Int. J. Dev. Biol. 46: 217-226 (2002)

Original Article

Multiple interactions between maternally-activated signalling pathways control *Xenopus nodal*-related genes

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ABSTRACT We have investigated the induction of the six *Xenopus nodal*-related genes, *Xnr1-Xnr6,* by maternal determinants. The β-catenin pathway was modelled by stimulation using Xwnt8, activin-like signalling was modelled by activin, and VegT action was studied by overexpression in animal cap explants. Combinations of factors were examined, and previously unrecognised interactions were revealed in animal caps and whole embryos. For the induction of *Xnr5* and *Xnr6* in whole embryos, using a β-catenin antisense morpholino oligonucleotide or a dominant negative XTcf3, we have demonstrated an absolute permissive requirement for the β-catenin/Tcf pathway, in addition to the requirement for VegT action. In animal caps *Xnr5* and *Xnr6* are induced in response to VegT overexpression, and this induction is dependent upon the concomitant activation of the β-catenin pathway that VegT initiates in animal caps. For the induction of *Xnr3,* VegT interacts negatively so as to inhibit the induction otherwise observed with wnt-signalling alone. The negative effect of VegT is not the result of a general inhibition of wnt-signalling, and does not result from an inhibition of wnt-induced *siamois* expression. A 294 bp proximal promoter fragment of the *Xnr3* gene is sufficient to mediate the negative effect of VegT. Further experiments, employing cycloheximide to examine the dependence of *Xnr* gene expression upon proteins translated after the mid-blastula stage, demonstrated that *Xnrs 4, 5* and *6* are 'primary' *Xnr* genes whose expression in the late blastula is solely dependent upon factors present before the mid-blastula stage.

KEY WORDS: *Xnr, nodal-related gene, β-catenin, VegT, activin, morpholino*

Introduction

The molecular events leading to the formation and patterning of the endoderm and mesoderm in *Xenopus* have been elucidated progressively in recent years (reviewed in Yasuo and Lemaire, 2001). Current models involve a relatively small number of maternally-supplied localised molecules, which initiate the transcription of a number of early zygotic genes. The essential and pivotal role of the transcription factor VegT, whose maternal mRNA is vegetally localised (Zhang and King 1996), has become apparent through experiments involving the technique of antisense oligonucleotide-depletion of the maternal mRNA (Zhang *et al.*, 1998; Kofron *et al.*, 1999; Xanthos *et al.*, 2001). Probably acting in combination with vegetally-localised maternal mRNA encoding a TGFβ family member, VegT activates the zygotic transcription of a number of genes which are necessary for formation of the endoderm (Yasuo and Lemaire, 1999, Clements *et al.*, 1999; Xanthos *et al.*, 2001), and formation of the mesoderm (Clements *et al.*, 1999; Kofron *et al.*, 1999). On the dorsal side of the embryo, the localised action of the dishevelled/β-catenin signalling pathway participates in a chain of events leading to formation of the Spemann organiser (Schneider *et al.*,1996; Carnac *et al.*, 1996; Kessler 1997; Larabell *et al.*, 1997; Miller *et al.*, 1999; Nishita *et al.*, 2000).

A common feature of recent models of endodermal patterning and dorsal mesoderm formation is the positive combined action in the vegetal-dorsal region, where the signals intersect, of two or more signals comprising (i) dorsally-localised β-catenin activity, and (ii) one or more either maternal (Weeks and Melton, 1987 Fukui *et al.*, 1994; Oda *et al.*, 1995) or zygotic vegetally -localised activin-like TGFβ signals (Watabe *et al.*, 1995; Cui *et al.*, 1996; Clements *et al.*, 1999; Zorn *et al.,* 1999; Agius *et al.*, 2000; Nishita *et al.*, 2000, Takahashi *et al.*, 2000). Thus, for example, the overlap of dorsal signalling and vegetally-localised Smad2 activity has been proposed to result in a co-operative interaction that activates expression of the *siamois* gene, spatially restricting siamois to its correct location (Crease *et al.*, 1998).

Downstream of VegT, there is evidence that a network of genes including multiple *TGF*β family members participate in endoderm and mesoderm formation. Participating *TGF*β family members

Abbreviations used in this paper: CHX, cycloheximide; dn, dominant negative; RT-PCR, reverse transcription polymerase chain reaction.

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0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

include genes encoding activin, derriere, and members of the family of nodal-related molecules, the Xnrs (Clements *et al*., 1999; Sun *et al*., 1999; Agius *et al*., 2000; Takahashi *et al*., 2000). The transcriptional activation of the *Xnr* genes by maternal signalling pathways is the subject of experiments reported here.

