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Endodermal Nodal-related signals and mesoderm induction in *Xenopus*

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SUMMARY

In *Xenopus*, mesoderm induction by endoderm at the blastula stage is well documented, but the molecular nature of the endogenous inductive signals remains unknown. The carboxy-terminal fragment of Cerberus, designated Cer-S, provides a specific secreted antagonist of mesoderm-inducing *Xenopus* Nodal-Related (Xnr) factors. Cer-S does not inhibit signalling by other mesoderm inducers such as Activin, Derrière, Vg1 and BMP4, nor by the neural inducer Xnr3. In the present study we show that Cer-S blocks the induction of both dorsal and ventral mesoderm in animal-vegetal Nieuwkoop-type recombinants. During blastula stages *Xnr1*, *Xnr2* and *Xnr4* are expressed in a dorsal to ventral gradient in endodermal cells. Dose-response experiments using *cer-S* mRNA injections support the existence of an endogenous activity gradient of Xnrs. Xnr expression at blastula can be activated by the vegetal determinants VegT and Vg1 acting in synergy with dorsal β-catenin. The data support a modified model for mesoderm induction in *Xenopus*, in which mesoderm induction is mediated by a gradient of multiple Nodal-related signals released by endoderm at the blastula stage.

Key words: Mesoderm induction, Nodal, Xnr, Cerberus, TGF-β, Derrière, Activin, VegT, β-catenin, Vg1, Cer-S

INTRODUCTION

The amphibian embryo provides a much utilized model system to study the early phases of embryonic patterning. The pioneering work of Nieuwkoop, Slack and colleagues has led to the current three-signal model of mesoderm induction and patterning (reviewed by Nieuwkoop, 1973; Slack, 1991a; Kessler and Melton, 1994; Heasman, 1997). Using recombinants of blastula endodermal and ectodermal explants, it was shown that mesoderm is generated via inductive signals from endoderm (Nieuwkoop, 1969; Slack, 1991b). The endoderm is thought to release two signals: first, a uniform or ventral endodermal signal that induces ventral mesoderm such as lateral plate, mesenchyme and blood and, second, a signal emanating from dorsal endoderm that induces dorsal organizer tissue in the overlying mesoderm (Nieuwkoop, 1969, 1973; Boterenbrood and Nieuwkoop, 1973; Harland and Gerhart, 1997; Heasman, 1997). The third signal in this model, also called the horizontal signal, emanates from dorsal organizer tissue during gastrulation and is able to induce the differentiation of dorsal histotypes such as notochord, somites and kidney in ventral mesodermal cells (Smith and Slack, 1983; Harland and Gerhart, 1997; Heasman, 1997). A number of molecules secreted by Spemann's organizer are thought to participate in this third signal (De Robertis et al., 1997; Harland and Gerhart, 1997).

A recent advance in the field has been the realization that the generation of mesoderm-inducing signals by endoderm is dependent on the activity of maternally encoded transcriptional regulators. Vegetal explants depleted of *β-catenin* mRNA by antisense oligodeoxynucleotides are unable to release Nieuwkoop's dorsal signal (Heasman et al., 1994). In a recent study, it was shown that the signal initiated by the *β-catenin* pathway is not released until after the midblastula transition (Wylie et al., 1996). This finding, based on the use of heterochronic animal-vegetal conjugates, is important for the present analysis because it demonstrated that, contrary to the previously held view, mesoderm induction may be mediated by zygotically expressed genes (Heasman, 1997). Oligonucleotide depletion experiments have also shown that *VegT*, a transcription factor maternally stored in the vegetal hemisphere of the *Xenopus* egg, is required for the release of both the ventral and dorsal mesoderm-inducing signals by vegetal explants (Zhang et al., 1998).

Despite intensive efforts, the molecules mediating the first two inductive signals of the *Xenopus* model remain unknown. Many growth factors with mesoderm-inducing activity have been identified to date, such as FGF, Activin, Vg1, *Xenopus* Nodal-related factors (Xnrs) and Derrière (Slack, 1991a; Smith, 1995; Thomsen and Melton, 1993; Jones et al., 1995; Sun et al., 1999). There is evidence suggesting that neither Activin nor FGF are likely to be endogenous mesoderm inducers released by endoderm, since mesoderm induction could not be blocked by Follistatin (an Activin and BMP inhibitor) nor by FGF blocking antibodies (Slack, 1991b). Interestingly, Activin applied at increasing doses to blastula

animal cap cells is able to induce the entire range of mesodermal derivatives, with ventral cell types at low and dorsal ones at high doses (Green and Smith, 1990; Green et al., 1992; Smith, 1995). Other known mesoderm-inducing factors of the transforming growth factor beta (TGF-β) superfamily – Vg1, Xnrs and Derrière – share with Activin this ability to induce both ventral and dorsal mesoderm.

In mouse and zebrafish, genetic studies strongly suggest that Nodal signalling pathways play a central role in mesoderm formation (Zhou et al., 1993; Conlon et al., 1994; Waldtrip et al., 1998; Nomura and Li, 1998; Song et al., 1999; Sampath et al., 1998; Rebagliati et al., 1998; Feldman et al., 1998; Gritsman et al., 1999). In *Xenopus*, four Xnr genes have been identified to date. Three of them, *Xnr1*, *Xnr2* and *Xnr4* are mesoderm-inducing factors (Jones et al., 1995; Joseph and Melton, 1997; Osada and Wright, 1999). *Xnr3*, despite sharing sequence similarities with other Xnrs, has very different activities since it lacks mesoderm-inducing capacity, acts as a neural inducer, and is under different regulatory control (Smith et al., 1995; Ecochard et al., 1995; Hansen et al., 1997; McKendry et al., 1997). The existence of three mesoderm-inducing Xnr genes, and possibly additional ones not yet identified, makes *Xenopus* loss-of-function studies difficult.

Cerberus is a head-inducing secreted factor (Bouwmeester et al., 1996) that acts as a multifunctional antagonist of Nodal, BMP (Bone Morphogenetic Proteins) and Wnt signals (Hsu et al., 1998; Piccolo et al., 1999). A carboxy-terminal fragment of Cerberus, called Cerberus-short (Cer-S), lacks the anti-Wnt and anti-BMP activities but retains full anti-Xnr1 activity (Piccolo et al., 1999). In biochemical studies, Cer-S was found to bind Xnr1, but not Activin nor Vg1 proteins (Piccolo et al., 1999).

In the present study we use the *cer-S* reagent as a specific inhibitor of Xnr signals. In Nieuwkoop animal-vegetal recombinants, *cer-S* was able to block mesoderm induction by both dorsal and ventral endodermal fragments. It was also found that endogenous *Xnr1*, *Xnr2* and *Xnr4* are expressed zygotically in blastula endoderm in a graded fashion with a maximum in dorsal endoderm, i.e., at the right time and place to mediate mesoderm induction. The expression of *Xnr1*, *Xnr2* and *Xnr4* transcripts starts at midblastula and can be activated synergistically by the vegetally localized determinants *VegT* and *Vg1* (Zhang et al., 1998; Joseph and Melton, 1998) acting together with the dorsal determinant *β-catenin* (Heasman et al., 1994; Schneider et al., 1996). These results lead us to propose that the first two signals of the *Xenopus* three-signal model could be provided by a gradient of Nodal-related signals released by the endoderm during the blastula stage.

MATERIALS AND METHODS

Synthetic mRNAs

The plasmids pCS2-*cer-S*, pCS2-*Xnr1*, pCS2-*Xnr2*, pCS2-*A-Vg1*, pCS2-*A-mouse-nodal*, pCS2-*tALK-4*, pCS2-*derrière* and pCS2-*β-catenin* were linearized with *Not*I, and pCS2-*VegT-En*^{R} and pCS2-*Sia-En*^{R} were linearized with *Sac*II; all were transcribed with SP6 RNA polymerase (Piccolo et al., 1999). pcDNA-Δ*N-XTcf-3* was linearized with *Xba*I and transcribed with T7 polymerase. Δ*N-XTcf*-3 and *derrière* plasmids were gifts from Drs H. Clevers and H. L. Sive respectively. pCS2-*En-VegT* was constructed essentially as described by Horb and Thomsen (1997) and pCS2-*tAlk-4* was constructed as described by Chang et al. (1997).

