The Wnt/β-Catenin Pathway Posteriorizes Neural Tissue in Xenopus by an Indirect Mechanism Requiring FGF Signalling

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In order to identify factors involved in posteriorization of the central nervous system, we undertook a functional screen in Xenopus animal cap explants which involved coinjecting noggin RNA together with pools of RNA from a chick somite cDNA library. In the course of this screen, we isolated a clone encoding a truncated form of β-catenin, which induced posterior neural and dorsal mesodermal markers when coinjected with noggin in animal caps. Similar results were obtained with Xwnt-8 and Xwnt-3a, suggesting that these effects are a consequence of activating the canonical Wnt signalling pathway. To investigate whether the activation of posterior neural markers requires mesoderm induction, we performed experiments using a chimeric inducible form of β-catenin. Activation of this protein during blastula stages resulted in the induction of both posterior neural and mesodermal markers, while activation during gastrula stages induced only posterior neural markers. We show that this posteriorizing activity occurs by an indirect and non-cell-autonomous mechanism requiring FGF signalling.© 2001 Academic Press

Key Words: Xenopus; A/P patterning; Wnt signalling; β-catenin; FGF signalling.

INTRODUCTION

Nieuwkoop's classical "activation/transformation" model proposes that two phases of signalling, arising from the mesoderm, are instrumental in generating anterior-posterior (A/P) pattern in the developing central nervous system. An initial "activation" signal inducing only anterior neural tissue (forebrain) is followed by a "transformation" phase, where subsequent signalling specifies more posterior neural character (midbrain, hindbrain, spinal cord). The posteriorizing signal(s) has been proposed to be distributed as a spatial or temporal gradient, with the highest concentration inducing the most posterior structures (Nieuwkoop, 1952; Slack and Tannahill, 1992).

Several candidate molecules for the initial neuralizing signal have been identified. These include: noggin (Lamb et al., 1993), chordin (Piccolo et al., 1996; Sasai et al., 1995), Xnr3 (Hansen et al., 1997), cerberus (Bouwmeester et al., 1996; Piccolo et al., 1999), and follistatin (Hemmati-Brivanlou et al., 1994), all of which act as antagonists of BMP signalling (Wilson and Hemmati-Brivanlou, 1997). Furthermore, evidence from chick supports an essential role for fibroblast growth factor (FGF) signalling in combination with Wnt signalling for full neural induction (Streit et al., 2000; Wilson et al., 2000, 2001). Candidates for the transforming signal include retinoic acid (RA) (Blumberg et al., 1997; Kolm et al., 1997), FGFRs (Cox and Hemmati-Brivanlou, 1995, 1997), and members of the Wnt family (McGrew et al., 1995, 1997). However, the precise role of each of these pathways and how they are

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unpublished results) were coinjected with to initiate the proper A-P patterns of somitic mesoderm are required in normal development to regulate analyses have also revealed that signals from somitic mesoderm and posterior markers. Neural A/P character, induced by factors from the somite which alone induces tissue of an anterior character (Lamb et al., 1997; Itasaki et al., 1997; Muhr et al., 1997; Yost et al., 1997; Woo and Fraser, 1997). In addition, transgenic regulatory analyses have also revealed that signals from somitic mesoderm are required in normal development to initiate the proper A-P patterns of Hoxb4 expression in the neural tube (Gould et al., 1998). This demonstrates that paraxial mesoderm is an important source of posteriorizing signals that contribute to the generation of neural patterning. Therefore, to identify factors in somitic mesoderm involved in the process of posteriorization, we performed a functional screen using Xenopus animal cap explants. Pools of synthetic RNA from a chick somite cDNA library (N.I. et al., unpublished results) were coinjected with noggin RNA, which alone induces tissue of an anterior character (Lamb et al., 1993). Explants were assayed for changes in their neural A/P character, induced by factors from the somite RNA pools, using a variety of posterior markers.

One clone isolated from this screen encoded a truncated form of \( \beta \)-catenin, a cytoskeletal component and an intracellular mediator of Wnt signalling. Cytosolic \( \beta \)-catenin is degraded by the proteosome using a mechanism that requires phosphorylation by GSK-3. Stimulation of cells by Wnt proteins leads to the inhibition of GSK-3 activity and stabilization of \( \beta \)-catenin, which subsequently translocates into the nucleus. There, it forms a complex with members of the TCF/LEF family of transcription factors and activates the expression of target genes (Cadigan and Nusse, 1997). The \( \beta \)-catenin clone isolated from our screen encoded an amino-terminal truncated protein lacking the first 87 amino acids, which contain the phosphorylation sites essential for regulation by GSK3 (Lu et al., 1997; Yost et al., 1996). This suggests that the protein product of this variant might be stable and capable of maintaining high levels of Wnt signalling through the canonical pathway.