There are six documented *Xnr* genes in *Xenopus laevis.* The protein products of *Xnrs 1,2,4,5* and *6* are all capable of inducing mesoderm (Jones *et al*., 1995; Lustig *et al*., 1996, Joseph and Melton, 1997; Takahashi *et al.,*2000). The Xnr3 protein is structurally diverse from the other five Xnrs. Unlike them, Xnr3 does not display mesoderm-inducing properties but it is a neural-inducing factor (Hansen *et al.,*1997), acting at least in part by antagonising BMP activity (Glinka *et al*., 1996). None of the *Xnr* genes is expressed as maternal mRNA. Transcription of all six *Xnrs* is activated at about the mid-blastula stage, and all except *Xnr3* are expressed in vegetal cells of the blastula (Jones *et al.,* 1995; Smith *et al*., 1995; Joseph and Melton, 1997, Agius *et al*., 2000; Takahashi *et al*., 2000). For *Xnrs1* and *2* this early expression is relatively low level, with transcript amounts peaking considerably later than maximal *Xnr 4, 5* and *6* expression (Agius *et al*,. 2000; Takahashi *et al*., 2000). All except *Xnr3* are expressed predominantly in dorsal vegetal domains at the late blastula stage (Agius *et al*,. 2000; Takahashi *et al*., 2000), whereas *Xnr3* transcripts are found in the dorsal marginal region and are absent from the vegetal region (Takahashi *et al*., 2000). At gastrula stages *Xnrs 1* and *2* are weakly expressed in a dorsal marginal domain that appears to be wider than Spemann's organiser (Jones *et al*., 1995). *Xnr 3* and *4* expression is localised to the organiser (Ecochard *et al*., 1995; Smith *et al.,* 1995; Joseph and Melton 1997), and in the case of *Xnr3* expression also extends into the deep marginal and vegetal cells, nearly down to the vegetal pole (Darras *et al*., 1997). *Xnr5* expression was undetectable in the gastrula embryo, whereas *Xnr6* is expressed below the dorsal lip of the blastopore (Takahashi *et al*., 2000). Only *Xnr1* is expressed in later, tailbud stage, embryos. At tailbud stages *Xnr1* is expressed in two small posterior domains and then in a large asymmetric domain in the left lateral plate mesoderm (Lustig *et al*., 1996), where it performs an essential conserved role in establishing the body's left-right asymmetry (Levin and Mercola 1998; Collignon *et al*., 1996; Sampath *et al*., 1997).

The induction of the *Xnr* genes by maternal determinants has been investigated previously, often using the isolated blastula ectoderm explant ('animal cap') assay. Induction of *Xnr* genes by the β-catenin pathway has been modelled by stimulation of the pathway using Xwnt8, which is known to activate the 'canonical' β-catenin/TCF pathway in Xenopus (Heasman *et al*., 1994; Darken and Wilson, 2001; Hamilton *et al*., 2001). The induction by maternal TGFβ family members has been modelled by Vg1 and activin (Clements *et al.,* 1999; Yasuo and Lemaire, 1999; Agius *et al*., 2000). The transcription factor VegT has been shown to activate expression of *Xnrs 1, 2, 4, 5* and *6* (Clements *et al*., 1999; Hyde and Old, 2000; Takahashi *et al*., 2000), but does not induce *Xnr3* (Hyde and Old, 2000). Because of the importance of Xnr signalling in early embryos, we have set out to investigate systematically the induction of the six *Xnr* genes by maternal determinants. In particular, we have sought to better understand the patterning of the early embryo by investigating the interactions between combinations of maternal determinants in inducing the six *Xnr* genes. So although our experiments overlap with previously published work, our systematic study of all six genes together, using combinations of inducing factors, is novel and reveals previously unrecognised interactions.

These six genes make up a gene family in *Xenopus laevis*. The genes have individual induction properties which ultimately we would like to understand in terms of their DNA sequences. We and others have previously investigated promoter elements responsible for the induction of *Xnr1* by VegT (Kofron *et al*., 1999; Hyde and Old, 2000), and FAST-responsive sequences within the first intron of the *Xnr1* gene have been identified that mediate the gene's activin/nodal responsiveness and left-side -specific expression (Osada *et al*., 2000). For the *Xnr3* gene, DNA sequences responsible for the wnt-mediated induction have been identified (McKendry *et al*., 1997). A comparison between the *Xnr1* and *Xnr3* promoters identified some notable conserved features, including wnt response elements, as well as differences such as the presence of T-box sites in the promoter of *Xnr1* but not *Xnr3* (Hyde and Old, 2000). The absence of T-box sites from the comparable region of the *Xnr3* promoter is consistent with the non-responsiveness of the *Xnr3* gene to transcriptional activation by VegT (Hyde and Old, 2000). This comparison between *Xnr1* and *Xnr3*, with the apparent conservation of certain promoter elements, encouraged the hope that the proximal promoter sequences of the *Xnr* genes might be interpretable in terms of the genes' combinatorial regulation, further motivating the experiments reported here.

Results

To study further the responses of the *Xnr* family of genes to signalling pathways known to be active in establishing and patterning the embryonic germ layers, and to explore systematically the possibility of combinatorial effects of such signalling pathways, we first examined the induction of the *Xnr* genes in blastula ectoderm (animal cap) explants cut from embryos that had been injected at the one-cell stage with mRNAs encoding Xwnt8, activin, or VegT, singly or in combination. The transcriptional responses of the six members of the nodal-related gene family, *Xnr1* through *Xnr6,* were analysed by RT-PCR. All the experiments were repeated several times, with reproducible outcomes.

The RT-PCR analysis was performed at stage 9 (late blastula, shortly after the initiation of zygotic transcription at the mid-blastula transition), and at stage 10 (blastopore formation, early gastrula). By stage 10 adequate time has elapsed for genes encoding possible secondary signalling proteins to have been transcribed and translated, and to have exerted their effects. Among these secondary signalling proteins are the multiple TGFβ family members that have been shown to be part of the network of zygotically-expressed endoderm-inducing and mesoderm-inducing factors initiated by the VegT transcription factor (Clements *et al*., 1999; Kofron *et al*., 1999; Agius *et al*., 2000; Xanthos *et al*., 2001).

It is immediately apparent from a comparison of the analyses of *Xnr* gene induction at stages 9 and 10 (Fig. 1) that there are a number of clear differences between them, especially in the combinatorial effects (to be discussed in detail below) of the inducing factors at the two developmental stages. For example, *Xnr6* expression shows strong synergy with all three inducing factors at stage 9, but almost no synergy at stage 10. The interactions are evidently dynamic, changing substantially and rapidly as development proceeds.

