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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 noncell-autonomous mechanism requiring FGF signalling.

***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), FGFs (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995), 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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integrated during the establishment of A/P pattern is not well understood.

Tissue transplantation experiments have revealed that somites and paraxial mesoderm can reprogramme the expression of *Hox* genes and other axial markers, thus inducing a more posterior neural character (Gould *et al.*, 1998; Grapin-Botton *et al.*, 1997; Itasaki *et al.*, 1996; Muhr *et al.*, 1997, 1999; 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 β-catenin, a cytoskeletal component and an intracellular mediator of Wnt signalling. Cytosolic β-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 β-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 β-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 β-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 β-catenin induces changes in the A/P character of noggin-treated animal caps. We demonstrate that truncated β-catenin induces both posterior neural and dorsal mesodermal markers when coinjected 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 β-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/β-catenin pathway occurs by a noncell-autonomous mechanism, which requires cell-to-cell contact. Finally, we show that downstream of the Wnt/β-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-LEFΔNβCTA, LEFΔNβCTA (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.*, 1991), *XFD* (Amaya *et al.*, 1991). 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 μM 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 μM DEX.

Histology, β-Gal Staining, and Whole-Mount in Situ Hybridization

For histological analyses, specimens were fixed, sectioned, and stained as described (Green *et al.*, 1990). For β-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 $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 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′-CCTCAACAAGTGCTTCGTCA, 5′-TAAAGGTGAGTCCGGTGGAG; *Krox-20*: 5′-CCTTTGATTCAGATGAGCGGAG, 5′CGACATGCTGCAGCTCAGGTT; *Xhox3*: 5′-GTACCTCAACCAACGGCCTA, 5′-GGACTCGGGAGAAGGGTAAC; *Xbra*: 5′-CACCGAGAAGGAGCTGAAGGTTAG, 5′-TGCCACAAAGTCCAGCAGAACC; *α-globin*: 5′-TGCTGTCTCACACCATCCAGG, 5′-TCTGTACTTGGAGGTGAGGACG; *Siamois*: 5′-GAGCCTCAGGTCAGCAAAAC, 5′-GGTACTGGTGGCTGGAGAAA; *Xnr3*: 5′-TCCACTTGTGCAGTTCCACAG-, 5′-ATCTCTTCATGGTGCCTCAGG; *eFGF*: 5′-TATGAATGCAAAGGGGAAGC, 5′-GTGGCAAGAAATGGGTCAGT; *FGF3*: 5′-TGGAGGGGTTTACGAACATC, 5′-CCCTTTGATGGCAACAATTC; *FGF8*: 5′-TTAAAGGTGCGGAGACTGGT, 5′-TGTCTTCGACCCTTTCCTTG.

Western Blotting

Protein was extracted from animal caps as described (Cunliffe and Smith, 1994). Two animal cap equivalents of protein were analysed by SDS–PAGE (15% acrylamide gel) and then blotted by wet electrophoretic transfer to a PVDF membrane. This membrane was reacted with mouse anti-HA monoclonal antibody 12CA5 (Boehringer) and subsequently with anti-mouse IgG conjugated with alkaline phosphatase (Sigma). Detection was carried out by using NBT and BCIP.

RESULTS

Induction of Posterior Neural and Mesodermal Markers by Components of the Wnt Pathway

Previous reports show that β-catenin and other components of the Wnt pathway cooperate with noggin and induce posterior neural markers in animal caps in the absence of mesoderm (McGrew *et al.*, 1995, 1997). In contrast, we find that coinjection of increasing RNA concentrations of truncated *β-catenin* with *noggin* induces both posterior neural and dorsal mesoderm markers (Fig. 1). At low concentrations of *β-catenin* RNA (20 pg/embryo), we observe induction of *En-2*, *Krox-20*, and *Hoxb9* (Fig. 1, lane 5), indicating a posteriorization of the noggin-induced neural tissue. At higher concentrations of *β-catenin* RNA (100 and 500 pg/embryo), we observe induction of *muscle actin*, demonstrating that the posteriorization of neural tissue is accompanied by formation of dorsal mesoderm. At high concentrations, *β-catenin* alone induces *NCAM* and *Hoxb9* but not *muscle actin*.