The isolation of this variant of \( \beta \)-catenin in a screen for posteriorization provides further support for the involvement of the Wnt signalling pathway in A/P patterning of neural tissue. Hence, in this study, we investigated the mode of action by which \( \beta \)-catenin induces changes in the A/P character of noggin-treated animal caps. We demonstrated that truncated \( \beta \)-catenin induces both posterior neural and dorsal mesodermal markers when co-injected with noggin. Similar results were obtained with Xwnt8 and Xwnt3a, suggesting that these activities may be a general property of Wnt signalling. Furthermore, we utilized an inducible form of \( \beta \)-catenin to precisely control the timing of events and see whether it was possible to induce posterior neural markers in the absence of mesoderm during gastrula stages. We demonstrate that posteriorization mediated by the Wnt/\( \beta \)-catenin pathway occurs by a non-cell-autonomous mechanism, which requires cell-to-cell contact. Finally, we show that downstream of the Wnt/\( \beta \)-catenin pathway, FGF signalling is necessary for induction of posterior neural markers, providing another link in the coordinated activities of these pathways.

**MATERIALS AND METHODS**

### DNA Constructs and RNA Synthesis

To prepare GR-LEF, LNFβCTA, LNFβCTA (Vleminckx et al., 1999) was amplified by PCR and fused in frame to the C terminus of the ligand binding domain of the human glucocorticoid receptor with a pentaglycine bridge (details upon request). Other constructs were as published: noggin (Smith and Harland, 1992), Xwnt8 (Christian et al., 1993), XFD (Amaya et al., 1993). Plasmids were linearized and used for in vitro synthesis of capped RNA by standard methods.

**Xenopus Embryos, Microinjection, and Animal Cap Explants**

Xenopus embryos were obtained by in vitro fertilization (Smith and Slack, 1983), chemically dejellied using 2% cysteine hydrochloride (pH 7.8–8.1), maintained in 10% normal amphibian medium (NAM; Slack, 1984) and staged according to Nieuwkoop and Faber (1967). Xenopus embryos were injected in 75% NAM containing 4% Ficoll type 400 (Sigma). Synthetic RNA was injected in a volume of 10 nl. Care was taken with respect to both the size of the animal caps and the stage of dissection, as previous work has shown that the ability of Wnt8 to trigger mesoderm formation in presumptive ectoderm can result from large caps or caps from later stages (stage 10) (Sokol, 1993). To avoid this problem, all Xenopus animal caps were dissected at stage 8 and the territory cut comprised less than 50% of the animal hemisphere, as recommended by Sive et al. (2000). Caps were cultured in 75% NAM. Treatment with dexamethasone (Sigma) was done at a final concentration of 1 \( \mu \)M in 75% NAM or 10% NAM for animal caps or whole embryos, respectively. Dissociation of animal cap cells was achieved by culturing in calcium- and magnesium-free medium (CMFM; Sargent et al., 1986) in the absence or presence of 1 \( \mu \)M DEX.

**Histology, \( \beta \)-Gal Staining, and Whole-Mount in Situ Hybridization**

For histological analyses, specimens were fixed, sectioned, and stained as described (Green et al., 1990). For \( \beta \)-gal staining, tissues were fixed in MEMFA for 1 h at room temperature, washed in PBS containing 0.01% Tween 20, and stained in PBS containing 1 mg/ml X-gal, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% NP-40. After staining, samples were refixed in MEMFA for 1 h and processed for in situ hybridization as described (Jones and Smith, 1999). Probes were BF-1 (Bourguignon et al., 1998), En-2 (Hemmati-Brivanlou et al., 1991), Krox-20 (Bradley et al., 1993), and Hoxb9 (Sharpe et al., 1987).