Embryo manipulations

Xenopus embryos obtained by in vitro fertilization were maintained in 0.1× Barth medium and staged according to Nieuwkoop and Faber (1994). RNA injections were performed at the 4-cell stage. At stage 8, vegetal explants were dissected in 1× Barth medium and allowed to heal for 10 minutes. Animal caps were cut in CMFM/LCMR 1:1 (Piccolo et al., 1999) and placed on top of vegetal explants in Terasaki plates (Nunc) for 2 hours before being either processed by RT-PCR or transferred into 0.3× Barth medium until stage 36 for histological analysis. For cycloheximide (CHX) treatment, caps were cut at stage 8, pretreated 30 minutes with CHX (25 μg/ml), treated for 90 minutes with 4 nM Vg1 protein (Piccolo et al., 1999) and CHX (25 μg/ml) and then processed for RT-PCR. This treatment with CHX inhibited [^{35}S]methionine and [^{35}S]cysteine incorporation into proteins by 94%. In situ hybridization was performed on hemisectioned embryos in order to visualize endodermal cells as described by Belo et al. (1997; http://www.lifesci.ucla.edu/hhmi/derobertis/index.html).

RT-PCR analysis

Primer sets used for RT-PCR are detailed below. *Activin βB*, 288 bp, forward 5′-CGGATCCAGTTTTACATTGAC-3′, reverse 5¢-CGAATTCTGCAGCACGAGTTC-3′, 30 cycles. *α-Actin*, 252 bp, forward 5′-TCCCTGTACGCTTCTGGTCGTA-3′, reverse 5′-TCTCAAAGTCCAAAGCCACATA-3′, 20 cycles. *Brachyury* (*XBra*), 319 bp, forward 5′-GCTGGAAGTATGTGAATGGAG-3′, reverse 5′-TTAAGTGCTGTAATCTCTTCA-3′, 25 cycles. *chordin* (*chd*), 267 bp, forward 5′-CCTCCAATCCAAGACTCCAGCAG-3′, reverse 5′-GGAGGAGGAGGAGCTTTGGGACAAG-3′, 25 cycles. *derrière*, 178 bp, forward 5′-GACAGCAAGATGAACAGGAA-3′, reverse 5′-CTACAAATGATCGATTGCCT-3′, 25 cycles. *Dickkopf1 (Dkk1)*, 255 bp, forward 5′-CACCAAGCACAGGAGGAA-3′, reverse 5′-TCAGGGAAGACCAGAGCA-3′, 25 cycles. *Elongation Factor α* (*EF1α)*, 221 bp, forward 5′-CCTGAACCACCCAGGCCAGATT-GGTG-3′, reverse 5′-GAGGGTAGTCAGAGAAGCTCTCCACG-3′, 20 cycles. *follistatin*, 230 bp, forward 5′-CAGTGCAGCGCT-GGAAAGAAAT-3′, reverse 5′-TGCGTTGCGGTAATTCACTTAC-3′, 30 cycles. *Frzb1*, 200 bp, forward 5′-GGAGATGCAGACTCC-TCTGTCA-3′, reverse 5′-GACCACTGAATGTAGCCAGGAC-3′, 25 cycles. *goosecoid* (*gsc*), 305 bp, forward 5′-CACACAAAGTCG-CAGAGTCTC-3′, reverse 5′-GGAGAGCAGAAGTTGGGGCCA-3′, 25 cycles. *NCAM*, 138 bp, forward 5′-GCGGGTACCTTCTAAT-AGTCA C-3′, reverse 5′-GGCTTGGCTGTGGTTCTGAAGG-3′, 25 cycles. *noggin*, 281 bp, forward 5′-AGTTGCAGATGTGGCTCT-3′, reverse 5′-AGTCCAAGAGTCTCAGCA-3′, 30 cycles. *Ornithine decarboxylase* (*ODC*), 228 bp, forward 5′-CAGCTAGCTGT-GGTGTGG-3′, reverse 5′-CAACATGGAAACTCACACC-3′, 25 cycles. *Siamois* (*sia*), 205 bp, forward 5′-AAGATAACTGGCA-TTCCTGAGC-3′, reverse 5′-GGTAGGGCTGTGTATTTGAAGG-3′, 25 cycles. *Vg1*, 160 bp, forward 5′-ATGCCTATTGCTTCTATTTGC-3′, reverse 5′-GGTTTACGATGGTTTCACTCA-3′, 25 cycles. *Xnr1*, 190 bp, forward 5′-AAGTCAAGTCCTCTGCCAAC-3′, reverse 5′-AGAGGTTTCCCATTTTCGAC-3′, 25 cycles. *Xnr2*, 211 bp, forward 5′-TAAGGGCTGAGGTTGAAGAAG-3′, reverse, 5′-CGGGGTCT-TCTGGTATCTGTC-3′, 25 cycles. *Xnr3*, 219 bp, forward 5′-CGAGTGCAAGAAGGTGGACA-3′, reverse 5′-ATCTTCATGGG-GACACAG GA-3′, 25 cycles. *Xnr4*, 300 bp, forward 5′-GAAATGGAGGTGATGGTAGAC-3′, reverse, 5′-GACCATCAT-CACTATCTGCTG-3′, 25 cycles.

RESULTS

Cer-S is an inhibitor of Xnrs and of organizer formation

The starting point for this investigation was the observation that *cer-S* mRNA microinjection blocked the formation of the

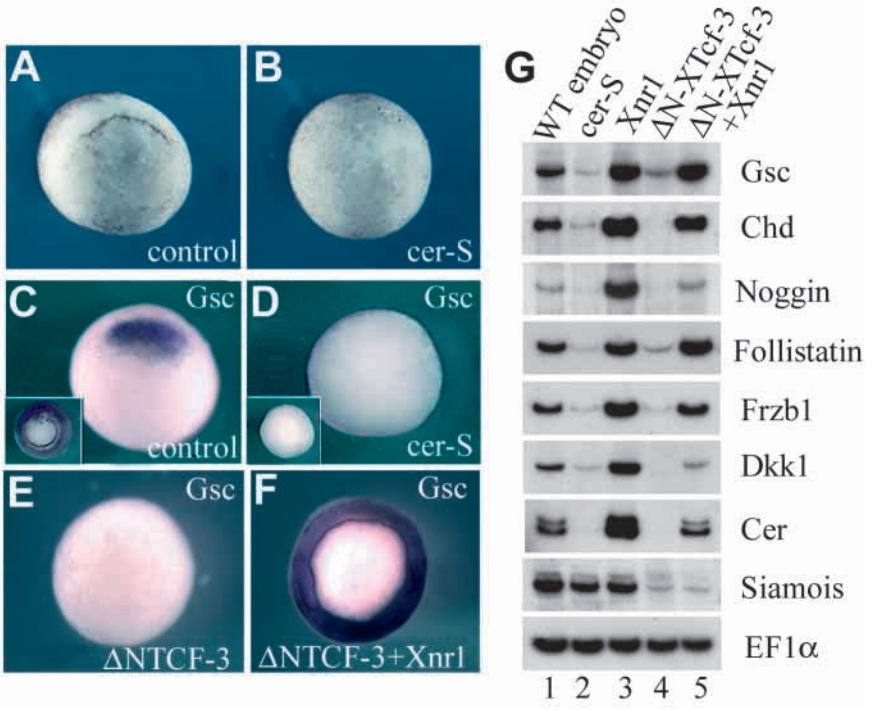

Fig. 1. Cer-S, a secreted inhibitor of Nodal-related factors, inhibits formation of Spemann's organizer. (A,B) *cer-S* mRNA (radial injection of 150 pg into each blastomere at the 4-cell stage) blocks dorsal lip formation. (C,D) *gsc* expression is blocked by *cer-S*, even after LiCl treatment that expands *gsc* expression to the entire mesoderm (insets). (E,F) Δ*N-XTcf-3* mRNA (800 pg radially) inhibits *goosecoid* expression, and co-injection of *Xnr1* (50 pg) restores it in the entire marginal zone. (G) RT-PCR analysis of Spemann organizer markers at stage 10. Lane 1: whole embryos. Lane 2: radial injection of *cer-S* (600 pg total) represses organizer genes. Lane 3: radial injection of *Xnr1* mRNA (50 pg) upregulates organizer markers. Lane 4: the β-catenin pathway antagonist Δ*N-XTcf-3* (800 pg) inhibits organizer markers, which are rescued by co-injection with 50 pg *Xnr1* (lane 5). *Xnr1* acts downstream of, or in parallel to, the β-catenin pathway. *Siamois* (Lemaire et al., 1995) is regulated by the β-catenin pathway (Brannon et al., 1997) independently of Xnr signals.

