12/101-positive cells at stage 26. MyoD staining was shown here in addition to 12/101 staining as it allows a better visualisation of the relative localisations of antibody and RLDx stainings, and demonstrates that muscle-specific staining was seen only in the progeny of the RLDx-labelled marginal zone cells and not in those of the animal cap. (C) Left panels, RLDx-labelled cells at stage 26. Right panels, 12/101-positive cells at stage 26. No MZ15 staining was ever observed (not shown and Table 1).

Table 1. Quantification of *Siamois*, *noggin* and *goosecoid* dorsalising activities in conjugate experiments

	% of muscles positive cells in conjugates	% of notochord-positive cells in conjugates	Number of positive conjugates
No mRNA	<1	0	2/20
noggin mRNA			
25 pg	30-40	0	7/8
50 pg	40-50	0	15/15
100 pg	50-60	0	15/15
Siamois mRNA			
25 pg	5-10	0	6/8
50 pg	30-40	0	15/15
100 pg	40-50	0	15/15
gsc mRNA			
100 pg	0	0	0/10
500 pg	0	0	0/10

The percentage of muscle- or notochord-expressing cells per 'sandwich' was estimated by scoring the number of nuclei in 12/101- and RLDx-labelled or in MZ-15- and RLDx-labelled cells compared to the total number of nuclei in RLDx-labelled cells, on alternate 10 μm sections. Similar values were obtained for MyoD.

early embryos. Alternatively, *Siamois*-induced dorsalising molecule could cooperate with additional factors present in ventral marginal zone but not in animal cap cells.

Activation of a secreted dorsaliser in animal caps is not a general property of dorsalising homeobox genes: animal caps injected with 100 pg or 500 pg of mRNA for the homeobox gene *gsc* (Cho et al., 1991) failed to dorsalise ventral mesoderm in our assay (Fig. 4B, Table 1) although *gsc* mRNA used here induced partial secondary axes when injected into ventral blastomeres of 4-cell stage *Xenopus* embryos (not shown).

Finally, we tested the effect of activating the pre-MBT Wnt pathway on the dorsalising properties of animal caps. Surprisingly, injection of 10 pg of *Xwnt-8* mRNA in animal caps also conferred dorsalising activity to the injected caps (Fig. 4C).

From these results, we conclude that, in the absence of any vegetal or marginal factor, *Siamois*, like *Xwnt-8*, activates the secretion of a dorsalising factor by animal cap cells.

Molecular basis of dorsalisation by *Siamois*

In an attempt to identify target genes of *Siamois* involved in the dorsalising activity of *Siamois*-expressing caps, we overexpressed *Siamois* mRNA in animal caps and looked for effects on genes thought to play a role in the dorsoventral patterning of the embryo during the blastula and early gastrula stages (for a review see Dawid, 1994).

Animal caps injected with increasing doses of *Siamois* mRNA were dissected at stage 9, cultured until stage 10.25 or 10.5/11, and analysed by RNase protection for the expression of early genes with dorsalising, ventralising, or mesoderm-inducing activity. First, *Siamois* activates neither *Xbra*, an early trunk mesodermal marker (Smith et al., 1991), nor *Xlim.1*, a marker for both trunk and head mesoderm at this stage (Taira et al., 1992 and D. Caillol and P. L., unpublished results), thus strengthening our proposition that *Siamois* does not induce mesoderm in animal caps (Fig. 5A). Second, overexpression of *Siamois* induced the dorsal genes *gsc*, *chordin*, *Xnr-3* and to a lesser extent *noggin*, and repressed the ventralising gene *Bmp-4* (Fig. 5), demonstrating the acquisition by the injected caps of a dorsal character.