To determine whether the induction of mesoderm is a specific property of this truncated form of β-catenin, or a general property resulting from activation of the Wnt pathway, we tested *Xwnt8*, *Xwnt3a*, and full-length *Xenopus* β-catenin in the same assay. Animal caps from embryos injected with *noggin* and *Xwnt8* RNA express *muscle actin* and *Xbra* (Figs. 2A and 2B, lane 5) and exhibit extensive elongation movements typical of dorsal mesoderm (Fig. 2C). In contrast, explants from *Xwnt8*-injected embryos express *α-globin* and *Xhox3* (Figs. 2A and 2B, lane 6) and form vesicles indicating the presence of ventral mesoderm (Fig. 2C). *Xwnt8*-injected animal caps also express *NCAM* and *BF-1*, reflecting the induction of neural tissue as described previously (Baker *et al.*, 1999; Wessely *et al.*, 2001). 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.

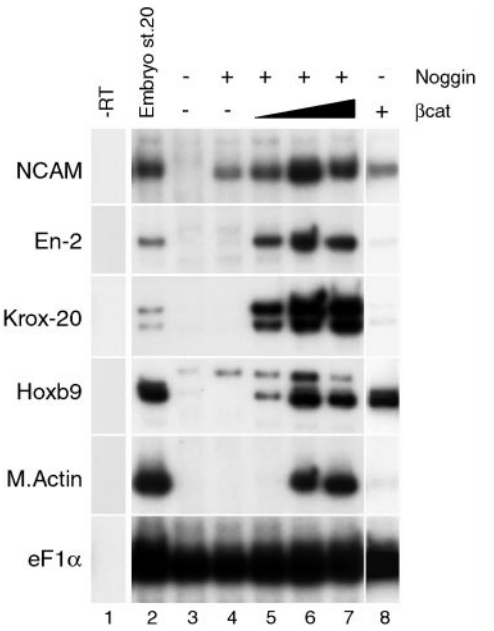

FIG. 1. An amino-terminal truncated β-catenin induces posterior neural and mesodermal markers in animal caps when coinjected with noggin. RT-PCR of animal caps injected with 500 pg/embryo of *noggin* RNA and 20 pg/embryo (lane 5), 100 pg/embryo (lane 6), 500 pg/embryo (lane 7) of a truncated *β-catenin*. Truncated *β-catenin* (500 pg/embryo) alone induced *NCAM* and *Hoxb9* but not *muscle actin* (lane 8). −RT (lane 1) is a negative control where no reverse transcriptase was added to embryo RNA during the RT reaction. Embryo RNA was used as positive control (lane 2). All samples were analysed at stage 20. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers. Animal caps injected only with *noggin* RNA (lane 4) expressed *NCAM*, but not posterior neural or mesodermal markers. *eF1α* was used as loading control.