dorsal lip and the expression of *goosecoid* and other Spemann organizer markers (Fig. 1A-D and G, lanes 1 and 2). Previous biochemical work had shown that the Cer-S secreted protein bound to Xnr1 protein (but not to other mesoderm inducing factors such as Activin, Vg1 and BMP4) and inhibited its activity in the subnanomolar range (Piccolo et al., 1999). To further test the specificity of the *cer-S* construct when injected as synthetic mRNA into embryos, we made use of an assay involving the formation of ectopic blastopore lips (Lustig et al., 1996). Various TGF-β family mRNAs were carefully titrated and the amount of mRNA required to induce ectopic blastopore lips after microinjection into a single animal blastomere at the 8-cell stage was determined. As shown in Fig. 2A to D′, the activities of *activin* (Green et al., 1992; Gurdon et al., 1995), *derrière* (Sun et al., 1999) and *A-Vg1* (Piccolo et al., 1999; Thomsen and Melton, 1993) mRNAs were unaffected by co-injection of *cer-S* mRNA, whereas *Xnr1* was blocked. In addition to *Xnr1* (50 pg), *Xnr2* (150 pg), Xnr4 (50 pg) and mouse *Nodal* (50 pg) mRNAs (Jones et al., 1995; Zhou et al., 1993; Joseph and Melton, 1997; Conlon et al., 1994) were also blocked in this assay (data not shown), demonstrating that *cer-S* is an inhibitor of multiple Xnrs. The differences in response observed between Xnrs and other TGF-β factors was not due to differences in the amount of mRNA injected, as all mRNAs were tritrated to the minimal dose required to induce ectopic lips. Furthermore, 150 pg of *Xnr1* or of *Xnr4* were still inhibited by co-injection of 150 pg *cer-S* mRNA, whereas *derrière* mRNA (150 pg) was not affected by the same dose of *cer-S* mRNA (Fig. 2 and data not shown).

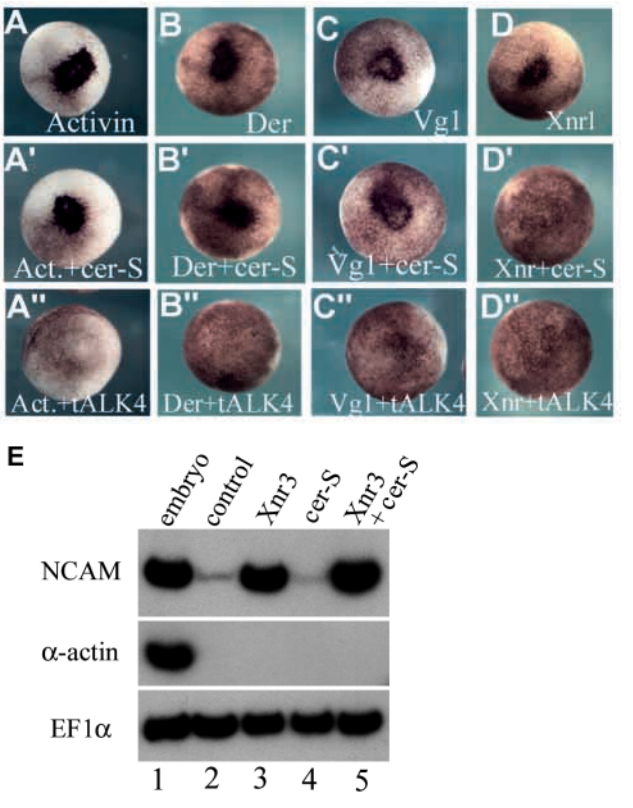

Fig. 2. Cer-S inhibits *Xnrs* but not *activin*, *derrière*, *Vg1* and *Xnr3*. Co-injection of *cer-S* mRNA (150 pg into a single animal blastomere) inhibited ectopic blastopore lip formation by *Xnr1* (50 pg, D′), but not by *activin* (30 pg), *derrière* (150 pg) or *A-Vg1* (50 pg) mRNA (A′-D′). *Xnr2* mRNA (150 pg) was also inhibited (data not shown). Although the doses used for each TGF-β mRNA differed, they all were titrated to elicit comparable biological responses. *tALK4* (800 pg) mRNA blocked all TGF-β mesoderm inducers tested (A″-D″). The ectopic blastopore lips are seen as a darker area in the injected animal cap region due to the apical constrictions of bottle cells (Lustig et al., 1996). (E) *Xnr3* (1.2 ng) is a neural (*NCAM*) but not mesodermal (*α-actin*) inducer in microinjected animal caps (lanes 2 and 3). *cer-S* mRNA (600 pg) does not inhibit *Xnr3* activity in animal caps (lanes 4 and 5). *EF1α* was used as a loading control.

Xnr3 is a gene related to Xnrs that functions as a neural inducer (Hansen et al., 1997). The finding that *cer-S* mRNA does not inhibit neural induction by *Xnr3* (Fig. 2E, compare lanes 3 and 5) strongly supports the view that *cer-S* is a specific antagonist of mesoderm-inducing Nodal-related factors. In addition, the inability of *cer-S* mRNA (600 pg) to induce N-CAM in animal caps (Fig. 2E, lane 4) is in agreement with the previous finding (Piccolo et al., 1999) that *cer-S* is devoid of anti-BMP activity. Although *cer-S* mRNA does not block signalling by the known TGF-β mesoderm inducers – *activin*, *derrière*, *Vg1* and *BMP4* – it is currently not possible to rule out the existence of an as yet undiscovered TGF-β mesoderm inducer that might be inhibited by *cer-S*. Keeping this caveat in mind, in this study *cer-S* mRNA is considered a specific anti-Xnr reagent.

The *cer-S* injection experiments (Fig. 1A-D and G, lanes 1 and 2) indicated that Nodal-related signals are required for the formation of the Spemann organizer at the gastrula stage. In the reciprocal experiment, radial injection of *Xnr1* mRNA into each blastomere of 4-cell embryos was sufficient to increase the expression of the organizer marker genes *goosecoid*, *chd*, *noggin*, *follistatin*, *Frzb1*, *Dkk1* and *cerberus* (Fig. 1G, lane 3). Since organizer formation requires an active *β-catenin* pathway (Heasman, 1997), we next asked whether *Xnr1* was able to rescue organizer tissue formation when this pathway was blocked. Microinjection of a specific inhibitor of the *β-catenin* pathway, *ΔN-XTcf-3* (Molenaar et al., 1996), blocked organizer formation, which could be rescued by co-injection of *Xnr1* mRNA (Fig. 1E,F and G, lanes 4 and 5).

These results suggest that Xnr signals are necessary and sufficient for formation of Spemann organizer tissue in the *Xenopus* gastrula. The ability of *Xnr1* mRNA to rescue organizer tissue in embryos injected with *ΔN-XTcf-3* further suggests that Xnrs act downstream or in parallel of β-catenin, mediating some of its biological activities. Results from genetic and microinjection experiments in zebrafish are consistent with this possibility (Fekany et al., 1999; Feldman et al., 1998).