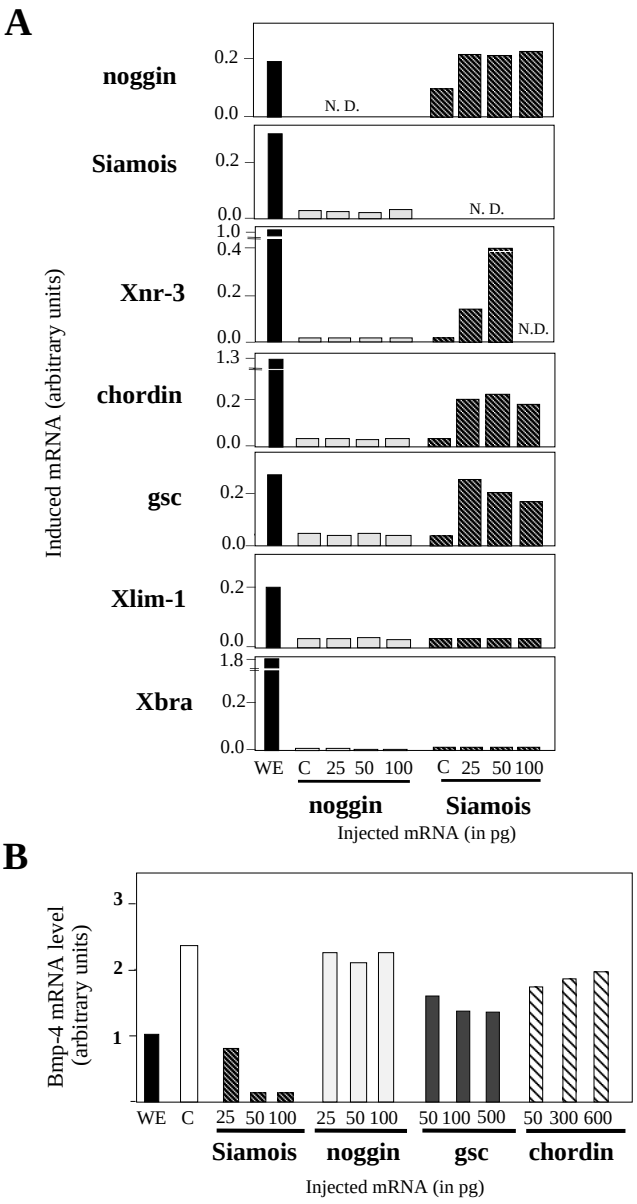


Fig. 5. *Siamois* triggers an organiser gene expression programme in animal cap explants. RNase protection assays using total RNA extracted from early gastrula animal cap sandwiches either uninjected (C) or injected with *Siamois*, *noggin*, *gsc* or *chordin* mRNAs. WE, whole embryo. (A) Effect of the injection of *Siamois* mRNA on the activation of *noggin*, *Siamois*, *chordin*, *gsc*, *Xlim.1*, *Xnr-3* and *Xbra*. (B) Effect of the overexpression of *Siamois*, *noggin*, *gsc* and *chordin* in animal caps on the steady state level of *Bmp-4* mRNA. To determine the level of *Siamois* mRNA, animal cap sandwiches were stopped at stage 10-10.25. Otherwise, the assays were performed on stage 10.5-11 sandwiches. The arbitrary units correspond to the ratio of the radioactive signal for the tested gene over that for the FGF receptor, used here as a loading control (10-15 animal caps per experimental point). ND, not done.

Interestingly, as *gsc*, *chordin*, *noggin* and *Xnr-3* mark both mesodermal and endordermal dorsal cells (Sasai et al., 1994; Vodicka and Gerhart, 1995; D. Caillol and P. L., unpublished results), activation of *gsc*, *chordin*, *Xnr-3* and *noggin* is not incompatible with our proposition that *Siamois*-expressing

animal cells do not form mesoderm and may suggest that they form anterior endoderm. In contrast to *Siamois*, *noggin* failed to regulate the expression of any of the above genes in animal caps (Fig. 5).

It has been previously reported that overexpression of *gsc* in the equatorial region of *Xenopus* embryos inhibits *Bmp-4* gene expression (Fainsod et al., 1994). Furthermore, *gsc* is able to induce *chordin*, which can antagonise *Bmp-4* activity (Sasai et al., 1995). We tested the possibility that repression of *Bmp-4* by *Siamois* in our system may be mediated by *gsc* and/or *chordin*. In animal caps, increasing concentrations of *gsc* or *chordin* mRNA did not affect the level of *Bmp-4* mRNA (Fig. 5B) suggesting that Siamois represses *Bmp-4* independently of the activation of *gsc* and *chordin*.

We conclude therefore that *Siamois* triggers an early dorsal genetic programme in animal caps in the absence of mesoderm. Furthermore, the activation of *noggin* and *chordin*, whose proteins can antagonise BMP-4 signalling (Sasai et al., 1995; Re'em Kalma et al., 1995) and the down-regulation of *Bmp-4*, suggest that the repression of the BMP-4 pathway may play a crucial role in the ability of *Siamois*-injected caps to dorsalise ventral mesoderm. This is further supported by the observation that the strength of this repression of *Bmp-4* increased with the amount of injected *Siamois* mRNA, thereby paralleling the efficiency of *Siamois*-expressing animal caps to dorsalise ventral mesoderm in our conjugate assay (Fig. 5B; Table 1).