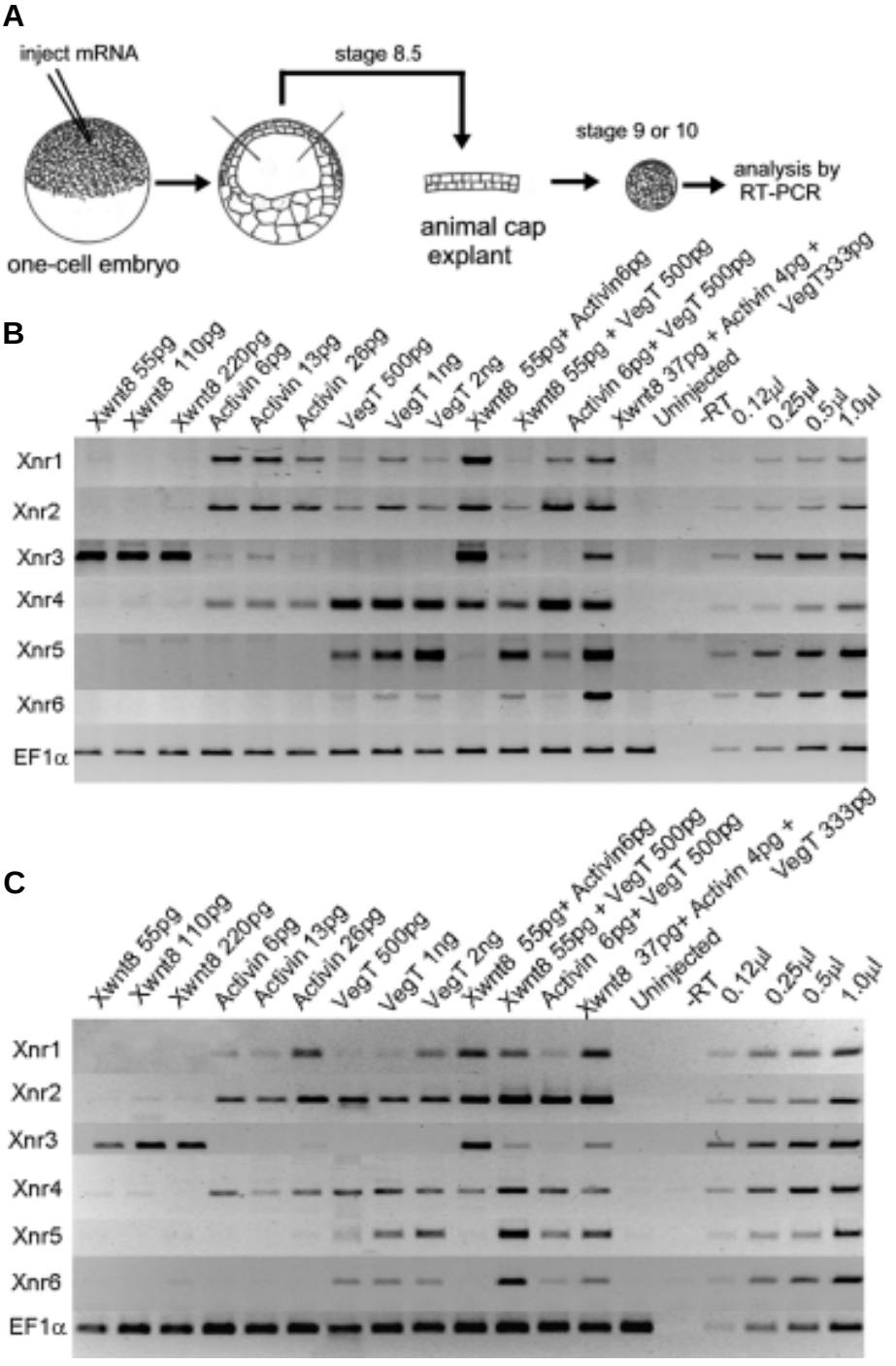


Fig. 1. Transcriptional responses of *Xnr* genes to *Xwnt8, activinβB* and *VegT* mRNAs singly or in combination. (A) *One-cell* Xenopus *embryos were injected with three different amounts of mRNA (covering a four-fold range) for* Xwnt8, activinβB *and* VegT. *Animal caps were dissected and cultured to stage 9 or 10, at which point RNA was prepared for RT-PCR analysis.* **(B)** *RT-PCR analysis at stage 9.* **(C)** *RT-PCR analysis at stage 10. Only* Xnr3 *was strongly induced by Xwnt 8. Activin induced* Xnr1,2 *and* 4. *VegT induced* Xnr1,2,4,5, *and* 6. *Xwnt8, activin and VegT mRNAs were injected pairwise and as a mixture of all 3 mRNAs. In pairwise combinations the amount of each mRNA was the same as the lowest amount used singly. Positive synergy was observed for* Xnr1,2 *and* 4 *in response to Xwnt8 and activin. The synergy for* Xnr4 *was seen at stage9 but not at stage 10. Synergy was seen for* Xnr4,5 *and* 6 *for Xwnt8 and VegT at stage 10. When all 3 factors were combined at stage 9* Xnr6 *displayed a strong synergistic induction. VegT showed a strong negative effect on the induction of* Xnr3 *by Xwnt8. Whole stage 9 or stage10 embryo cDNA was used for the linearity series.*