Cer-S blocks mesoderm induction in Nieuwkoop recombinants

In *Xenopus*, it is well established that Spemann organizer tissue

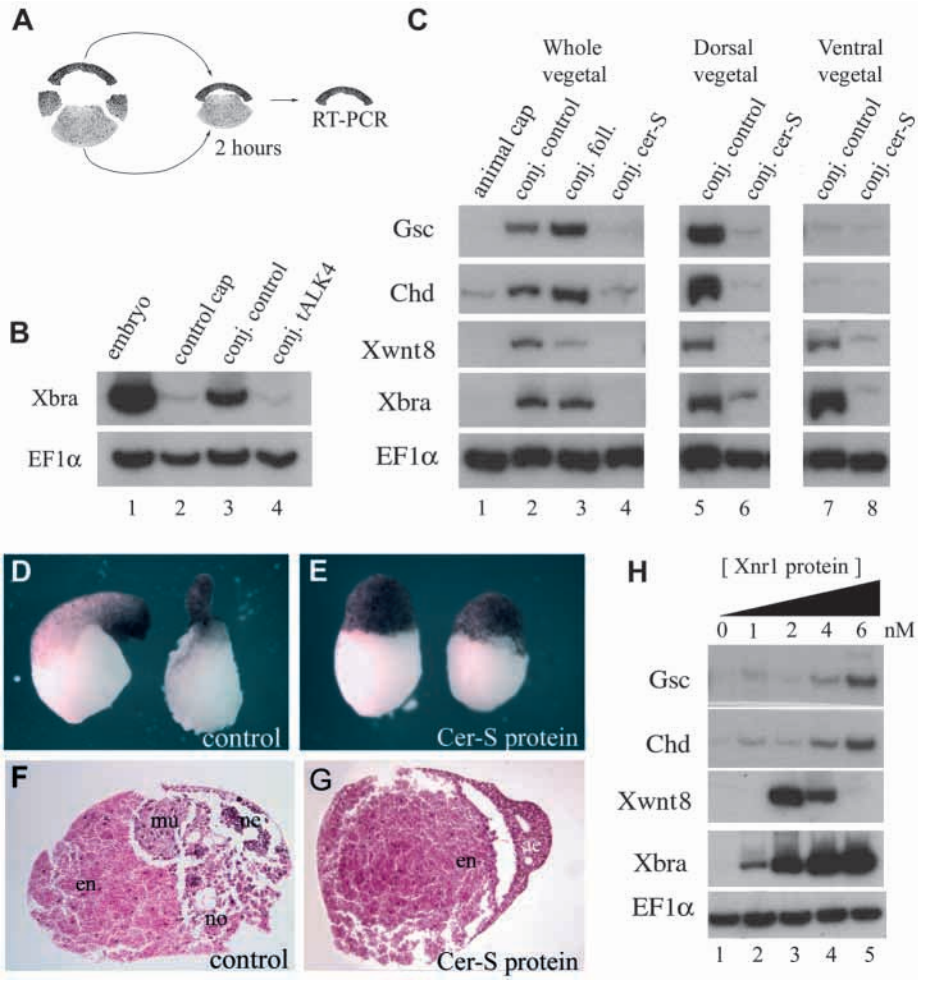

Fig. 3. The endogenous mesoderm-inducing signals are blocked by Cer-S in Nieuwkoop animal-vegetal conjugates. (A) Experimental design. (B) Microinjection of *tALK4* mRNA (*500* pg into each animal blastomere at 8-cell stage) blocks the response of animal caps to endogenous mesoderm-inducing signals (compare lanes 3 and 4). Caps were in contact with endoderm for 2 hours and are compared to control animal caps incubated without endoderm (lane 2). *EF1α* is a control for RNA recovery. (C) Lanes 1-4, Nieuwkoop recombinants of uninjected animal caps with vegetal pole explants injected with *follistatin* (2 ng) or *cer-S* (600 pg) mRNA. Note in lane 4 that *cer-S* blocks dorsal (*gsc*, *chd*), ventral (*Xwnt-8*) and pan-mesodermal (*Xbra*) markers, whereas in lane 3 *follistatin* mRNA has only a slight dorsalizing effect (total conjugates *n*=45, two experiments). This amount of *follistatin* mRNA was sufficient to abolish the activity of activin mRNA in co-injection assays (not shown). Lane 5, dorsal endoderm (Nieuwkoop center) induces preferentially the organizer markers *gsc* and *chd* (*n*=16, three independent experiments). Lane 7, ventral endoderm induces ventral markers *Xwnt-8* and the pan-mesodermal marker *Xbra* (*n*=17). Lanes 6 and 8, *cer-S* mRNA in the endodermal fragment prevents both dorsal and ventral mesoderm inductions (*n*=15 each). Conjugates were prepared between stage 8 and 8.5 and harvested for RNA after two hours. (D-G) External and histological morphology of vegetal fragments conjugated in the presence of control conditioned medium or of 20 nM Cer-S (Piccolo et al., 1999) protein and cultured until stage 36. Note that sections of the control contain muscle (mu), notochord (no) and some neural tissue (ne), whereas in the protein-treated sample the animal cap remains as atypical epidermis (ae) and endoderm (en) (*n*=26, three independent experiments). (H) Animal caps treated for two hours with control oocyte conditioned medium (lane 1) or with increasing doses (lanes 2-5) of Xnr1 protein (Piccolo et al., 1999). Increasing concentrations of Xnr1 protein induce first ventral and then dorsal mesodermal markers, producing thresholds after 2 hours in culture.

is induced by dorsal endoderm. The classical approach to study this inductive event is the Nieuwkoop animal-vegetal conjugate. We therefore used this experimental paradigm (Nieuwkoop, 1969; Wylie et al., 1996) to investigate the nature of the endogenous mesoderm-inducing signals (Fig. 3A).

We first asked whether a TGF-β-like signal secreted by endoderm is required for mesoderm induction. To this end, a truncated activin type IB receptor (Chang et al., 1997), tALK4, was expressed in the animal cap cells that receive the signal. As shown in Fig. 3B, *tALK4* mRNA blocked induction of the pan-mesodermal marker *Xbra* by the endogenous endodermal signal. This implicated a requirement for TGF-β signalling in Nieuwkoop conjugates after only 2 hours of contact, but did not distinguish which factor was involved, since tALK4 was able to block signalling by the mesoderm inducing factors *activin*, *derrière*, *A-Vg1* and *Xnr1* (Fig. 2A″ to D″).

We next tested whether the endodermal signal required Nodal-related factors by microinjecting *cer-S* mRNA into the vegetal pole of early embryos. Endodermal explants from these embryos were prepared at stage 8 to 8.5, recombined with uninjected animal caps and analyzed only after 2 hours of contact with vegetal explants; i.e., during the period in which mesoderm induction occurs in vivo (Wylie et al., 1996). PCR was carried out in the animal cap fragments as described by Wylie et al. (1996); as a control for accuracy of the dissections, vegetal fragments were analyzed for the mesodermal markers *Xbra* and *Xwnt8*, which were not expressed in either uninjected or *cer-S* mRNA-injected vegetal explants (not shown). It was found that in these Nieuwkoop recombinants *cer-S* inhibited not only the induction of the organizer markers *goosecoid* and *chd*, but also the ventral marker *Xwnt8* and the pan-mesodermal marker *Xbra* (Fig. 3C, compare lanes 2 and 4). As a negative control we used *follistatin* mRNA (Fig. 3C, lane 3), an inhibitor of Activin and BMPs (Harland and Gerhart, 1997), which failed to prevent mesoderm induction in agreement with previous work (Slack, 1991b).

Since dorsal and ventral endoderm have different inductive activities (Boterenbrood and Nieuwkoop, 1973), we extended these results using dorsal-vegetal or ventral-vegetal endodermal explants, which induced predominantly dorsal or ventral mesoderm, respectively, in animal cap recombinants (Fig. 3C, lanes 5 and 7). Expression of *cer-S* mRNA in endoderm resulted in the inhibition of mesoderm induction by both dorsal and ventral endodermal fragments (Fig. 3C, lanes 6 and 8).