**RNA Isolation and RT-PCR**

Fifteen animal caps or two embryos were processed for RNA isolation by using the TRIzol reagent (Gibco BRL) according to.
the manufacturer's instructions. RT-PCR was performed as described (Wilson and Melton, 1994) and PCR primers for eF1α, NCAM, En-2, Hoxb9, and muscle actin were described previously (Hemmati-Brivanlou and Melton, 1994). Other primers are as follows: BF-1: 5′-CCCTCAACAAGTGCTTCGGA, 5′-TCAGAATGCTGGGAGTTG; Krox-20: 5′-CCCTCAACAAGTGCTTCGGA, 5′-TCAGAATGCTGGGAGTTG; Hoxa3: 5′-GTACCTCAACCAAGGCGCTCA, 5′-GGACTCGAGGAGAAGGGTAAC; Xbra: 5′-CCCTCAACAAGTGCTTCGGA, 5′-TCAGAATGCTGGGAGTTG; TGAAGGTTAG, 5′-CCCTCAACAAGTGCTTCGGA, 5′-TCAGAATGCTGGGAGTTG; Xwnt8-2C). In contrast, explants from Xwnt8-injected embryos express α-globin and Xho3 (Figs. 2A and 2B, lane 6) and form vesicles indicating the presence of ventral mesoderm (Fig. 2C). The expression of Hoxb9 may reflect the induction of posterior neural tissue or lateral plate mesoderm as it marks both of these tissues. Similar results were obtained when Xwnt3A and full-length Xβ-catenin were tested (data not shown). Therefore, a common feature of activating the Wnt pathway appears to be its ability to induce both neural and mesodermal markers.

An Inducible Form of β-Catenin, GR-LEFΔNβCTA, Can Posteriorize Neural Tissue in the Absence of Mesoderm

These results raise the question of whether the change in A/P character of the neural tissue is a direct consequence of
the activation of the Wnt pathway or is secondary to the induction of mesoderm in the animal cap. To address this point, we designed experiments to test whether the two processes could be separated. We generated a dexamethasone (DEX)-inducible form of β-catenin, in which the LEF-1 DNA binding domain and the C-terminal trans-activation...
domain of β-catenin (LEF\(\beta\)CTA) (Vleminkx et al., 1999) were fused to the ligand binding domain of the human glucocorticoid receptor, creating GR-LEF\(\beta\)CTA. This should create a variant where the trans-activation domain is dependent upon the presence of DEX ligand to potentiate its activity. In agreement with this, GR-LEF\(\beta\)CTA induced the formation of double axes and expression of the direct target genes siamois (Brannon et al., 1997) and Xnr3 (McKendry et al., 1997) in animal caps only after exposure to DEX (data not shown).

Animal caps lose their capacity to respond to mesoderm-inducing factors such as activin and FGF during gastrula stages (Green et al., 1990). If Wnt-mediated induction of posterior neural markers is independent of mesoderm formation, the activation of GR-LEF\(\beta\)CTA during gastrula stages should lead to the induction of posterior neural markers in the absence of mesoderm. Therefore, we injected GR-LEF\(\beta\)CTA RNA into the animal pole of 2-cell stage Xenopus embryos with and without noggin RNA and isolated animal caps at stage 8. We then added DEX at different stages, cultured the animal caps until late neurula stages, and examined gene expression by RT-PCR. In animals injected with noggin only, posterior neural markers including En-2 and Krox-20 were induced at stage 9 (Figs. 3A and 3B, lane 6). Consistent with the idea of posteriorization, it also suppressed the anterior neural marker BF-1. However, when DEX was added at stage 12, while En-2 and Krox-20 were still induced, Hoxb9 and mesodermal markers were not induced and BF-1 was only partially suppressed (Figs. 3A and 3B, lane 7). No posteriorization or mesoderm induction was observed when DEX was added at stage 15 (Fig. 3A, lane 8) or when DEX was not present in the culture medium (Figs. 3A and 3B, lane 5). These results show that Wnt signalling can posteriorize neural tissue in the absence of mesoderm in a stage-specific manner.

We also investigated the properties of the inducible β-catenin variant in the absence of noggin. In animal caps injected only with GR-LEF\(\beta\)CTA, DEX addition at stage 9 resulted in the induction of NCAM, BF-1, Hoxb9, and mesodermal markers with ventral character (Figs. 3A, lane 10 and 3B, lane 9). Addition of DEX at later stages did not induce any neural or mesodermal markers (Figs. 3A, lanes 11 and 12 and 3B, lane 10). To rule out the possibility that distinct activities of GR-LEF\(\beta\)CTA at different stages were a consequence of degradation of the protein, we analysed the protein levels by Western blot using an anti HA antibody against a tag present in the construct (Fig. 3C). The levels of GR-LEF\(\beta\)CTA remain stable until at least stage 16. This is consistent with other reports, indicating that GR fusion proteins are remarkably stable (Kolm and Sive, 1995; Tada et al., 1997).