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

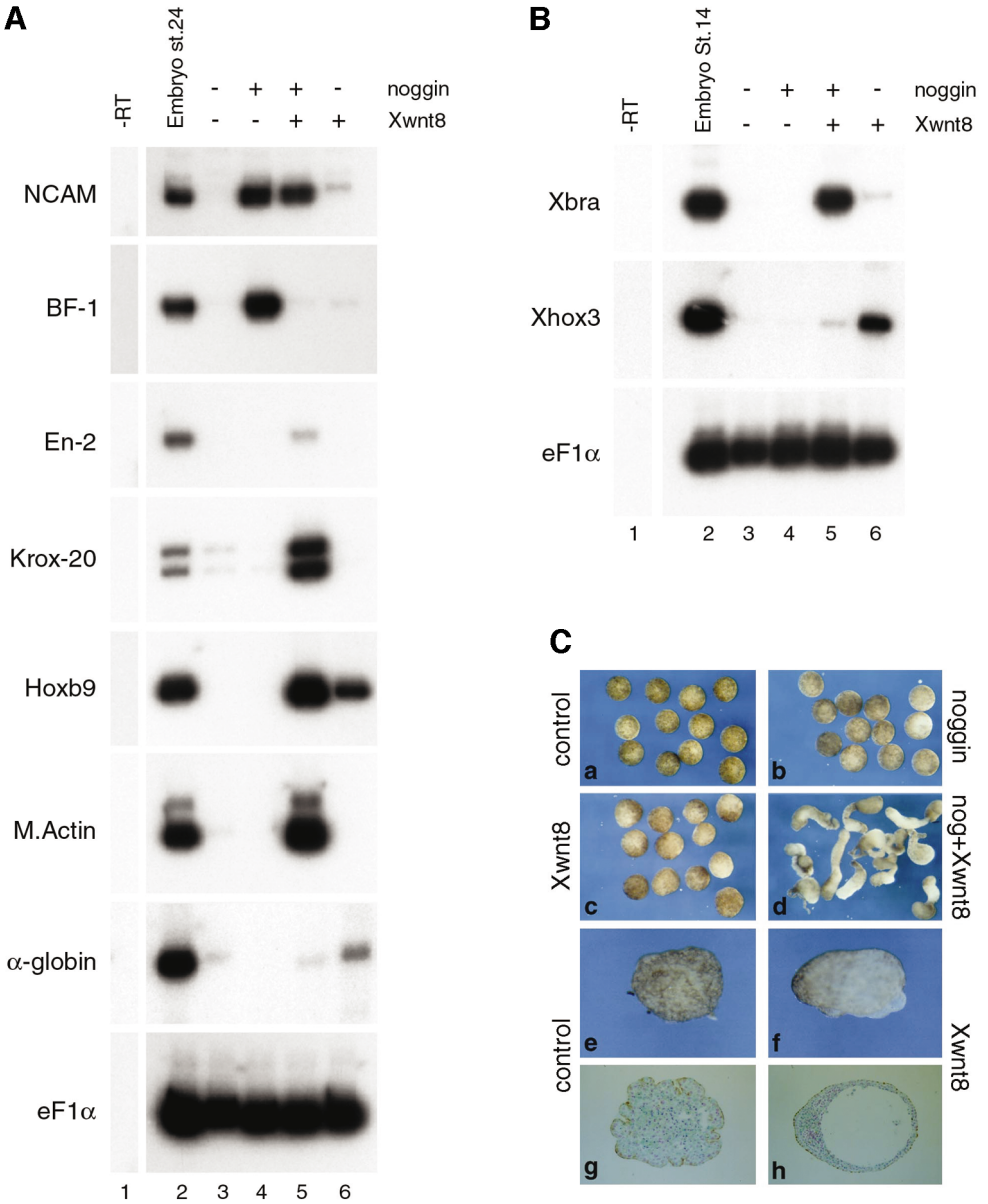

FIG. 2. Xwnt8 induces posterior neural and dorsal mesodermal markers in animal caps when coinjected with noggin and ventral mesodermal markers when injected alone. (A) RT-PCR of animal caps assayed when sibling embryos reached stage 24. Animal caps from uninjected embryos (lane 3) did not express neural 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. Posterior neural markers and the dorsal mesodermal marker *muscle actin* were induced in explants coinjected with *noggin* and *Xwnt8* (50 pg/embryo; lane 5). *Xwnt8* RNA alone (lane 6) induced the ventral mesoderm marker *α-globin* and *Hoxb9*. (B) RT-PCR of animal caps assayed at stage 14 to examine mesodermal markers. *Xwnt8* induced *Xhox3* and coinjection of *noggin* resulted in an increase in the expression of *Xbra* and a reduction in *Xhox3* expression. Embryo RNA was used as a positive control and −RT is embryo RNA processed without reverse transcriptase and served as a negative control. *eF1α* was used as loading control. (C) Morphology of animal caps fixed either at stage 18 (a–d) or stage 38 (e, f). Explants from uninjected (a), *noggin*-injected (b), and *Xwnt8*-injected (c) embryos did not elongate. Animal caps coinjected with *noggin* and *Xwnt8* elongated (d). Animal caps from uninjected embryos (e, g) did not form vesicles, while those injected with *Xwnt8* RNA (f, h) formed vesicles characteristic of ventral mesoderm.

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 (LEFΔNβCTA) (Vleminckx *et al.*, 1999) were fused to the ligand binding domain of the human glucocorticoid receptor, creating GR-LEFΔNβCTA. 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-LEFΔNβCTA 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-LEFΔNβCTA during gastrula stages should lead to the induction of posterior neural markers in the absence of mesoderm. Therefore, we injected *GR-LEFΔNβCTA* 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 animal caps injected with *noggin* and *GR-LEFΔNβCTA*, addition of DEX at stage 9 induced the posterior neural markers *En-2*, *Krox-20*, and *Hoxb9* and dorsal mesodermal markers (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-LEFΔNβCTA*, 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-LEFΔNβCTA 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-LEFΔNβCTA 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-LEFΔNβCTA is restricted to late blastula stages. Furthermore, activation of GR-LEFΔNβCTA 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-LEFΔNβCTA in Intact Xenopus Embryos

To investigate the consequences of activating GR-LEFΔNβCTA in whole embryos, *GR-LEFΔNβCTA* 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-LEFΔNβCTA 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-LEFΔNβCTA is similar to the response in intact embryos.