As the endogenous mesoderm-inducing signal is released at the blastula stage (Wylie et al., 1996), we tested whether Cer-S protein could block mesoderm induction when added at this time. Animal-vegetal explants were recombined in the presence of 20 nM Cer-S protein (Piccolo et al., 1999) at midblastula (stage 8). The resulting conjugates failed to form either dorsal or ventral mesoderm, with the animal cap

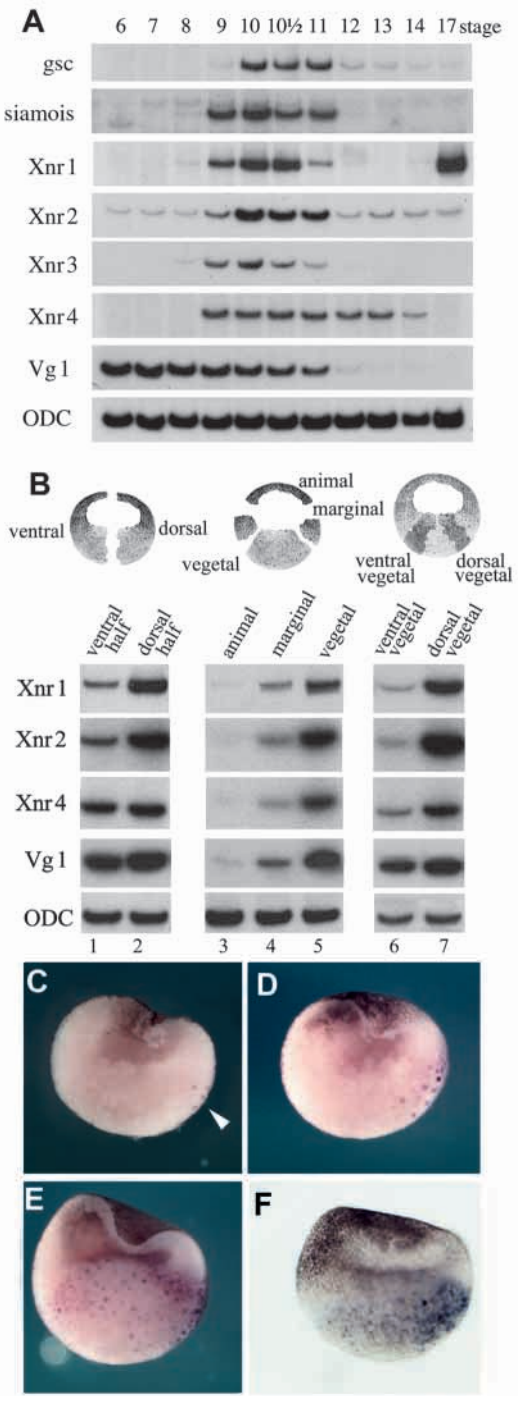

Fig. 4. Endogenous Xnrs are expressed at the right time and place to function as mesoderm inducers. (A) Time course of gene expression analyzed by RT-PCR at various developmental stages (Nieuwkoop and Faber, 1994). The mesoderm inducers *Xnr1*, *Xnr2* and *Xnr4* start zygotic expression at the same time as *Siamois* and *Xnr3* (which are expressed immediately after midblastula and are direct targets of β-catenin regulation). *ODC* is used as a loading control.
(B) Dissections of embryos at stage 9 showing that *Xnr1*, *Xnr2* and *Xnr4* are expressed in the endoderm and at higher levels dorsally than ventrally. *Vg1* is expressed uniformly in the vegetal pole.
(C-F) *Xnr1* in situ hybridizations of blastula stage embryos showing a gradient of expression in endoderm. (C) Stage 8 embryo showing a few nuclei stained in the dorsal vegetal mass (arrowhead). (D) Stage 8.5 blastula embryo in which *Xnr1* expression has expanded into neighboring vegetal cells. (E) Stage 9 blastula embryo displaying graded *Xnr1* expression throughout the embryonic endoderm.
(F) External view of a stage 9 embryo cleared in Murray's solution in order to visualize *Xnr1* staining in the vegetal hemisphere. In this embryo, the ventral side, with its more pigmented animal cap, can be clearly distinguished from the less pigmented dorsal side. Note that *Xnr1* expression on the dorsal side is of longer duration, in addition to reaching higher levels than in ventral endoderm.

differentiating as epidermis and the vegetal fragment as endoderm (Fig. 3D-G).

In the reciprocal gain-of-function experiment, Xnr1 protein was added to stage 8 animal caps and incubated for 2 hours. At low concentrations (2 nM) Xnr1 protein induced ventral mesoderm and at higher doses (6 nM) dorsal mesoderm, producing sharp thresholds after only two hours of incubation (Fig. 3H, lanes 3-5). These results confirm and extend previous work of Jones et al. (1995), who used injected *Xnr2* mRNA. The loss- and gain-of-function experiments presented here indicate that Nodal-related signals are necessary and sufficient for the induction of both dorsal and ventral mesoderm at the blastula stage.

Graded expression of Xnrs in blastula endoderm

To determine whether Xnrs are expressed at the right time and place to mediate endogenous inductive activities during the blastula stage, we re-examined their expression patterns. Using RT-PCR analysis, *Xnr1*, *Xnr2* and *Xnr4* were found to be expressed after midblastula, starting to accumulate at the same time as early zygotic genes transcribed at the midblastula transition (Fig. 4A) such as *siamois* (Lemaire et al., 1995; Brannon et al., 1997) and *Xnr3* (Hansen et al., 1997; McKendry et al., 1997) that are direct targets of early β-catenin signalling. Organizer-specific markers such as *goosecoid* start to be expressed after Xnrs (Fig. 4A). At stage 9, when mesoderm induction takes place, embryo dissections showed that *Xnr1*, *Xnr2* and *Xnr4* transcripts were enriched in the dorsal half and in the vegetal region of the embryo (Fig. 4B). Previously, Xnr expression was detected in blastula endoderm, but was thought to be uniform (Jones et al., 1995; Yasuo and Lemaire, 1999; Clements et al., 1999). Our results suggested a possible dorsal to ventral gradient composed of multiple Nodal-related factors in the endoderm of the blastula.

To confirm the existence of graded Xnr signals in the endoderm of the blastula, the expression of *Xnr1* was re-examined using a more sensitive in situ hybridization procedure, in which the embryos are fixed and hemisectioned facilitating the penetration of the probe into endodermal cells. As can be seen in Fig. 4C, *Xnr1* expression started at midblastula in superficial large yolky endodermal cells, on one side of the embryo. Using regularly cleaving pigmented embryos with distinct dorsoventral polarity, we established that these cells were located in the dorsal side. The expressing cells correspond to the superficial cells in which nuclear translocation of *β-catenin* was first discovered by Schneider et al. (1996). At stage 8.5, *Xnr1* transcripts expanded to deeper neighboring cells (Fig. 4D). At stage 9, *Xnr1* expression was detected throughout the vegetal mass, while still displaying a dorsal to ventral gradient expression (Fig. 4E). This graded expression at stage 9 was also seen in external views of embryos rendered transparent by treatment with Murray's solution (Fig. 4F). By the gastrula stage *Xnr1* transcripts became undetectable in the endoderm and were found instead in the dorsal marginal zone as described previously (Jones et al., 1995 and data not shown).