Transcriptional Responses of Xnr *Genes to XWnt8, Activin, or VegT, Singly*

Preliminary experiments (not shown) were performed to determine the minimal amounts of Xwnt8, activin, and VegT synthetic mRNAs that were required to give clear transcriptional activation of responding *Xnr* genes in animal cap explants. The amounts used subsequently were based on these preliminary experiments so as to give dosage-dependent responses of the caps to each of the three mRNAs, as made evident by graded induction of at least one responding *Xnr* gene (although simple dose-dependence was not observed for all *Xnr* genes at each developmental stage; see below). In the experiments shown in Fig. 1, for each mRNA a set of three mRNA doubling amounts was injected, covering a fourfold range. For comparisons of the levels of induction achieved in animal caps with the amount of *Xnr* gene transcript found naturally in whole embryos, we note that the amount of animal cap RNA analysed was the same as the amount of RNA analysed from whole embryos in the '1.0 µl' track of each linearity series shown in the figures.

Figure 1B (analysis at stage 9), and Fig. 1C (analysis at stage 10) show that *Xnr3* was strongly induced by Xwnt8 in animal cap explants, as expected from previous work (Smith *et al.*, 1995; McKendry *et al.*, 1997). None of the other five *Xnr* genes was induced in these caps. This absence of induction of *Xnr1, 2, 4, 5, and 6,* by Xwnt8 is consistent with the fact that Xwnt8 cannot induce mesoderm formation in early blastula animal caps (Sokol 1993), whereas each of these five Xnrs is capable of inducing mesoderm robustly in animal caps (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000).

The non-responsiveness of the endogenous *Xnr1* gene contrasts with our previous work showing that a 616 bp fragment of the *Xnr1* promoter does indeed respond to wnt signalling in a transient assay, using a luciferase reporter, in which DNA was injected into early embryos (Hyde and Old, 2000). The *Xnr1* promoter fragment used in that transient assay included a 14 bp sequence that is identical to a functional 'wnt responsive' element shown also to be present in the *Xnr3* promoter (Hyde and Old, 2000; McKendry *et al.*, 1997). This element does not in fact bind Lef1/Tcf (McKendry *et al.*, 1997), but possibly mediates transcriptional activation of X*nr3* through the action of the homeodomain proteins siamois and twin, which are induced directly by the wnt/β-catenin pathway (Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Laurent *et al.*, 1997). It therefore

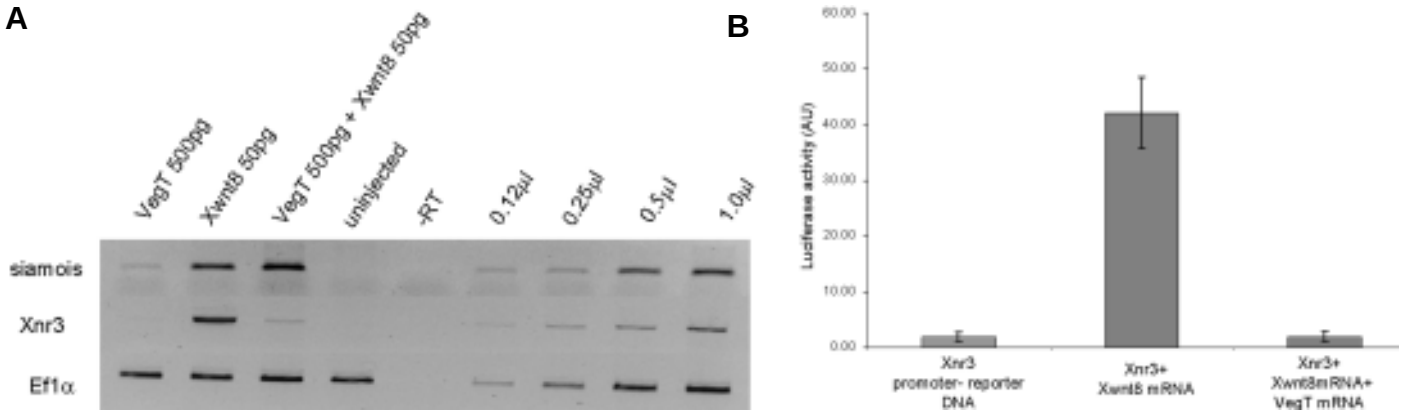


Fig. 2. The negative effect of VegT on *Xnr3* induction. (A) *The negative effect of VegT does not act through inhibition of siamois induction. One-cell* Xenopus *embryos were injected with* Xwnt8 *mRNA (50 pg), VegT mRNA (500 pg) or a mixture of the two mRNAs. Animal caps were harvested at stage 9 for RT-PCR analysis. Xwnt8 induced both* Xnr3 *and* siamois *expression. VegT inhibited the Xwnt8-induction of* Xnr3 *but not* siamois. **(B)** *VegT has a strong negative effect on the transcriptional activation, by Xwnt8, of the* Xnr3 *promoter. A 294 bp promoter fragment was linked to a* luciferase *reporter gene. The promoter-reporter DNA (30 pg) was injected into one-cell* Xenopus *embryos with or without Xwnt8 (1 ng) and VegT (2 ng) mRNA. Embryos were harvested at stage11 to assay luciferase activity. Samples of ten embryos were analysed and each bar represents the average of duplicate samples. The induction of* Xnr3 *by Xwnt8 and the negative effect of VegT were confirmed by multiple independent experiments, although the extent of induction/inhibition varied between experiments.*

appears that the sensitive transient assay of the *Xnr1* promoter fragment can reveal responses that are different from those of endogenous genes, either because the promoter fragment in question lacks negatively-acting DNA elements, or because there is a finite basal level of ('leaky') expression in sensitive transient assays, and this basal level is detectably stimulated by transcriptional activators that may not normally act alone (see below).