Induction of the Posterior Neural Markers by GR-LEFΔNβCTA 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-LEFΔNβCTA* 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

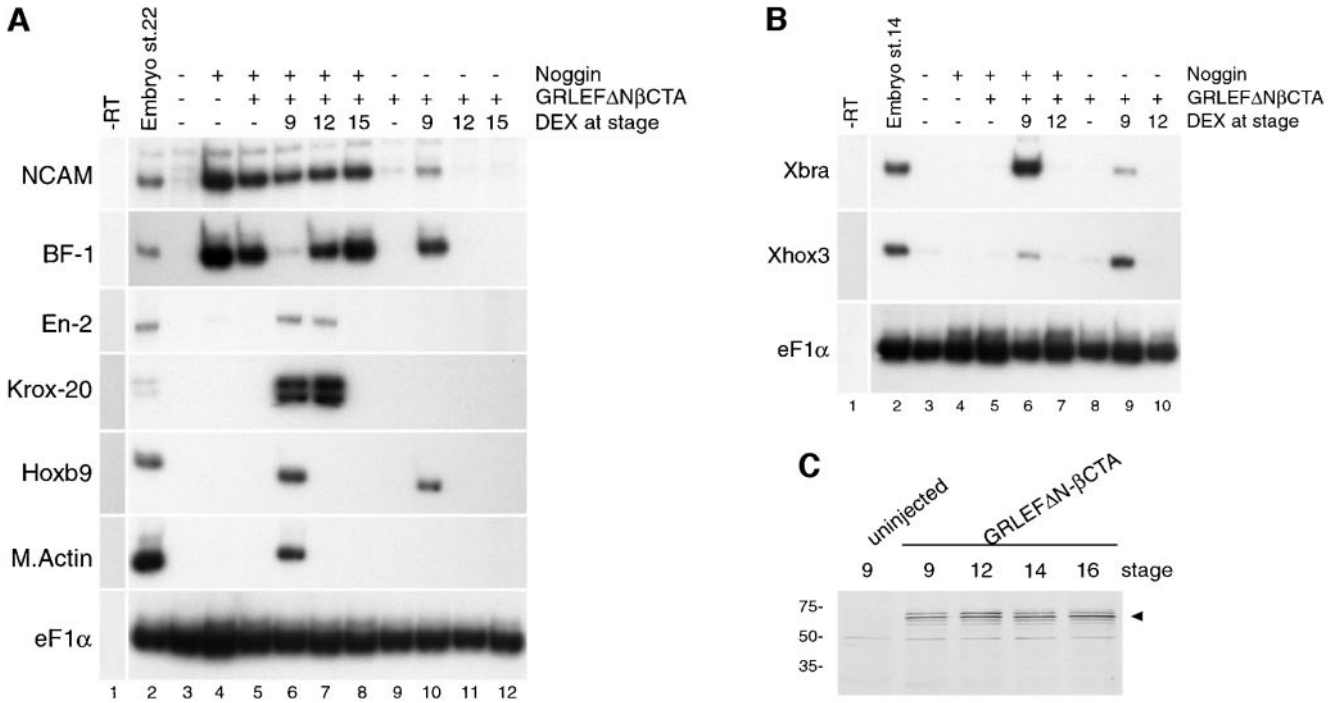

FIG. 3. An inducible form of β-catenin, GR-LEFΔNβCTA, results in expression of both posterior neural and mesodermal markers when activated at stage 9, but only posterior neural markers when activated at stage 12 in coinjections with noggin. (A) RT-PCR of animal caps analysed at stage 22. Animal caps 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. Posterior neural and mesodermal markers were detected in tissues from embryos coinjected 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.

stained area. This suggests that a noncell-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* and *GR-LEFΔNβCTA* RNA were cultured in the presence or absence of DEX in calcium- and magnesium-free medium (CMFM), which causes dissociation of cell-to-cell contact. As a positive control, intact animal caps expressed posterior markers (Fig. 6A, lanes 6 and 7) when DEX was added to the culture medium. Dissociation of animal cap cells completely blocked the induction of the posterior neural markers and *muscle actin* and the suppression of *BF-1* (Fig. 6A, lanes 9 and 10). Induction of the known direct targets of the Wnt pathway, *siamois* and *Xnr3*, was not affected by the dissociation treatment (Fig. 6B, lanes 5, 6 and 7, 8). These results demonstrate that the induction of posterior neural markers by GR-LEFΔNβCTA occurs in a noncell-autonomous manner and requires cell-to-cell contact.