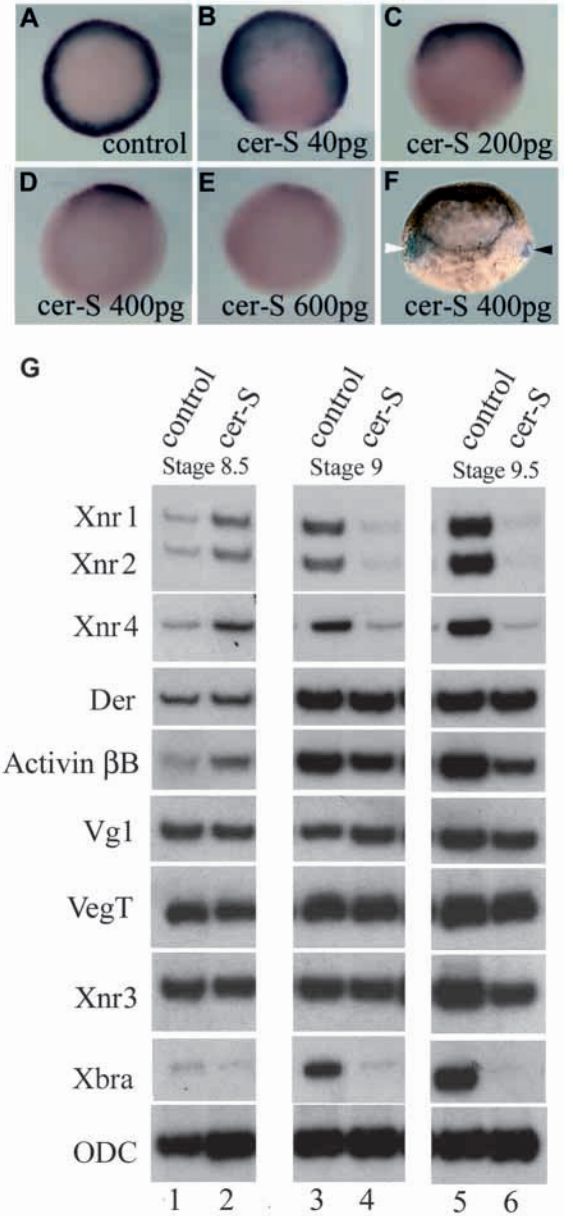

Fig. 5. Injections of *cer-S* mRNA dose-dependently reduce Xbra expression in gastrula embryos. Embryos are injected radially in the vegetal pole at the 4 cell stage, then processed for *Xbra* in situ staining at stage 10.5. (A) Control uninjected embryo, *Xbra* is expressed as a mesodermal ring. (B-E) Embryos injected with increasing amounts of *cer-S* mRNA, showing graded reduction of the *Xbra* expression domain. (F) Embryos injected vegetally with 400 pg of *cer-S* mRNA at the 4-cell stage and with *lacZ* lineage tracer mRNA into blastomere C4 at the 32-cell stage. In this lateral view the white arrowhead indicates *lacZ* in the ventral side (note that the pigment in the animal cap also marks the ventral side) and the black arrowhead points to the expression of *Xbra* transcripts on the dorsal side (n=51). (G) RT-PCR analysis of *Xenopus* embryos injected with 600 pg of *cer-S* mRNA. RNAs were harvested from uninjected controls or *cer-S*-injected embryos at one-hour intervals at stages 8.5 (lanes 1, 2), 9.0 (lanes 3, 4) and 9.5 (lanes 5, 6). *Xnr1*, *2* and *4* transcripts are initially not inhibited by *cer-S* (lanes 1, 2), but are decreased at later stages (a positive feedback loop for Nodal-related gene expression has been described; Meno et al., 1999). Importantly, the levels of *derrière*, *Vg1*, *VegT* and *Xnr3* remained unchanged, and *activin βB* was only partially decreased. Note that *cer-S* mRNA inhibited the initial expression of *Xbra* and that *cer-S* can inhibit *Xbra* transcriptional activation even in the presence of *derrière*, *activin* and *Vg1* transcripts.

We conclude that Xnrs are expressed at the correct time and place to participate in mesoderm induction by endoderm. In the case of *Xnr1*, the in situ hybridizations suggest that a gradient of activity could be established not only by increased mRNA levels on the dorsal side, but also by the longer duration of its expression in dorsal endoderm.

cer-S blocks *Xbra* expression in a dose-dependent way

To test a possible gradient of Xnr activity, we examined the response of the mesodermal ring of *Xbra* expression to increasing doses of *cer-S*. Vegetal injection of *cer-S* mRNA into each blastomere at the 4-cell stage (Fig. 5A) caused a dose-dependent reduction of the extent of *Xbra* expression in the marginal zone at the gastrula stage (Fig. 5B-F). At the highest concentrations (150 pg per blastomere) *Xbra* expression was abolished. This experimental design follows on the footsteps of Thisse and Thisse (1999), who applied it to the inhibition of zebrafish mesoderm formation by *antivin*, a TGF-β type molecule that can block both *activin* and *nodal* signalling via interactions with activin receptors (Meno et al., 1999). Using *lacZ* mRNA as a lineage tracer, it was found that at intermediate doses *Xbra* is inhibited in the ventral side of the embryo (Fig. 4F). Since low doses inhibit ventrally and high doses dorsally, these results strongly support the idea that a dorsal-ventral gradient of Xnr activity exists in vivo.

Recent studies involving the dissociation and reaggregation of *Xenopus* embryos have shown that some aspects of endoderm development require cell-cell interactions (Yasuo and Lemaire, 1999; Clements et al., 1999). To test whether *cer-S* mRNA affected the post-midblastula expression of known TGF-β mesoderm-inducing candidates, we analyzed embryos injected radially with 150 pg *cer-S* mRNA. As shown in Fig. 5G, the initial expression of *Xnr1*, *Xnr2* and *Xnr4* was not inhibited by *cer-S* at stage 8.5, but was decreased at later blastula stages. This inhibition can be explained by the positive feedback loop proposed for Nodal-related genes in zebrafish (Meno et al., 1999). Importantly, the expression of *derrière* (Sun et al., 1999) was not affected, and *activin βB* (Dohrmann et al., 1993) was only partially decreased by *cer-S* mRNA injection. Transcript levels of *VegT*, *Vg1* and *Xnr3* were not affected, whereas initial expression of *Xbra* at blastula stages was blocked by *cer-S* mRNA (Fig. 5G).

These microinjection experiments show a dose-dependent inhibition of *Xbra* by *cer-S* in intact embryos. It is noteworthy that the block in mesoderm formation takes place in the