Overall, these results show that the competence for induction of neural tissue by GR-LEF\(\beta\)CTA is restricted to late blastula stages. Furthermore, activation of GR-LEF\(\beta\)CTA at stage 9 has the ability to induce ventral mesoderm, which is dorsalized in the presence of noggin. This is consistent with our earlier experiments using the truncated form of β-catenin and Xwnt8 and confirms that the chimeric variant functions as an inducible modulator of Wnt signalling.

**Activation of GR-LEF\(\beta\)CTA in Intact Xenopus Embryos**

To investigate the consequences of activating GR-LEF\(\beta\)CTA in whole embryos, GR-LEF\(\beta\)CTA and β-gal RNAs were coinjected in one animal/dorsal blastomere at the 8-cell stage. Embryos were cultured in the presence or absence of DEX and gene expression was analysed by in situ hybridization. DEX addition at stage 9 suppresses BF-1 expression (Fig. 4B) and shifts the expression domains of En-2, Krox-20, and Hoxb9 anteriorly (Figs. 4F, 4J, and 4N, respectively). Addition of DEX at stage 12 resulted in similar effects on the expression of BF-1, En-2, and Krox-20 (Figs. 4C, 4G, and 4K), but Hoxb9 was unaffected (Fig. 4O). Neural markers were unchanged by the addition of DEX at stage 15 or in the absence of DEX by comparison to the uninjected sides of the embryos (Figs. 4A, 4D, 4E, 4H, 4I, 4L, 4M, and 4P). These findings show that ectopic expression of GR-LEF\(\beta\)CTA in whole embryos results in neural markers being expressed in more anterior locations, suggesting that anterior tissues acquire a more posterior character. Furthermore, the competence of animal caps for the posteriorizing action of GR-LEF\(\beta\)CTA is similar to the response in intact embryos.

**Induction of the Posterior Neural Markers by GR-LEF\(\beta\)CTA Occurs in a Noncell-Autonomous Manner**

Activation of the Wnt/β-catenin signalling pathway can induce posterior neural markers and suppress anterior character in both noggin-injected animal caps and in whole embryos. However, the molecular mechanism by which the pathway causes these effects is not clear. It has been suggested that En-2 is directly regulated by the Wnt pathway, in a mechanism that is dependent on TCF sites present in the En-2 promoter (McGrew et al., 1999). The induction of other posterior neural markers may occur directly or indirectly. To address this point, we investigated whether the induction of En-2 and Krox-20 by β-catenin occurs in a cell-autonomous manner. First, noggin and GR-LEF\(\beta\)CTA were coinjected in one animal blastomere of 16-cell stage embryos with β-gal RNA as a lineage tracer. Animal caps were dissected at stage 8, cultured in the presence or absence of DEX, fixed at stage 20, and processed for β-gal staining followed by in situ hybridization with En-2 or Krox-20 probes. In the absence of DEX, no induction of En-2 or Krox-20 occurs (Figs. 5A and 5B). Addition of DEX at stage 9 (Figs. 5C and 5D) or stage 12 (Figs. 5E and 5F) resulted in the induction of En-2 and Krox-20. In all cases, induction of these genes occurred outside of the β-gal
stained area. This suggests that a non-cell-autonomous mechanism may be operating in the induction of these genes by GR-LEFΔNβCTA.