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

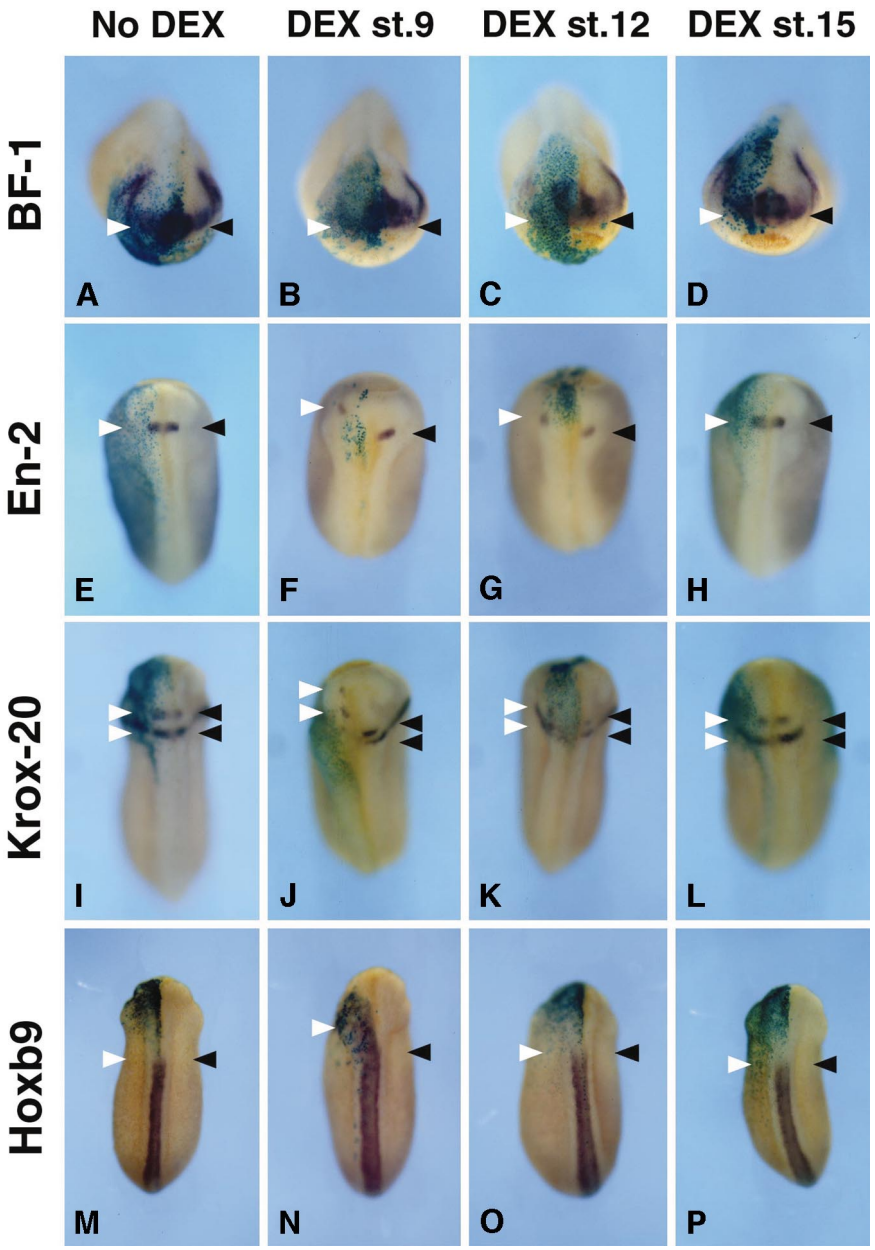

FIG. 4. Activation of GR-LEFΔNβCTA in intact *Xenopus* embryos results in posteriorization of the neural tube. One animal/dorsal blastomere of 8-cell stage embryos was injected with 50 pg of *GR-LEFΔNβCTA* RNA and 200 pg of *β-gal* RNA. Embryos were fixed at stages 18–22, processed for β-gal staining to reveal the injected side (light blue staining) and for whole-mount *in situ* hybridization with probes specific for *BF-1* (A–D), *En-2* (E–H), *Krox-20* (I–L), and *Hoxb9* (M–P) (purple staining). With no addition of DEX, the markers show an identical pattern of expression on the injected and uninjected sides (A, 24/25; E, 20/20; I, 20/20; M, 24/24). When DEX was added at stage 9, an anterior shift of the markers resulted (B, 25/30; F, 26/30; J, 25/28; N, 20/32). Addition of DEX at stage 12 affected *BF-1* (C, 20/32), *En-2* (G, 22/28), and *Krox-20* (K, 17/25) but not *Hoxb9* (O, 3/29). Addition of DEX at stage 15 did not affect any of the markers (D, 3/28; H, 2/27; L, 2/28; P, 1/25). The position of the markers is indicated with a white arrowhead on the injected side and a black arrowhead on the control side. Panels A–D are frontal views (dorsal is up) all other panels are dorsal views (anterior is up).

Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). 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-

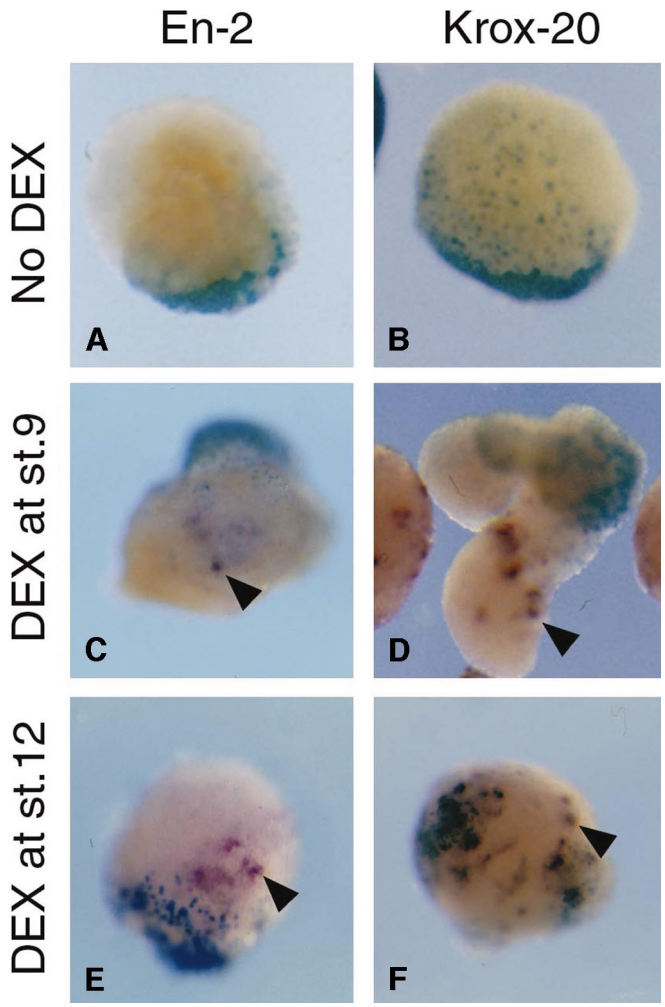

FIG. 5. Induction of *En-2* and *Krox-20* by GR-LEFΔNβCTA occurs in a noncell-autonomous manner. One animal blastomere of 16-cell stage embryos was injected with 500 pg *noggin* RNA, 50 pg *GR-LEFΔNβCTA* RNA, and 200 pg *β-gal* RNA as a lineage tracer. Animal caps were dissected at stage 8, cultured in the absence or presence of DEX, fixed at stage 20, stained for β-gal (light blue staining), and processed for whole-mount *in situ* hybridization with probes specific for *En-2* (A, C, and E) and *Krox-20* (B, D, and F) (purple staining). When DEX was not added to the culture media, induction of *En-2* (A, 0/25) or *Krox-20* (B, 0/30) was not observed. Addition of DEX at stages 9 and 12 resulted in the induction of *En-2* (C, 22/26 and E, 18/24) and *Krox-20* (D, 26/32 and F, 22/28) in a noncell-autonomous manner. Arrowheads indicate patches where the markers were induced outside the cells expressing the lineage tracer.

tion of the posterior neural markers by Wnt/β-catenin signalling, we coinjected embryos with *noggin*, *GR-LEFΔNβCTA*, and *XFD*, a truncated FGF receptor 1, which blocks FGF signalling (Amaya *et al.*, 1991). Animal caps were isolated at stage 8 and cultured in the presence or absence of DEX. In caps coinjected with *noggin* and *GR-LEFΔNβCTA*, addition of DEX at stage 9 led to the induction of the posterior neural markers and *muscle actin* (Fig. 7A, lane 6). Coinjection of 100 pg/embryo of *XFD* RNA suppressed both the expression of *muscle actin* and posteriorization of the explants. The expression of *En-2*, *Krox-20*, and *Hoxb9* was reduced, while expression of *BF-1* recovered (Fig. 7A, lane 7). Increasing the amount of *XFD* RNA to 500 pg/embryo resulted in a stronger suppression of the posterior neural markers (Fig. 7A, lane 8). In explants cultured in DEX from stage 12, the presence of *XFD* led to a complete suppression of *En-2* and *Krox20* (Fig. 7A, lanes 10 and 11). In summary, inhibiting FGF signalling with *XFD* suppressed the posteriorizing effect of *GR-LEFΔNβCTA*.