Competence of ventral mesoderm for dorsalisation by animal caps overexpressing *Siamois*, *chordin* or *noggin*

To test if secretion of chordin or noggin could account for the dorsalising activity of *Siamois*-injected caps, we have used the fact that ventral mesoderm progressively looses its competence to become dorsalised during gastrulation (Lettice and Slack, 1993). If *Siamois* acts in animal caps mainly through the secretion of *noggin* or *chordin*, one would expect that ventral mesoderm would simultaneously lose its competence to be dorsalised by *Siamois*-, *noggin*- or *chordin*-expressing caps. The assay used to address this issue is schematised in Fig. 6A. Animal caps expressing *Siamois*, *noggin* or *chordin* were isolated at stage 9 and conjugated with RLDx-labelled ventral mesoderm of increasing age. The competence of early (stage 10.25), mid (stage 12) or late (stage 13) gastrula ventral mesoderm to become dorsalised by the injected caps was then determined by looking for the presence of muscle cells in the conjugates. Stage 9 animal caps were used in these experiments because the ability of *Siamois*-injected caps to dorsalise early mesoderm was maximal at that stage (data not shown). Nearly all (n=58/60) ventral mesoderm explants combined at stage 10.25 or stage 12 with *Siamois*- or *noggin*-expressing animal caps were dorsalised as demonstrated by the presence of muscle cells in the mesodermal part of the explant (Fig. 6B). The proportion of muscle cells in the labelled mesoderm ranged from about 30 to 60% depending on the amount of RNA injected (25 pg or 100 pg) (Fig. 6B). Animal caps injected with 600 pg of *chordin* mRNA could also dorsalise stage 10.25 or 12 ventral mesoderm. This activity, however, appeared to be weaker than that elicited by *noggin* or *Siamois* as conjugates presented about 30% of differentiated muscle cells in the labelled mesoderm (Fig. 6B). Animal caps overexpressing *Siamois* markedly failed to dorsalise stage 13 ventral mesoderm (Fig. 6B): in 9 out of 10 explants analysed, the conjugates contained only ~1-5% of muscle cells. In contrast, animal caps dissected from embryos injected with *noggin* mRNA or chordin mRNA dorsalised stage 13 ventral mesoderm (Fig. 6B).

The different capacities of *Siamois*-injected caps on the one hand and of *chordin*- or *noggin*-injected caps on the other hand to dorsalise stage 13 ventral mesoderm suggest that secretion of these two latter molecules is not sufficient to account for the dorsalising properties of *Siamois*-injected caps. Three TGF-β related molecules with dorsalising potential, *Xnr-1*, *Xnr-2* and *Xnr-3*, have been isolated recently (Jones et al., 1995; Smith et al., 1995). Of these, only *Xnr-3* is activated by *Siamois* in animal caps (Fig. 5A and data not shown).

As *Xnr-3* is not activated by *noggin* in animal caps (this study; Smith et al., 1995), we tested whether the state of activation of *Xnr-3* in animal caps may account for the qualitative difference in dorsalising activity of caps injected with *Siamois* or *noggin* mRNA. Injection of up to 1 ng of *Xnr-3* mRNA promoted elongation of the caps but failed to confer dorsalising activity (data not shown), suggesting that *Xnr-3* dorsalising activity does not account for the difference in dorsalising activity of *Siamois*- or *noggin*-expressing animal cap cells.

Siamois-injected animal caps contain neural and proneural cells and secrete a neuralising factor

In addition to being able to dorsalise ventral mesoderm cells, organiser cells have the property of inducing neural tissue in overlaying ectoderm (reviewed in Kessler and Melton, 1994). We tested if *Siamois*-expressing caps also had this property. Injection of as little as 10 pg of *Siamois* mRNA in animal blastomeres and explantation of the injected animal caps at stage 9 resulted in the appearance in the injected caps of large cement glands, an anterior proneural structure (Sive et al., 1989) (16/17 caps analysed, Fig. 7A). In addition, a large proportion of injected animal caps contained cells expressing the panneural marker N-CAM (7/17 caps injected with 10 pg of *Siamois* mRNA and 3/3 caps injected with up to 100 pg of *Siamois* mRNA, Fig. 7B).