Activin efficiently induced *Xnr1, 2,* and *4,* but not *3, 5,* or *6* (a slight induction of *Xnr3* is apparent at stage 9). The dose-responses of the caps at the two developmental stages are complex. This may reflect the observation that the highest dose of activin had a profound effect upon the caps, visible as a pale coloration owing to the pigment redistribution that accompanies bottle cell formation (Kurth and Hausen, 2000). As expected from previous data, VegT induced *Xnr1, 2, 4, 5,* and 6 (Clements *et al.*, 1999; Takahashi *et al.*, 2000), but not *Xnr3* (Hyde and Old, 2000).

Transcriptional Responses of Xnr *Genes to Combinations of Factors: Positive and Negative Interactions*

Xwnt8, activin, and VegT mRNAs were injected in all pairwise combinations, and also as a mixture of all three mRNAs (Fig. 1). In the pairwise combinations, the amount of each component injected was the same as the lowest amount used in each set of single-mRNA injections, e.g. the 'Xwnt8 plus VegT' mixture contained 55 pg Xwnt8 mRNA plus 500 pg VegT mRNA.

Clear positive synergy was observed for *Xnr1, 2,* and *4,* in response to Xwnt8 plus activin. Xwnt8 alone did not induce these three *Xnr* genes, but Xwnt8 was capable of potentiating the responses to activin for each of them. In the case of the *Xnr4* gene, the synergy was evident at stage 9 (Fig. 1B), but not stage 10 (Fig. 1C). The combination of Xwnt8 plus activin was not effective in inducing *Xnrs 5* and *6*.

Xwnt8 and VegT were synergistic for induction of *Xnr4, 5, and 6,* even though Xwnt8 alone did not induce these genes. For *Xnr5* and *6,* strong synergy was apparent at stage 10, and this result is consistent with previous findings that β-catenin potentiates VegT in inducing these two genes (Takahashi *et al.*, 2000). When all three factors, Xwnt8, activin, and VegT were combined, at stage 9 *Xnr6* displayed very strong synergistic induction above that the relatively weak induction produced by VegT alone, and even above the synergistic induction by VegT and Xwnt8 together, even though Xwnt8 and activin were incapable of inducing the gene either alone or when combined as a pair.

In addition to these positive synergistic effects of VegT and Xwnt8, there was a simultaneous strong negative effect of VegT upon the ability of Xwnt8 to induce *Xnr3*. In the presence of VegT, the characteristically robust induction of *Xnr3* by Xwnt8 was dramatically reduced, in caps analysed at stage 9 (Fig. 1B). This negative interaction was stronger at stage 9 than at stage 10, where a weaker but still substantial negative effect of VegT was evident (Fig. 1C). A possible mechanism for the inhibitory effect of VegT could be mediated through inhibition of *siamois* expression. The *siamois* gene is a direct target of wnt-signalling, and siamois induces *Xnr3* (Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Kessler 1997), so an inhibition of *siamois* expression might cause a loss of *Xnr3* induction. We therefore asked if VegT led to a reduction in siamois induction in animal caps coinjected with *Xwnt8* and *VegT* mRNAs. The analysis of *siamois* expression (Fig. 2A), shows that the expected induction of *siamois* by wnt signalling is not reduced by expression of VegT in the animal caps. This indicates that the reduction of *Xnr3* expression in these animal caps is not due to a failure of *siamois* expression.

VegT Inhibits the Ability of Xwnt8 to Activate Transcription from a 294 bp Region of the Xnr3 *Promoter*

A general inhibition of wnt activity as a mechanism for the strong inhibition of *Xnr3* induction by VegT is excluded because in the same animal caps where the negative interaction with respect to *Xnr3* induction occurred, the combination of VegT and XWnt8 was positively synergistic for the induction of *Xnrs 4*, *5*, and *6*. To examine this further, we asked if the proximal promoter region of the *Xnr3* gene mediated the negative interaction.

The wnt-inducibility of the *Xnr3* promoter has been studied previously. A 294 bp DNA region adjacent to the transcriptional start site of the gene was shown to direct wnt-inducible transcription, and wnt-responsive sequence elements were identified within this promoter region. Two of these elements bound LEF-1/TCF proteins (one element was a high affinity site, one a low affinity site). A third element did not bind these proteins, but contains a homeodomain-binding consensus sequence (McKendry *et al.*, 1997). Since the homeodomain protein siamois is an inducer of *Xnr3* (Kessler 1997), and siamois is itself a direct target of the wnt pathway (Carnac *et al.*, 1996; Brannon and Kimelman 1996)), it is possible that siamois and/or related proteins act through this third element.

We cloned the 294 bp *Xnr3* promoter fragment upstream of a luciferase reporter gene to create a *Xnr3::luciferase* promoter-reporter plasmid construct. Plasmid DNA was injected into 1 cell embryos near the animal pole, coinjected together with *Xwnt8* mRNA alone, or with *VegT* mRNA. Injected embryos were allowed to develop until stage 11 and harvested to assay luciferase activity (Fig. 2B). Embryos receiving the *Xwnt8* mRNA expressed about 20-fold more luciferase than those without *Xwnt8* mRNA, consistent with previous findings (McKendry *et al.*, 1997). When *VegT* mRNA was coinjected into these embryos in addition to the plasmid DNA and *Xwnt8* mRNA, the luciferase activity was similar to the uninduced level obtained with plasmid DNA alone, indicating an inhibition of wnt-induction. Thus the reporter assays confirmed the inhibitory effect of VegT upon wnt-induction of *Xnr3*, and showed that the effect is mediated by DNA sequences within the 294 bp proximal promoter region.