Concomitantly, we tested whether cell communication within the animal cap is required for the induction of neural markers. Animal caps from embryos injected with noggin RNA and GR-LEFΔNβCTA RNA were cultured in the presence or absence of DEX at stage 9 (lane 6). Posteriorization occurred in the absence of mesoderm induction when DEX was added at stage 12 (lane 7). When DEX was added at stage 15 (lane 8), neither posteriorization nor mesoderm induction occurred. In the absence of noggin, activation of GR-LEFΔNβCTA at stage 9 but not at later stages induced the expression of NCAM, BF-1, and Hoxb9 (lane 10). (B) RT-PCR of animal caps assayed for mesodermal markers at stage 14. Mesodermal markers were detected in caps expressing GR-LEFΔNβCTA when DEX was added at stage 9 (lanes 6 and 9) but not at stage 12 (lanes 7 and 10). Noggin dorsalized the mesoderm induced by GR-LEFΔNβCTA, as the expression of Xbra increased and Xhox3 diminished. -RT is a negative control, embryo is a positive control, and eF1α served as loading control. (C) GR-LEFΔNβCTA protein levels are similar between blastula and neurula stages. Embryos were injected with 50 pg/embryo of GR-LEFΔNβCTA RNA, ectodermal explants were dissected at stage 8 and collected at the indicated stages. Two cap equivalents of protein were analysed by Western blotting using the mouse anti-HA monoclonal antibody 12CA5 after SDS-PAGE. The band corresponding to GR-LEFΔNβCTA is indicated by an arrowhead and the positions of molecular mass markers (kDa) are indicated on the left of the panel.

FIG. 3. An inducible form of β-catenin, GR-LEFΔNβCTA, results in expression of both posterior neural and mesodermal markers when activated at stage 9, but only posterior neural markers when activated at stage 12 in coinjections with noggin. (A) RT-PCR of animal caps analysed at stage 22. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from embryos injected with noggin RNA (500 pg/embryo; lane 4) expressed NCAM and BF-1 but not posterior neural or mesodermal markers. Prior neural and mesodermal markers were detected in tissues from embryos co-injected with noggin RNA and GR-LEFΔNβCTA RNA (50 pg/embryo; lanes 5–8) after the addition of DEX at stage 9 (lane 6). Posteriorization occurred in the absence of mesoderm induction when DEX was applied at stage 12 (lane 7). When DEX was added at stage 15 (lane 8), neither posteriorization nor mesoderm induction occurred. In the absence of noggin, activation of GR-LEFΔNβCTA at stage 9 but not at later stages induced the expression of NCAM, BF-1, and Hoxb9 (lane 10). (B) RT-PCR of animal caps assayed for mesodermal markers at stage 14. Mesodermal markers were detected in caps expressing GR-LEFΔNβCTA when DEX was added at stage 9 (lanes 6 and 9) but not at stage 12 (lanes 7 and 10). Noggin dorsalized the mesoderm induced by GR-LEFΔNβCTA, as the expression of Xbra increased and Xhox3 diminished. -RT is a negative control, embryo is a positive control, and eF1α served as loading control. (C) GR-LEFΔNβCTA protein levels are similar between blastula and neurula stages. Embryos were injected with 50 pg/embryo of GR-LEFΔNβCTA RNA, ectodermal explants were dissected at stage 8 and collected at the indicated stages. Two cap equivalents of protein were analysed by Western blotting using the mouse anti-HA monoclonal antibody 12CA5 after SDS-PAGE. The band corresponding to GR-LEFΔNβCTA is indicated by an arrowhead and the positions of molecular mass markers (kDa) are indicated on the left of the panel.

The Wnt/β-Catenin Pathway in Xenopus

FGF Signalling is Required for the Induction of Posterior Neural Markers by GR-LEFΔNβCTA

The indirect nature of the posteriorisation by β-catenin raises a question as to the identity of the signals that mediate these inductive events. Possible candidates include members of the FGF family of secreted proteins, which induce posterior neural markers in noggin-injected animal caps and posteriorize neural plate explants (Cox and
Recent evidence has also shown that Wnt and FGF signalling work in concert to regulate early events in neural induction (Wilson et al., 2001). Furthermore, loss-of-function experiments have shown that FGF signalling is required for posterior neural development (Holowacz and Sokol, 1999; Ribisi et al., 2000).

To ask whether FGF signalling is required for the induc-
With probes specific for En-2 staining), and processed for whole-mount in situ hybridization (B, D, and F) or stage 12 (Fig. 7C, lanes 6 and 7), increasing the amount of XFD RNA to 500 pg/embryo resulted in a stronger suppression of the posterior neural markers (Fig. 7A, lanes 6 and 7). Increasing amounts of SU5402 suppressed the induction of posterior neural markers either when added at stage 9 (Fig. 7B, lanes 6 and 7) or stage 12 (Fig. 7C, lanes 6 and 7), providing further evidence for the requirement of FGF signalling in the induction of posterior neural markers by GR-LEFΔNβCTA.