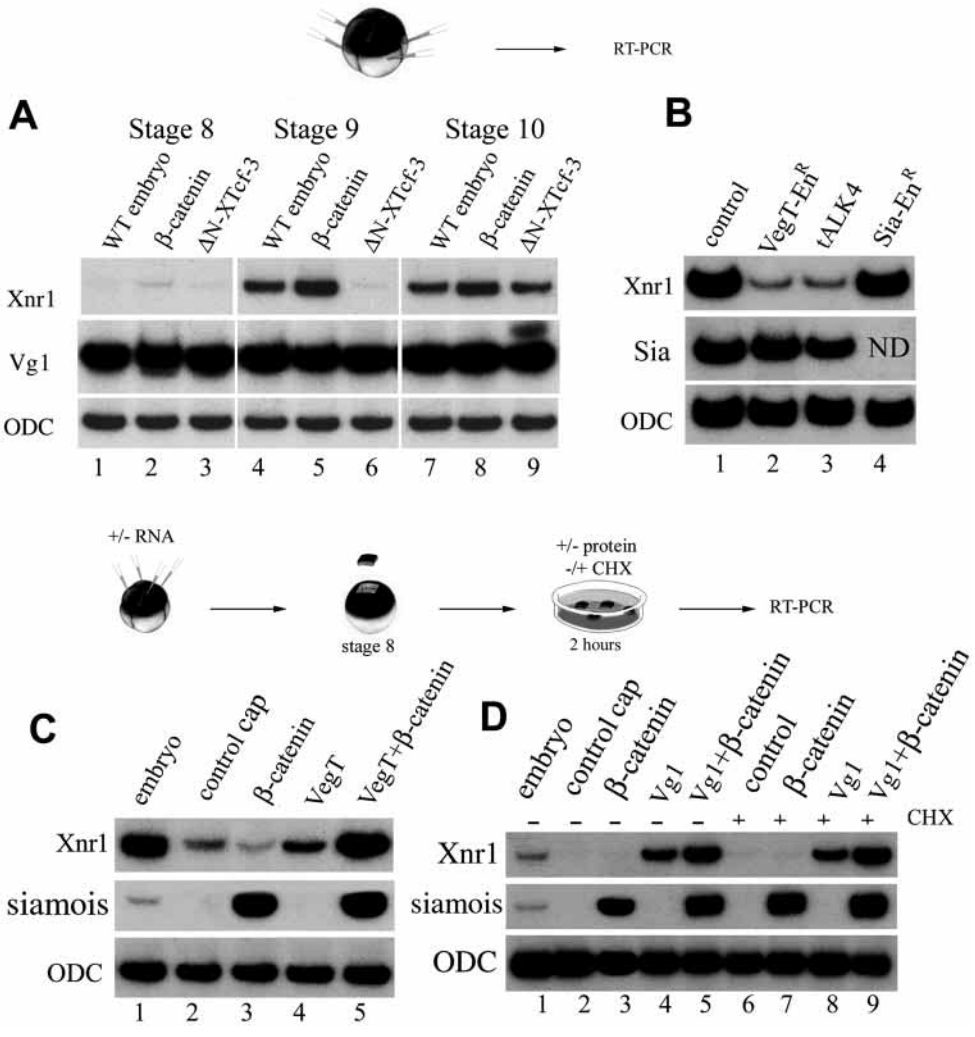

Fig. 6. Zygotic expression of *Xnr1* is regulated by β-catenin, VegT and Vg1. (A) *Xnr1* is upregulated by *β-catenin* (200 pg) and inhibited by *ΔN-XTcf-3* mRNA (800 pg) at stage 9 (lanes 4-6) in radially injected embryos. At stage 10, however, *Xnr1* transcripts are expressed even in the presence of *ΔN-XTcf-3*; this is consistent with the formation of ventral mesoderm in these ventralized embryos. Maternal *Vg1* is not affected. (B) Whole embryos injected radially at 4-cell stage with 800 pg *VegT-En^R* mRNA (lane 2), 2 ng of *tALK4* (lane 3), or 120 pg of *Sia-En^R* (lane 4) and analyzed by RT-PCR at stage 9. *Xnr1* requires VegT activity and TGF-β-like signalling for expression at the blastula stage. *Xnr1* expression at blastula is not dependent on Siamois activity since it is not blocked by *Sia-En^R* mRNA. (C) Animal cap experiments showing that *VegT* mRNA (200 pg) can weakly induce *Xnr1* and synergizes with *β-catenin* (100 pg) mRNA (lanes 4 and 5). *β-catenin* mRNA on its own is unable to induce *Xnr1* in animal caps (lane 3). (D) Treatment of animal caps with 4 nM Vg1 protein at stage 8 for 2 hours induces *Xnr1*, and *β-catenin* (100 pg mRNA) enhances this induction (lanes 2-5). The same response was obtained after blocking protein synthesis with cycloheximide (lanes 6-9), indicating that *Xnr1* is a primary response gene to Vg1 protein. Although only results for *Xnr1* transcripts are shown, *Xnr2* and *Xnr4* were also analyzed in all samples of A, C and D with comparable results (data not shown).

presence of continued expression of the putative mesoderm-inducing mRNAs *derrière*, *activin* and *Vg1* at the blastula stage (Fig. 5G). The results indicate that mesoderm induction in *Xenopus* requires an endogenous activity gradient of *Xnrs*.

The maternal determinants *β-catenin*, *VegT* and *Vg1* regulate Xnr expression

Oligonucleotide depletion experiments have demonstrated that two maternal mRNAs, *β-catenin* and *VegT*, are required for the production of zygotic mesoderm-inducing signals in Nieuwkoop conjugates (Wylie et al., 1996; Zhang et al., 1998). To investigate whether these intracellular molecules function as upstream regulators of Xnr expression at the blastula stage, we performed the gain- and loss-of-function analyses shown in Fig. 6. At stage 9, expression of *Xnr1* in whole embryos was increased by *β-catenin* mRNA and decreased by dominant-negative *ΔN-XTcf-3* mRNA injections (Fig. 6A, lanes 4-6). This requirement of the β-catenin pathway for Xnr expression was transient and was no longer seen at stage 10 (Fig. 6A, lanes 7-9), indicating the existence of additional, β-catenin independent, regulatory mechanisms.

Injection of a dominant-negative construct consisting of the engrailed (En) repressor domain fused to the VegT transcription factor (Horb and Thomsen, 1997), *VegT-En*^{R}, repressed expression of *Xnr1*, *Xnr2* and *Xnr4* in stage 9 embryos (Fig. 6B, lanes 1 and 2 and data not shown). This suggested a requirement for VegT in the regulation of Xnr expression. In animal cap explants, overexpression of *VegT* (but not of *β-catenin* alone) led to weak expression of *Xnr1* at stage 9 (Fig. 6C). This induction was potentiated by co-injection of *β-catenin* and *VegT* mRNAs (Fig. 6C, lanes 4 and 5), suggesting that *VegT* and *β-catenin* act synergistically.

Injection of *tALK4* mRNA (Chang et al., 1997) into whole embryos revealed that zygotic expression of *Xnr1*, *Xnr2* and *Xnr4* at the blastula stage also requires a TGF-β-like signal (Fig. 6B, lane 3 and data not shown). The best endogenous candidate molecule for this signal is Vg1, whose mRNA is maternal and localized (like VegT) uniformly in the vegetal pole of the egg (Kessler and Melton, 1994). Mature Vg1 protein secreted by microinjected oocytes (Piccolo et al., 1999) was added to stage 8 animal caps for 2 hours. During this period, *Xnr1* mRNA was induced by Vg1 protein and this response was potentiated in *β-catenin*-injected explants (Fig. 6D, lanes 4 and 5). *Xnr1* induction was a primary response to Vg1 protein, since it also took place in the presence of 25 μg/ml cycloheximide (Fig. 6D, lanes 8 and 9), which in these experiments inhibited protein synthesis by 94%. The results suggest that vegetally localized cytoplasmic determinants such as *VegT* and *Vg1* may generate a gradient of expression of mesoderm-inducing Xnrs in blastula endoderm by synergizing, directly or indirectly, with the dorsal determinant *β-catenin*.

DISCUSSION

The cerberus-short reagent provides an antagonist of multiple Xnrs, which was used here to study the mechanism of mesoderm induction in *Xenopus*. We find, first, that Cer-S either as injected mRNA or as soluble protein added at the blastula stage is able to block both the dorsal and the ventral mesoderm inducing signals released by endodermal explants. Second, endogenous Xnrs are expressed during blastula in a graded fashion in endoderm. Xnr1 expression starts on the dorsal side at midblastula and from there expands to the rest of the endoderm (Fig. 4C-E). Thus, dorsal endoderm (also known as the Nieuwkoop center) expresses *Xnr1* not only at higher levels but also for a longer period of time than ventral endoderm during the blastula stage. Third, microinjection of *cer-S* mRNA into embryos causes a dose-dependent inhibition of *Xbra* expression in embryos in the presence of endogenous *derrière*, *activin* and *Vg1* mRNAs (Fig. 5). Since ventral *Xbra* is blocked at low doses and dorsal expression at high doses of *cer-S* mRNA, this result is consistent with the requirement of an Xnr gradient of activity for induction of mesoderm. Fourth, maternal determinants such as *VegT*, *Vg1* and *β-catenin* can cooperate in the zygotic activation of Xnr expression at the blastula stage.