To test if neural or proneural differentiation is a cell-autonomous property of *Siamois*-expressing cells or results from the secretion by these cells of a neural inducer, we conjugated stage 9 animal caps injected with various amounts of *Siamois* mRNA with stage 9 animal caps injected with the lineage tracer RLDx alone and looked for the presence of RLDx-positive cells in the induced cement glands or N-CAM-positive cells (Fig. 7C-E). Analysis of 10 conjugates injected with up to 100 pg of *Siamois* mRNA and positive for N-CAM staining showed that N-CAM-positive cells were never found in RLDx-positive cells. In contrast, in all animal conjugates injected with 10 pg, 33 pg or 100 pg of *Siamois* mRNA (n=30), the induced cement gland contained RLDx-positive cells (Fig. 7D,E), indicating that overexpression of *Siamois* in animal cap cells conferred on them cement gland-inducing ability.

DISCUSSION

Relationships between *Siamois*, the pre-MBT Wnt pathway and mesoderm induction

Formation of a functional Organiser has been proposed to

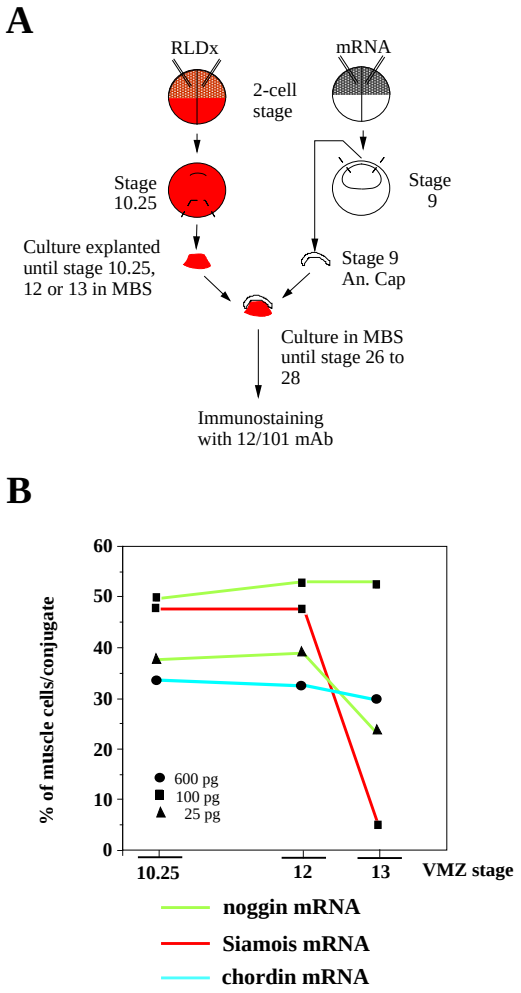


Fig. 6. The windows of competence for dorsalisation by *Siamois*, *noggin* and *chordin* are different. (A) Diagram of heterochronic experiments. Pieces of ventral mesoderm tissue were dissected from embryos previously injected with the lineage tracer RLDx (red). They were conjugated with stage 9 animal caps from embryos injected with the indicated amounts of *Siamois*, *noggin* or *chordin* mRNAs either immediately after dissection or after culture in MBS until the equivalent of stage 12 or 13. The 'sandwiches' were cultured until their mesoderm component reached the equivalent of stage 26-28, fixed, sectioned and analysed for muscle by immunostaining with the muscle specific antibody 12/101. (B) Quantification of the percentage of muscle cells in heterochronic mesoderm-ectoderm conjugates. The percentage of muscle cells per sandwich was estimated by calculating the ratio of the nuclei of 12/101-positive cells over the total number of nuclei of RLDx-positive cells. The stage 9 animal caps used in the sandwiches were derived from embryos injected with 25 pg (triangles) or 100 pg (squares) of mRNAs for *Siamois* or *noggin* or with 600 pg (circles) of *chordin* mRNA. Each point on the graph represents the average muscle content in 10 analysed conjugates. VMZ, ventral marginal zone.