These results suggest that Wnts might work in part through activation of FGFs. To investigate whether FGFs themselves are induced in animal caps following activation of GR-LEFΔNβCTA, animal caps explanted from embryos injected with noggin and GR-LEFΔNβCTA RNA were analysed by RT-PCR. Activation of GR-LEFΔNβCTA at stages 9 and 12 induced FGF3 and FGF8 (Fig. 7C, lanes 6 and 7), while eFGF was induced only when DEX was added at stage 9. In the absence of noggin, FGFs are only induced when GR-LEFΔNβCTA is activated at stage 9 (Fig. 7C, lane 9).

These results show that FGFs are induced in response to Wnt/β-catenin signalling. This suggests that FGFs are a critical aspect of the noncell-autonomous inductive events following activation of Wnt/β-catenin pathway.

**DISCUSSION**

In this paper, we show that members of the Wnt pathway cooperate with noggin to induce posterior neural and dorsal mesodermal markers in animal caps. The use of an inducible form of β-catenin enabled us to demonstrate that during gastrula stages Wnt/β-catenin signalling can induce posterior neural markers in the absence of mesoderm. Furthermore, our results show that these activities occur by an indirect and noncell-autonomous mechanism requiring cell-to-cell contact. FGF signalling appears to be part of this
mechanism as we have shown that FGFs are upregulated by an inducible form of β-catenin and the induction of posterior neural markers by Wnt/β-catenin pathway requires FGF signalling. These findings raise a number of interesting issues with respect to Wnt signalling and A/P neural patterning in vertebrate embryos.

Wnt/β-Catenin Signalling in Neural Patterning and Mesoderm Induction

In this study, Xwnt8, β-catenin, and truncated β-catenin functioned in a similar manner when coexpressed with noggin in animal caps. Posterior markers such as En-2, Krox-20, and Hoxb9 were induced together with the dorsal mesoderm marker muscle actin. Members of the Wnt pathway have previously been shown to induce posterior neural markers in the absence of mesoderm (McGrew et al., 1995, 1997). The basis of these different results is not clear, but could be due to the amount of RNA injected. We observe the induction of posterior markers in the absence of mesoderm with low concentrations of β-catenin RNA. Wnt signalling has been reported to induce either dorsal (Sokol, 1993) or ventral mesoderm (Chakrabarti et al., 1992; Christian and Moon, 1993) in animal caps. These differences have been related to the size of the animal cap and the time at which the explant is isolated (Sokol, 1993). In our assays, activation of the Wnt/β-catenin signalling pathway in animal caps induced ventral mesoderm and coinjection of noggin led to the dorsalisation of this mesoderm, as reported previously (Lamb et al., 1993; Smith and Harland, 1992). Our results in explants confirm recent observations in whole embryos, where activation of the Wnt/β-catenin signalling pathway at late blastula stages, by the use of an heat-shock inducible system, led to the ectopic expression of ventral mesodermal markers (Hamilton et al., 2001).

These findings prompted us to ask whether or not the induction of the posterior neural markers by members of the Wnt pathway is dependent on the induction of mesoderm. Our results with the inducible GR-LEFΔNβCTA construct clearly show that, at stage 12, posteriorization of neural tissue can occur independently of mesoderm formation. Furthermore, the competence of the animal cap to respond to the posteriorizing effects of GR-LEFΔNβCTA ends by midneurula stages (Fig. 3A). The animal cap anal-

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**FIG. 6.** Induction of posterior neural markers by GR-LEFΔNβCTA requires cell-to-cell contact. (A) RT-PCR analysis showing that intact explants from embryos coinjected with noggin (500 pg/embryo) and GR-LEFΔNβCTA (50 pg/embryo) RNA expressed posterior neural and mesodermal markers when DEX was added at stage 9 (lane 6) and only posterior neural markers when DEX was added at stage 12 (lane 7). Dissociation of the explants blocked the induction of the posterior neural and mesodermal markers (lanes 9 and 10). As a negative control, DEX was not added to the culture medium (lanes 5 and 8). (B) Dissociation of animal cap cells did not affect the induction of direct targets of TCF/β-catenin. Activation of GR-LEFΔNβCTA at stage 9 induced Xnr3 and Xnr3 in both intact (lane 6) and dissociated (lane 8) explants. As a negative control, DEX was not added to the culture medium (lanes 5 and 7). Animal caps were dissected at stage 8 and cultured intact (in 75% NAM) or dissociated in calcium- and magnesium-free medium (CMFM) until stage 11 (B) or stage 22 (A). Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while noggin-injected explants expressed only anterior neural markers. eF1α served as loading control, –RT as a negative control, and embryo RNA as a positive control.
analysis correlates with results in whole embryos, where unilateral injections of GR-LEFbCTA caused anterior shifts in the pattern of expression of the posterior neural markers analysed. In addition, our findings are consistent with reports showing deletion of anterior structures caused by expression of Xwnt8 DNA (Christian and Moon, 1993; Fredieu et al., 1997) or by ubiquitous expression of Xwnt8 in transgenic Xenopus embryos under an inducible heat-shock promoter (Wheeler et al., 2000).