Effects of Disrupting Signalling Pathways upon Xnr *Gene Expression in Whole Embryos*

The animal cap experiments described above displayed a number of interactions between signalling pathways that are known to be active in establishing and patterning the mesoderm and endoderm. In order systematically to examine the roles of the signalling pathways in inducing *Xnr* gene expression in whole embryos, we have exploited a dominant negative derivative (Molenaar *et al.*, 1996) of Xtcf3, dnXTcf3, which disrupts the function of the β-catenin/Tcf signalling pathway. To disrupt activin signalling we have used the truncated ActRIIB receptor construct, tAR (Hemmati-Brivanlou and Melton, 1992). This dominant negative activin receptor has broad specificity, inhibiting signalling by a number of TGFβ family members including, activin, Vg1, and all the Xnrs excluding Xnr3 (Kessler and Melton, 1994; Schulte-Merker *et al.*, 1994, Clements *et al.*, 1999; Takahashi *et al.,* 2000)

The roles of maternal VegT action in inducing *Xnr* genes have been comprehensively examined by others in loss-of-function experiments using the oligonucleotide-directed mRNA depletion technique (Kofron *et al.*, 1999; Xanthos *et al.*, 2001), and have not been pursued by further loss-of-function experiments here.

The effectiveness of each of the dominant negative constructs, dnXtcf and tAR, was first established by injecting their synthetic mRNAs into animal caps and assessing their ability to prevent the action of co-injected Xwnt8 mRNA, or activin mRNA (Fig. 3A). As expected dnXtcf almost completely abolished the induction of *Xnr3* by Xwnt8, and tAR abolished or greatly reduced the induction of *Xnr1, 2,* and *4,* by activin.

The effects of the dominant negative constructs were then examined in whole embryos, at stage 9, following a vegetally-placed microinjection of their synthetic mRNAs into embryos at the 1-cell stage. Effects of the tAR were remarkably limited, the most substantial being a partial reduction in *Xnr1* and *Xnr2* expression (Fig. 3A). Effects on the other *Xnrs* were minor. As expected from previous work there was no reduction in expression of *Xnr5* or *Xnr6* (Takahashi *et al.*, 2000). In the case of *Xnr6* this lack of effect of tAR initially seemed surprising in view of our demonstration of the large synergistic contribution of activin to *Xnr6* induction, described above, that was observed when activin was combined with VegT and Xwnt8 in

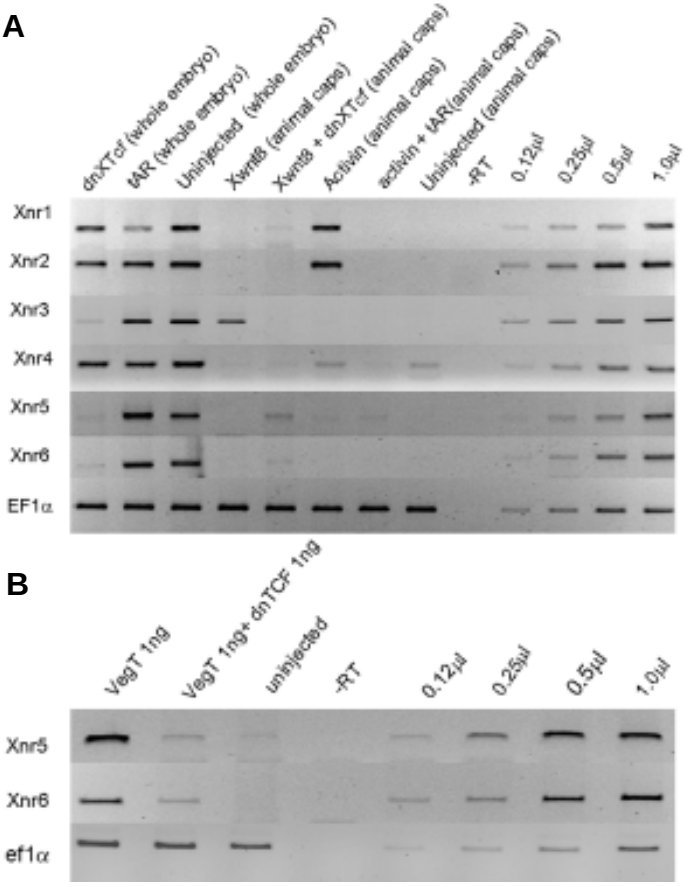


Fig. 3. Effect of dominant negative XTcf3 and a truncated activin receptor, tAR on *Xnr* gene expression. (A) *The effectiveness of the dominant negative constructs was established in animal cap assays. Dominant negative* XTcf3 *mRNA (500 pg) was coinjected with* Xwnt8 *mRNA (10 pg); and* tAR *mRNA (1 ng), with* activin *mRNA (13 pg). Animal caps were harvested at stage 9 for RT-PCR analysis. Dominant negative XTcf3 reduced the level of* Xnr3 *induction by Xwnt8. The induction of* Xnr1, 2 *and* 4 *by activin was suppressed by tAR. To test the effects in whole embryos, one-cell* Xenopus *embryos were injected vegetally with dominant negative* XTcf3 *mRNA (500 pg) or* tAR *mRNA (1 ng). Whole embryos were harvested at stage 9 for analysis by RT-PCR. In whole embryos,* Xnr1 *expression was reduced by tAR.* Xnr2 *and* 4 *expression showed minor reduction. Dominant negative XTcf3 substantially reduced* Xnr3, 5, *and* 6 *expression.* **(B)** *In animal caps, VegT induction of* Xnr5 *and* Xnr6 *is dependent on β-catenin activity in the cap. One cell stage* Xenopus *embryos were injected with* VegT *mRNA (1 ng), and half the embryos were subsequently injected with dominant negative* Xtcf3 *mRNA (1 ng). Animal caps were harvested at stage 9 for RT-PCR analysis. Dominant negative XTcf3 substantially reduced the induction of* Xnr5 *and* 6 *by VegT.*