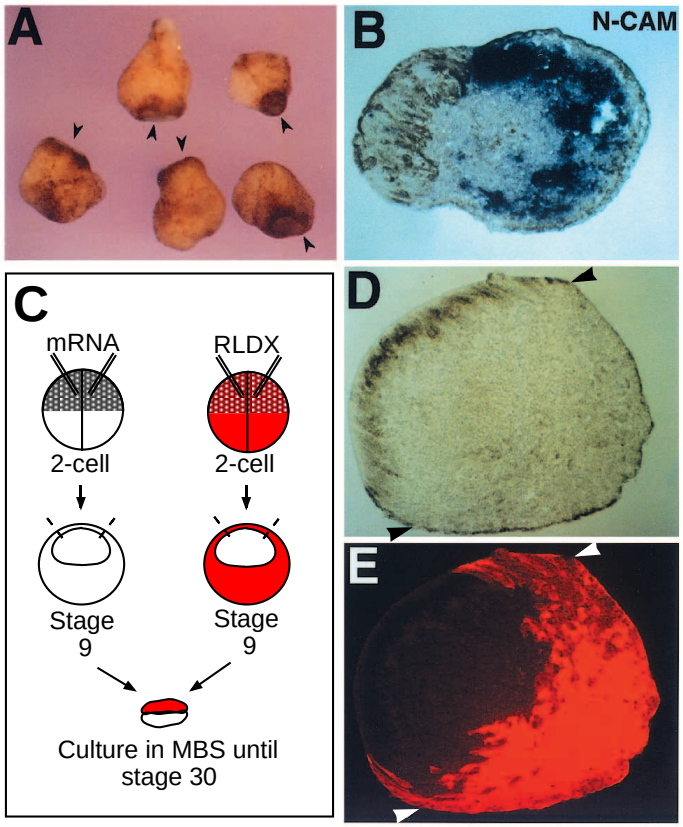


Fig. 7. Neuralisation of *Siamois*-expressing animal caps. (A) Animal caps were injected with 10 pg of *Siamois* mRNA, explanted at stage 9 and cultured in MBS as doublets until stage 30. Large cement glands formed in over 90% of the injected caps (arrowheads). (B) Section through a stage 30 animal cap injected with 100 pg of *Siamois* mRNA and stained with the antibody 4d, specific for the pan-neural marker N-CAM. (C) Diagrammatic representation of the experiment showing the cement gland inducing ability of *Siamois*. Animal caps injected with either *Siamois* mRNA (10, 33 or 100 pg) or the lineage tracer RLDx were excised and combined at stage 9 and cultured until stage 30. (D,E): Phase contrast (D) and fluorescent (E) view of a recombinant between an animal cap injected with 10 pg of *Siamois* mRNA and an RLDx-injected animal cap. Arrowheads mark the limits of the induced cement gland. Note the presence of RLDx-positive cells with the cement gland typical columnar shape.

result from the synergistic action of the pre-MBT Wnt-signalling pathway and mesoderm induction. Activation of *Siamois* (this study) and *Xnr-3* (Smith et al., 1995) in animal caps in response to Wnt signalling but not in response to mesoderm induction demonstrates that activation of the pre-MBT Wnt signalling on its own is sufficient to trigger a dorsal genetic programme during the blastula stages. Furthermore, as animal caps injected with *Xwnt-8* mRNA have dorsalising properties, our results suggest that, while a synergy with mesoderm induction is required for the differentiation of organiser cells into axial structures, activation of the pre-MBT Wnt pathway alone is sufficient for early embryonic cells to acquire organiser properties. In addition, our finding that *Siamois*, a target of the pre-MBT Wnt-signalling pathway, can cooperate with *Xbra*, an early target of the mesoderm induction pathway, to induce dorsal mesoderm strongly suggests that the synergy between the pre-MBT Wnt pathway and mesoderm induction takes place at the level of the early target genes of these two pathways.

Is *Siamois* a mediator of the pre-MBT Wnt-signalling pathway in the embryo?