XFD was introduced as RNA, which is translated and becomes active soon after injection. So, it was possible that the suppression of the posterior neural markers was due to an early effect on the competence of the animal cap to respond to the activation of *GR-LEFΔNβCTA*. To address this question, we used the specific inhibitor of the tyrosine kinase activity of the FGF receptor, SU5402 (Calbiochem) (Mohammadi *et al.*, 1997), which can be applied simultaneously with DEX. We injected embryos with *noggin* and *GR-LEFΔNβCTA* RNA, dissected animal caps at stage 8, and cultured them in the presence of DEX and SU5402. Increasing amounts of SU5402 suppressed the induction of posterior neural markers either when added at stage 9 (Fig. 7B, lanes 7 and 8) or stage 12 (Fig. 7C, lanes 10 and 11), 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

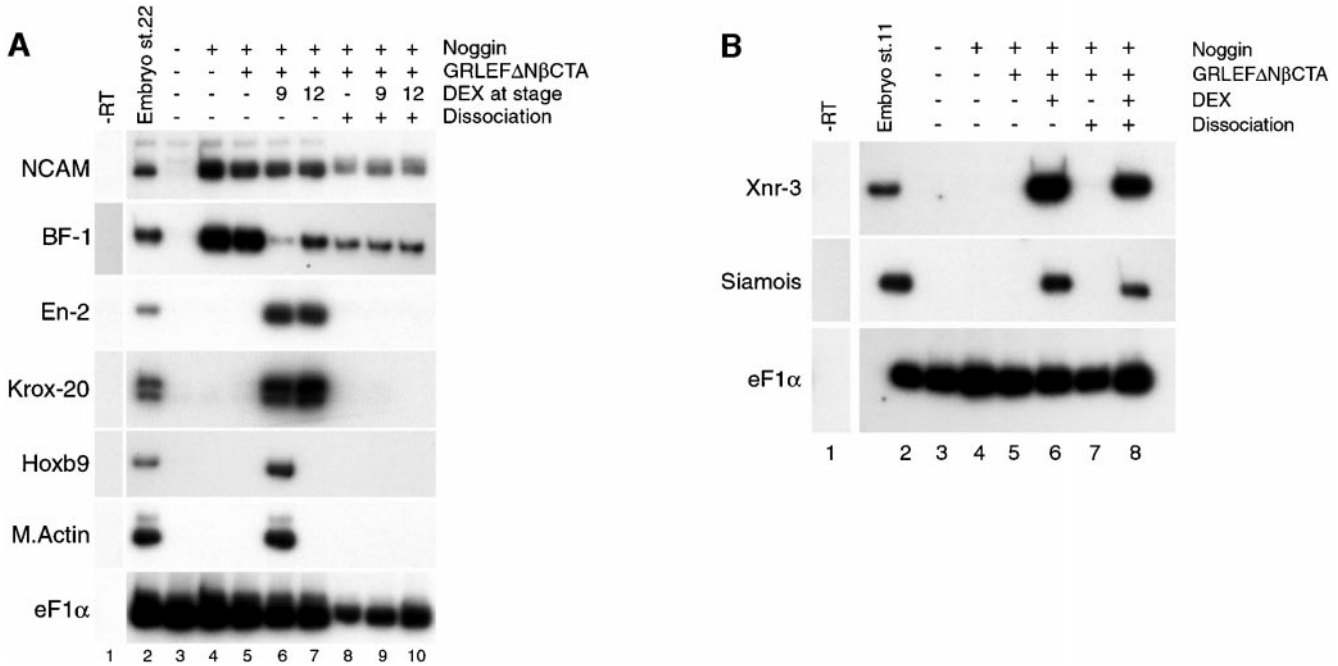

FIG. 6. Induction of posterior neural markers by GR-LEFΔNβCTA requires cell-to-cell contact. (A) RT-PCR analysis showing that intact explants from embryos coinjected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) RNA expressed posterior neural and mesodermal markers when DEX was added 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 *Siamois* 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.