These results suggest that the classical 3-signal model (Slack, 1991a; Heasman, 1997) for mesoderm induction in *Xenopus* could be modified in the way shown in Fig. 7. Maternal activities such as dorsal β-catenin and vegetal VegT and Vg1 cooperate to set up a zygotic dorsal to ventral gradient in the endoderm composed of multiple Xnrs at stage 9, when mesoderm induction takes place. At high Nodal-related concentrations, which require a functional β-catenin pathway

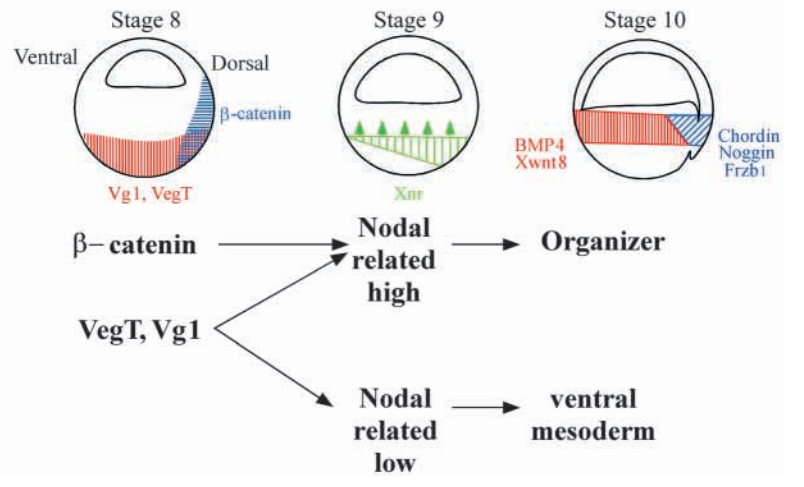

Fig. 7. Model of mesoderm induction at the blastula stage by a dorsal to ventral gradient composed of multiple Nodal-related genes expressed in endoderm. This simplified model concerns the nature of the endodermal signals, but does not address the function of important regulators such as *β-catenin* and *siamois* in the marginal zone of the embryo. The model has predictive value as it may help explain how molecules as dissimilar as *β-catenin*, *Vg1*, *Xnr1* and *Xnr2*, *noggin* and *chordin* mRNAs might rescue dorsal development via a sequential pathway when overexpressed in UV-ventralized embryos.

in the dorsal side of the embryo, the Spemann organizer (expressing genes such as *chordin*, *noggin* and *Frzb1*) is induced in overlying cells by early gastrula. In the ventral side, VegT and Vg1 would lead to the production of lower levels of Nodal-related signals, and ventral mesoderm (expressing genes such as *Xwnt8* and *BMP4*) would be induced. Similarly, in embryos ventralized by UV irradiation (Heasman, 1997) or by ΔN-XTcf-3 (Molenaar et al., 1996, Fig. 6A), the uniformly distributed VegT and Vg1 products would produce low levels of Xnrs sufficient to induce ventral mesoderm at the gastrula stage.

A particularly attractive aspect of the model in Fig. 7 is that it may help explain a long-standing puzzle in *Xenopus* embryology. A surprisingly large number of microinjected molecules are able to rescue, often completely, the UV ventralized phenotype that results from interfering with cortical rotation of the fertilized egg (Heasman, 1997). The UV-rescuing gene products include such diverse molecules as *β-catenin* (and other members of this signalling pathway; Harland and Gerhart, 1997), *Vg1* (Thomsen and Melton, 1993), *Xnr1* and *Xnr2* (Jones et al., 1995), *noggin* (Smith and Harland, 1992) and *chordin* (Sasai et al., 1994). Although one can argue that each of these diverse genes acts via different redundant pathways, their common UV-rescue activity may be easier to unravel if considered as part of a cascade of sequential gene activations. In this view, overexpression of *β-catenin* or *Vg1* would lead to high levels of Xnr expression in blastula endoderm, which in turn would mediate the induction of Spemann organizer in overlying cells, activing genes such as *noggin* and *chordin* that execute dorsal patterning at the gastrula stage (Fig. 7).

This sequential model of gene activation must be considered an oversimplification of the in vivo situation. It is likely that multiple signalling pathways synergize to pattern the gastrula. For example, our model does not take into account the role that β-catenin and its target genes *siamois* and *Xtwn* (Lemaire et al., 1995; Laurent et al., 1997) may have in the marginal zone itself. It is known that the promoter of the homeobox gene *goosecoid*, which is active in dorsal mesoderm (Steinbeisser et al., 1993), contains Siamois and Xtwn binding sites (Watabe et al., 1995; Laurent et al., 1997) in addition to a TGF-β responsive element. However, in embryos in which the function of *β-catenin* and expression of *siamois* are blocked by the inhibitor ΔN-XTcf-3, overexpression of *Xnr1* mRNA is sufficient to activate *goosecoid* and other organizer markers (Fig. 1). Furthermore, in animal cap experiments Xnr1 protein is able to induce ventral and dorsal mesodermal markers with sharp thresholds in the low nanomolar range (Fig. 3H). Since Activin, Vg1 and Derrière are not inhibited by Cer-S, the present experiments do not address whether these molecules may cooperate with Xnrs in mesoderm patterning in vivo.

There is ample genetic support for a critical role of Nodal-related molecules in mesoderm formation in many vertebrates. In mouse, mutations in the gene *nodal* result in embryos severely deficient in mesodermal tissues (Zhou et al., 1993; Conlon et al., 1994). It has been argued that, because some mutant embryos contain patches of *Brachyury* expression, mouse Nodal is involved in the maintenance rather than in the initiation of mesoderm induction (Conlon et al., 1994). In *Xenopus*, the present results suggest a requirement for Nodal-related signalling in the initial mesoderm induction by endoderm. In zebrafish, two Nodal-related genes, *cyclops* and *squint* have been identified to date (Sampath et al., 1998; Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998). Both mutations affect axial mesoderm and in double mutants the effects are synergistic, leading to the loss of *goosecoid* expression in the organizer and lack of head and trunk mesoderm (Feldman et al., 1998). In *cyclops*;*squint* double homozygotes a horseshoe of *Brachyury* expression persists, as is the case in *Xenopus* embryos injected with intermediate doses of *cer-S* mRNA. In *Xenopus*, high doses of *cer-S* can block Xbra expression. This difference between the two species might be explained by residual Xnr signals in the zebrafish mutants, or by subtle differences in the transcriptional control of *Brachyury*. For example, it has been reported that overexpression of *antivin* abolishes mesodermal expression of *snail1* and *eve1* in conditions in which some *Brachyury* expression persists in injected zebrafish embryos (Thisse and Thisse, 1999).

In *Xenopus*, a proteolytic cleavage mutant of *Xnr2* was shown to act as a dominant-negative agent in *Xenopus*, leading to inhibition of dorso-anterior endodermal markers and partial inhibition of mesodermal markers (Osada and Wright, 1999). Since the dominant-negative Xnr2 construct is active only in injected cells, the distribution of microinjected mRNA could play a role in the differences seen with the stronger effects reported here for *cer-S* mRNA. Cer-S is an antagonist of multiple Nodal-related signals that is secreted into the extracellular space and may achieve a more uniform distribution, causing, at high levels, a complete loss of mesoderm induction. It should be mentioned that Jones et al. (1996) reported that Xnr2 can only signal at short range in animal cap explants and that this range was increased in constructs in which processing and secretion was enhanced. Our results imply that Xnrs can diffuse from vegetal explants to animal caps recombinants. It is possible that endogenous Xnrs in endoderm are more efficiently processed, secreted and transported than Xnr2 expressed ectopically in animal caps.

The model of Xnr-mediated mesoderm induction in *Xenopus* (Fig. 7) is strongly supported by a recent study carried out by Kofron et al. (1999). In *VegT*-depleted embryos the induction of mesoderm is inhibited, and can be rescued by expression of *Xnr1* mRNA in vegetal fragments of Nieuwkoop conjugates. In addition, they demonstrated that *VegT* response sites exist in the *Xnr1* promoter.

It is noteworthy that studies using increasing concentrations of Activin had previously demonstrated that graded doses of a TGF-β family member were sufficient to induce and pattern ventral and dorsal mesoderm (Green and Smith, 1990; Green et al., 1992). The results presented here suggest that in *Xenopus* such a gradient of mesoderm-inducing factors can be provided by multiple Nodal-related signals expressed in the endoderm at the blastula stage.

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