In keeping with a possible role of *Siamois* as a mediator of the

pre-MBT Wnt-signalling pathway, *Xwnt-8* mRNA and *Siamois* mRNA have several properties in common: unlike *Xnr-3*, another target of the pre-MBT-signalling pathway, both can induce complete secondary axes when injected into the ventral marginal zone and both confer similar dorsalising properties on injected animal caps (Sokol et al., 1991; Lemaire et al., 1995; this study). However, several differences exist. For example, animal caps co-injected with *Siamois* and *Xbra* mRNA elongate (Fig. 2), but animal caps injected with *Xwnt-8* and *Xbra* mRNA do not (Cunliffe and Smith, 1993). Similarly, injection of *Siamois*, but not of *Xwnt-8*, mRNA into animal caps results in neural differentiation of ectodermal cells (this study, Cunliffe and Smith, 1993). However, activation of the pre-MBT Wnt-signalling pathway by overexpression of β-catenin in animal caps results in the activation of a cement gland marker (McGrew et al., 1995). An explanation for these differences may be that, although we used *Xwnt-8* mRNA as a convenient tool to mimick the activation of the pre-MBT Wnt-signalling pathway, it may also have other effects. Overexpression of *Xwnt-8* after the midblastula transition leads to a partial ventralisation of dorsal structures (Christian and Moon, 1993). Thus, injection of *Xwnt-8* mRNA may lead to two antagonistic sequential effects in animal tissue: until the midblastula transition the pre-MBT Wnt-signalling pathway is activated, thereby promoting dorsal development, while after the MBT, residual *Xwnt-8* mRNA or protein antagonises dorsal development. The differences observed between injection of *Siamois* and *Xwnt-8* mRNA could reflect the fact that overexpression of *Siamois* has the same consequences as the activation of the pre-MBT Wnt-signalling pathway without having the subsequent ventralising effects of *Xwnt-8*.

Goosecoid, chordin, Xnr-3 and Bmp-4 are target genes of Siamois

We have identified four potential target genes for *Siamois*: *gsc*, *chordin*, *Xnr3* and *Bmp-4*. Although our experimental system does not provide us with proof of a direct regulation, this possibility is not excluded: *chordin* and *gsc* accumulate shortly after *Siamois* in the dorsal-vegetal region of the late blastula (Sasai et al, 1994 ; Lemaire et al., 1995 ; Smith et al., 1995; D. Caillol and P. L., unpublished results) and the domain of expression of *Xnr3* also overlaps with that of *Siamois* in the dorsal vegetal epithelium (Lemaire et al., 1995; Smith et al., 1995). The amounts of injected *Siamois* mRNA necessary to regulate the expression of these genes (25-100 pg) are much higher than the level of endogenous *Siamois* mRNA present in dorsal vegetal cells (see Fig. 1 for a comparison between the levels of *gsc* and *Siamois* mRNA in whole embryos). While it is difficult to relate the amount of injected mRNA to the amount of protein produced in the embryo, this may suggest that, in vivo, *Siamois* cooperates with other vegetal factors to regulate these genes.

While the regulatory sequences for *chordin*, *Xnr-3* and *Bmp-4*, have not been characterised, the regulatory sequences of *gsc* have been analysed recently (Watabe et al., 1995). In this study, the authors identified a proximal and a distal regulatory element responsive to Wnt and activin signalling respectively. As *Siamois* is not activated by mesoderm inducers, regulation of *gsc* by *Siamois* is likely to be mediated by the Wnt-responsive element. This element, both in *Xenopus* and in the mouse, contains two potential ATTA homeodomain consensus binding sites (Watabe et al., 1995). It will therefore be interesting to test if *Siamois* can bind to these sequences and to analyse the effect of their mutation on *gsc* regulation.

Activation of *gsc* by *Siamois* is unlikely to play a significant role in the dorsalising activity of *Siamois*-injected caps: while the dorsalising ability of *Siamois*-injected animal caps increases with the amount of injected *Siamois* mRNA, the activation of *gsc* by *Siamois* is maximal at low concentrations of injected *Siamois* mRNA and decreases with higher doses. However, *gsc* dorsalising potential has been shown to be restricted to the vegetal and equatorial cells (Niehrs et al, 1994). Activation of *gsc* by *Siamois* in the marginal zone could therefore contribute to the normal function of *Siamois* in the embryo.