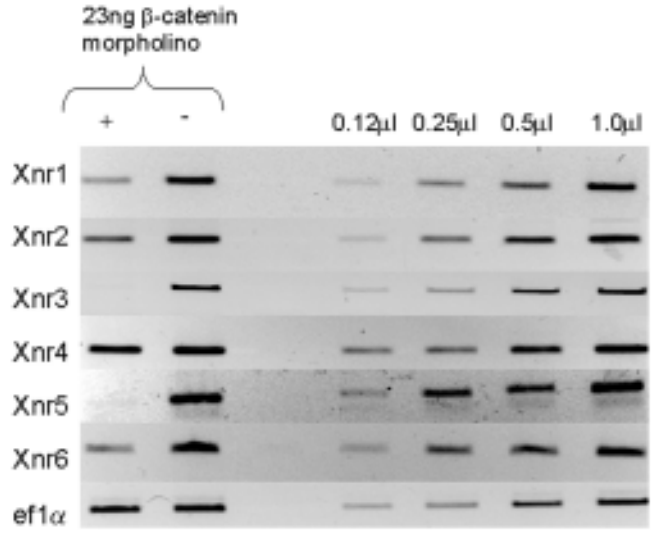
animal cap explants, analysed at stage 9. Thus, given (i) the presence of VegT protein throughout the vegetal region of blastula embryos (Stennard *et al.,* 1999), and (ii) an active dorsally-localised β-catenin pathway within the vegetal region, where (iii) activin-like TGFβ family members are also thought to be present (Weeks and Melton, 1987 Fukui *et al*., 1994; Oda *et al*., 1995), it might be expected that tAR would eliminate any synergistic contribution of the activin-like signalling to *Xnr6* expression. However, immunodetection of Smad2 phosphorylation (Lee *et al*., 2001), reveals that activin-like signalling is not detectable before stage 9. Therefore there is little or no temporal overlap between activin-like signalling and other pathways before stage 9, and hence little opportunity for synergy to occur at this stage.

Consistent with expectations from previous work (Smith *et al*., 1995; McKendry *et al*., 1997), dnXtcf caused a loss of *Xnr3* expression in whole embryos (Fig. 3A). The dnXtcf also caused an almost complete loss of *Xnr5* and *6* expression, despite the fact that these two genes are not induced by Xwnt8 in animal cap explants. We concluded from the animal cap experiments that Xwnt8 can act synergistically with VegT in inducing *Xnr5* and *6.* The effect of dnXtcf in whole embryos leads us to conclude further that the β-catenin/Tcf pathway is absolutely necessary for expression of *Xnr5* and *6* in whole embryos. Since Xwnt8 alone cannot induce *Xnr5* or *Xnr6*, the role of Xwnt8 can be described as permissive for the induction of these two *Xnrs*. This conclusion that wnt activity is necessary prompts the question: How does the induction of *Xnr5* and *Xnr6* occur at all in animal caps injected singly with *VegT* mRNA? We reason that induction of these two genes in animal caps is indeed dependent upon β-catenin activity, and that activation of the β-catenin pathway is another consequence of the VegT expression in the caps. It is known that VegT induces Xwnt8 (Zhang and King, 1996). To confirm the deduction that the induction of *Xnr5* and *Xnr6* by VegT in animal caps is dependent upon β-catenin activity, we examined the effect of dnXtcf upon the induction of the *Xnrs* by VegT in animal caps. As shown in Fig. 3B, our deduction is confirmed by the failure of VegT to induce the two *Xnrs* in the presence of the inhibitor of the β-catenin pathway.

The conclusion that VegT activates β-catenin/Tcf pathway in animal cap explants raises a further question: Xwnt8 and activation of the β-catenin/Tcf pathway robustly induce *Xnr3,* so why does VegT not lead to the induction of *Xnr3* in animal caps? This question is answered by the observation, described above for the pairwise combination of *VegT* and *Xwnt8* mRNAs, that VegT has a striking negative effect upon the induction of *Xnr3* by Xwnt8. So although the β-catenin/Tcf pathway is activated in the caps, this is not able to induce *Xnr3* in the presence of VegT.