**Mechanism of Induction of the Posterior Neural Markers by the Wnt/β-Catenin Pathway**

Together, our data show that Wnt signalling posteriorizes neural tissue by an indirect mechanism. The noncell-autonomous induction of En-2 and Krox-20 expression by GR-LEFbCTA in animal caps is consistent with the localized expression of GR-LEFbCTA in whole embryos resulting in an anterior displacement of posterior neural markers outside of the injected cells (Fig. 4). Dissociation of animal caps blocked the induction of posterior neural markers but did not block the induction of the known direct targets of the Wnt/β-catenin pathway, siamois and Xnr3.

It has been suggested that En-2 is directly regulated by the Wnt/β-catenin pathway, via LEF/TCF sites present in the En-2 promoter (McGrew et al., 1999). These sites are required for the activation of a reporter by noggin and Xwnt3a in animal caps, and may be essential for the Wnt1-dependent expression of En-2 during development. However, in mice, Wnt3 was shown to be required for the maintenance of En-2 expression but not for its initiation (Danielian and McMahon, 1996; McMahon et al., 1992). It is possible, however, that other Wnt family members may play a direct role in the initiation of En-2 expression. The activation of the posterior neural markers by an indirect mechanism as revealed by our results indicates that activation of En-2 expression by Wnt/β-catenin signalling may also occur independent of these Lef/TCF sites.

Recombination experiments in which dorsal mesoderm was cocultured with ectoderm (Fredieu et al., 1997) provide more evidence in support of an indirect mechanism for induction of posterior neural markers by the Wnt/β-catenin pathway. In these experiments, expression of β-catenin (or treatment with lithium) in the dorsal mesoderm reduced the capacity of the recombinants to form anterior structures such as eyes and cement gland. These authors suggested that the Wnt/β-catenin pathway might trigger the production of a “dominant posteriorizing morphogen” in the...
dorsal mesoderm, which would then act on the ectodermal/ neural tissue.

**FGF Signalling Is Required for Wnt/β-Catenin Posteriorization**

We have shown that FGF signalling is required for the induction of posterior neural markers by the Wnt/β-catenin pathway. XFD and a chemical inhibitor of FGF signalling, SU5402, both block the induction of posterior neural markers in animal caps injected with noggin and GR-LEF3NβCTA. In a previous report (McGrew et al., 1997), XFD was shown to block the repression of the anterior gene Otx-2, but not the induction of En-2 and Krox-20, when coinjected with noggin and Xwnt3a. In our experiments, increasing amounts of XFD RNA completely blocked the induction of all posterior markers, including En-2 and Krox-20, when GR-LEF3NβCTA was activated at stage 9 or stage 12. These results were confirmed by the ability of SU5402 to suppress the induction of the posterior neural markers when added simultaneously with DEX, suggesting that FGF signalling is required after the activation of GR-LEF3NβCTA.

Finally, we show that FGF3 and FGF8 are induced in animal caps as a consequence of the activation of GR-LEF3NβCTA. During late gastrula and early neurula stages, FGF3 (Tannahill et al., 1992) and FGF8 (Christen and Slack, 1997) are expressed in the posterior ectoderm and mesoderm, which is consistent with a role for these proteins as posteriorising factors. Xwnt8 is also expressed in the posterior/lateral mesoderm and has been suggested to induce expression of the posterior neural marker Pax3 (Bang et al., 1999). The requirement for FGF signalling and the induction of FGFs by Wnts suggests a model in which regulation of FGFs by Xwnt8 is essential for the promotion of posterior fates during embryonic development. In summary, our results have provided insight into some of the underlying mechanisms associated with the roles of Wnt signalling in A-P patterning and suggest that FGF signalling is an integral aspect of the posteriorizing action of the Wnt/β-catenin pathway.

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**REFERENCES**


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