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-

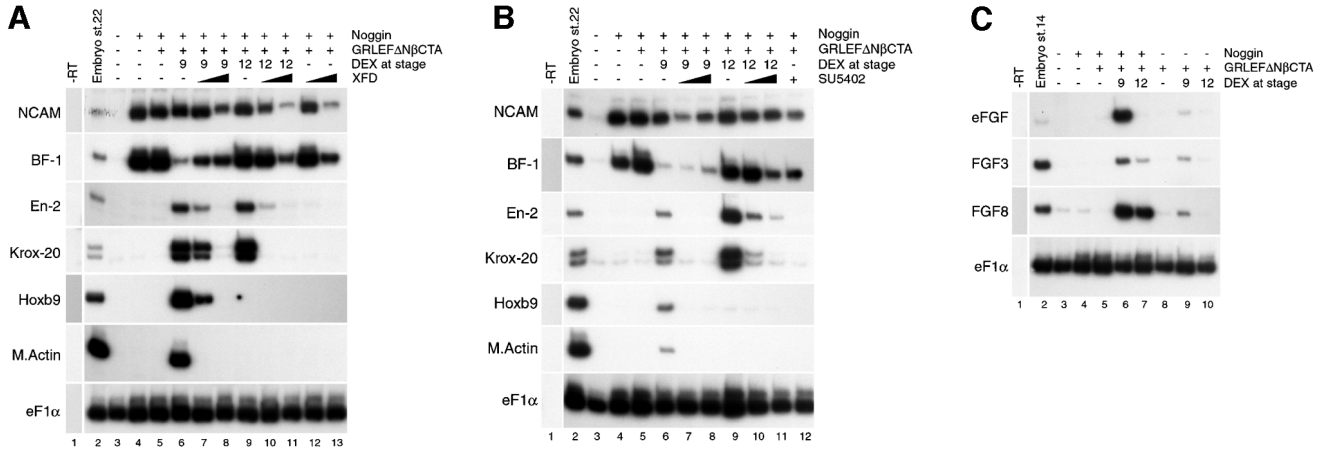

FIG. 7. FGF signalling is required for induction of posterior neural and mesodermal markers by GR-LEFΔNβCTA. (A) XFD suppressed the induction of the posterior neural and mesodermal markers by GR-LEFΔNβCTA. RT-PCR detected the induction of posterior and mesodermal markers in explants coinjected with *noggin* (500 pg/embryo) and *GR-LEFΔNβCTA* (50 pg/embryo) when DEX was added at stage 9 (lane 6). Coinjection of *XFD* RNA (100 pg/embryo) caused a reduction in the expression of *En-2*, *Krox-20*, and *Hoxb9*, suppressed *muscle actin*, and reestablished *BF-1* expression (lane 7). Coinjection of 500 pg/embryo *XFD* RNA resulted in the suppression of all posterior neural markers (lane 8). When DEX was added at stage 12, XFD blocked the induction of *En-2* and *Krox-20* (lanes 10 and 11). In the absence of DEX, 500 pg/embryo *XFD* RNA caused a reduction in the expression of *NCAM* and *BF1* (lane 13) (B) The FGF signalling inhibitor SU5402 blocked the induction of posterior neural markers when added simultaneously with DEX at stage 9 or stage 12. Both 10 μM (lanes 7 and 10) and 50 μM (lanes 8 and 11) of SU5402 suppressed the induction of posterior neural and mesodermal markers. Addition of 50 μM of SU5402 at stage 9 in the absence of DEX did not affect the expression of *NCAM* or *BF-1* (lane 12). (C) *FGFs* are induced by GR-LEFΔNβCTA. RT-PCR of animal caps analysed when sibling embryos reached stage 14 detected the expression of *eFGF*, *FGF3*, and *FGF8* when DEX was added at stage 9 (lanes 6 and 9). *FGF3* and *FGF8* expression was also detected when DEX was added at stage 12 (lane 7). −RT is a negative control and embryo is a positive control. *eF1α* served as loading control.

ysis correlates with results in whole embryos, where unilateral injections of GR-LEFΔNβCTA 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-LEFΔNβCTA in animal caps is consistent with the localized expression of GR-LEFΔNβCTA 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, *Wnt1* 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/$\bar{\beta}$-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-LEFΔNβCTA. 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-LEFΔNβCTA 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-LEFΔNβCTA.

Finally, we show that *FGF3* and *FGF8* are induced in animal caps as a consequence of the activation of GR-LEFΔNβCTA. 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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