Siamois and the BMP-4 pathway

Antagonising the BMP-4 pathway is sufficient to create an organiser (reviewed in Lemaire and Kodjabachian, 1996). For instance, *noggin* and *chordin*, two Spemann organiser genes, act as inhibitors of BMP-4 protein activity (Re'em Kalma et al., 1995; Sasai et al., 1995). The concerted repression of *Bmp-4* and activation of *chordin* and *noggin* gene expression by *Siamois* may account for the organising properties of this gene. However, two pieces of evidence suggest that *Siamois* also activates a parallel dorsalising pathway. First, the time of loss of competence of ventral mesoderm for dorsalisation by animal caps expressing either *noggin*, *chordin*, or *Siamois* differs (this study). This suggests that, although one of the consequences of the overexpression of *Siamois* in animal caps is to repress the BMP-4 pathway, the main dorsalising activity detected in our assay may be due to the activation of another dorsalising pathway. Second, secondary axes generated by overexpression of *noggin, chordin,* or a truncated BMP receptor in whole embryos generally lack the anterior-most structures, while *Siamois* efficiently induces secondary axes with a complete head (Graff et al., 1994; Sasai et al., 1994; Lemaire et al, 1995). This suggests that antagonising the BMP-4 pathway results in the creation of a trunk organiser while overexpression of *Siamois* induces both head and trunk organisers. Surprisingly, *Xlim.1*, the only gene shown to be required for head formation (Shawlot and Behringer, 1995) and a gene displaying similar effects as *Siamois* when overexpressed in animal caps (Taira et al., 1994; Sasai et al, 1995), is not activated by *Siamois* in these cells. *Xnr-3*, because of its ability to dorsalise gastrula ventral mesoderm, its expression in dorsal vegetal cells and its activation by *Siamois* in animal caps may account for the dorsalising activity of *Siamois*. However, *Xnr-3*-expressing animal caps fail to dorsalise ventral mesoderm (data not shown), suggesting that if *Xnr-3* plays a role in the organising properties conferred by *Siamois* it probably requires a co-factor.

Neural induction by *Siamois*

Repression of the BMP-4 pathway in ectoderm by overexpressing *noggin*, *chordin* or dominant negative forms of a BMP-4 receptor or of the ligand itself is sufficient to convert this tissue into cement gland and neural tissue (reviewed in Lemaire and Kodjabachian, 1996). Neuralisation by *Siamois* may therefore reflect the repression of the BMP-4 pathway in *Siamois*-expressing ectoderm. It is so far unclear whether *Siamois*-expressing cells are directly converted into neural

tissue: while our data indicate that *Siamois*-expressing caps do not form mesoderm, they do not exclude the possibility that they contain some dorsal endoderm. Indeed, activation of the dorsal mesendodermal genes, *gsc*, *chordin*, *noggin* and *Xnr-3*, but not of the mesodermal genes, *Xlim.1* and *Xbra*, may suggest the presence of early endodermal cells in *Siamois*-injected caps, a possibility that will need further investigation.

Our finding that cement gland induction by *Siamois* can occur in a non cell-autonomous fashion is in keeping with a role for *Siamois* in neural induction during normal development: the progeny of cells expressing *Siamois* at the early gastrula stage will form during gastrulation the anterior mesendoderm, located underneath the ectoderm most highly specified for cement gland formation (Sive et al., 1989) and expressing *chordin*. However, the inducing ability of anterior mesendoderm is questionable as Sive and colleagues (1989) have shown that explanted anterior mesendoderm from mid-gastrula lacks neural and cement gland-inducing ability, while Sharpe and Gurdon (1990) showed that it harboured anterior neural inducing ability. Therefore further studies will be necessary to determine precisely the potential role of *Siamois* in neural induction during development.

Siamois may define the late blastula organiser

On the basis that injection of *Siamois* mRNA into ventral-vegetal cells could induce a complete secondary axis to which the injected cells do not participate, we proposed that *Siamois* may confer a Nieuwkoop centre-like activity on embryonic cells (Lemaire et al., 1995). This is in keeping with our finding that *Siamois* is a target of Wnt signalling but not of mesoderm induction (this study). However, while the Nieuwkoop centre is thought to act during the blastula stages, the results presented in this article indicate that signals activated by *Siamois* are able to dorsalise gastrula mesoderm and induce neural structures. While it is not ruled out that Nieuwkoop centre signals have properties different from expected, an alternative interpretation of our results is that *Siamois* is involved in conferring organising properties on late blastula or early gastrula vegetal cells. Indeed, these cells have been proposed by Gerhart and collaborators (1991), to constitute the late blastula organiser, an intermediate organising centre acting between Nieuwkoop centre and Spemann organiser.

In summary, the findings reported in this article suggest that *Siamois* may be an important mediator of the Pre-MBT Wnt-signalling pathway and indicate that overexpression of *Siamois* in embryonic cells confers upon them two organiser properties in the absence of recognisable mesoderm: ability to dorsalise ventral mesoderm during gastrulation and to induce neural tissue. Therefore mesoderm induction, while being necessary for the differentiation of organiser cells into dorsal mesoderm, may play a more minor role than the Pre-MBT Wnt-signalling pathway in the acquisition by these cells of early organising potential.

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