Xnr *Gene Expression in Embryos Depleted of β-Catenin*

As a further approach towards examining the roles of β-catenin signalling in whole embryos, we have exploited morpholino oligonucleotide methodology to inhibit translation of maternal β-catenin mRNA (Heasman *et al*., 2000). Vegetal injection at 1-cell stage of 23ng of antisense morpholino oligonucleotide directed against β-catenin mRNA resulted in embryos which developed with a characteristic lack of dorsal development (not shown). Analysis of *Xnr* gene expression in injected embryos at stage 9 revealed the expected absence of *Xnr3* expression (Fig. 4). Expression of *Xnr5* was also almost abolished, *Xnr1* and *Xnr6* transcripts were substantially reduced (to 12-25 % control values), *Xnr2* partially reduced, and *Xnr4* essentially unaltered. These effects are broadly



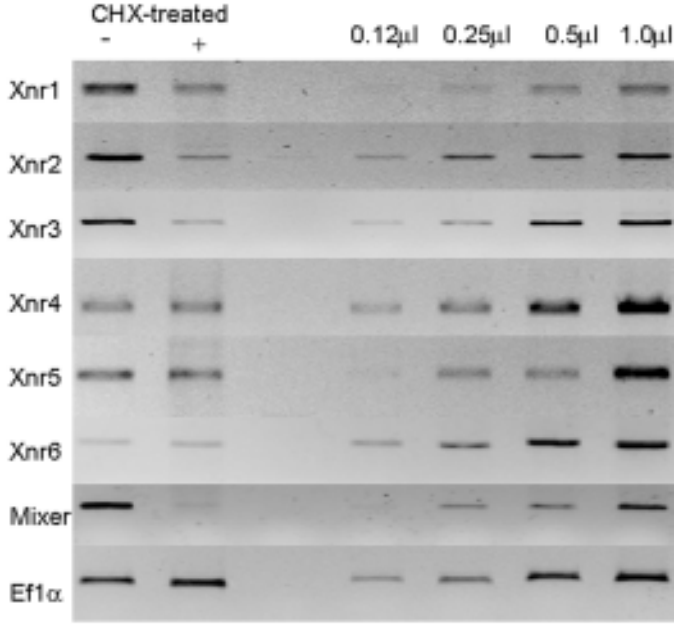


Fig. 4. (Left) Effect of a β-catenin antisense morpholino oligonucleotide on *Xnr* gene expression. *Embryos were injected vegetally at the one-cell stage with a β-catenin morpholino antisense oligonucleotide, and then harvested at stage 9 for RT-PCR analysis.* Xnr3 *and* Xnr5 *expression was abolished;* Xnr1 *and* 6 *were substantially reduced and* Xnr4 *was unaffected by the morpholino.*

Fig. 5. (Right) Effects of inhibiting protein synthesis on expression of *Xnr* genes. *Embryos were treated continuously with cycloheximide (CHX) from stage 7 onwards, and analysed by RT-PCR at stage 9, in parallel with control, untreated embryos.* Mixer *expression is dependent on protein synthesis from zygotic transcripts and was completely lost after CHX treatment.* Xnr1, 2 *and* 3 *gene expression was significantly reduced by CHX treatment, indicating that they require zygotic proteins.* Xnr 4, 5 *and* 6 *expression was unaffected.*

TABLE 1

INDUCING FACTORS AND THEIR INTERACTIONS, FOR INDIVIDUAL *Xnr* GENES IN THE ANIMAL CAP ASSAY

Gene	*Factors which induce the gene in animal cap explants*
Xnr1	VegT; or activin (synergised by β-catenin)
Xnr2	VegT (synergised by β-catenin); or activin (synergised by β-catenin)
Xnr3	β-catenin in absence of VegT (VegT inhibits)
Xnr4	VegT; or activin (synergised by β-catenin)
Xnr5	VegT plus β-catenin (both essential)
Xnr6	VegT plus β-catenin (both essential) (synergised by activin, stage 9)

similar to those observed with dnXtcf, but they are not identical. *Xnr6* was less sensitive than *Xnr5* to the morpholino, an effect not seen with dnXtcf for reasons that may reflect unknown different specificities of the two methods for interfering with β-catenin signalling. Taken together with the previous results, we conclude that β-catenin signalling is required for normal expression of *Xnr5* and *Xnr6*, but not for expression of *Xnr4.*

Effects of Inhibiting Protein Synthesis upon Expression of Xnr *Genes*

We have examined the effect of inhibiting the translation of zygotically expressed mRNAs by treating embryos with cycloheximide continuously from developmental stage 7, and analysing expression of the *Xnr* genes, at stage 9, by RT-PCR. The effectiveness of this treatment was demonstrated (Fig. 5) by the almost complete absence of *Mixer* transcripts, a gene whose expression is known to be dependent upon protein synthesis from zygotic transcripts and subsequent signalling by secreted factors, probably including TGFβ family members (Yasuo and Lemaire, 1999).

In cycloheximide-treated embryos, the amount of expression of *Xnrs 4, 5,* and *6*, was essentially unaffected, whereas expression of *Xnr 1, 2,* and *3* was substantially reduced by about 50%. The effects upon *Xnr1* and *2* are consistent with previous suggestions that the early expression of these two genes is partly dependent upon (zygotic) expression of other *Xnr* genes such as *Xnrs 4*, *5*, and *6* (Clements *et al*., 1999; Kofron *et al*., 1999; Agius *et al*., 2000; Takahashi *et al*., 2000). That expression of the three *Xnr* genes *4*, *5*, and *6* in late blastula embryos is almost totally insensitive to the cycloheximide treatment indicates that their expression is entirely or largely controlled by signalling proteins and/or transcription factors whose synthesis takes place before the onset of zygotic transcription.

Discussion

Combinations of Factors: Conclusions

From our analysis of interactions of maternal signalling pathways, as they affect *Xnr* gene expression, we have made a number of novel observations. The results are relevant to emerging models of the molecular circuitry of germ layer formation and patterning in early *Xenopus* embryos. Understanding of this molecular circuitry is currently at a very basic level with regard to the potential for, and probable importance of, interactions between signalling pathways.

We have shown that β-catenin signalling is a requirement for the induction of *Xnr 5* and *6* by VegT, and we have concluded that β-catenin signalling and activin together have the ability to make a very large synergistic contribution to the early induction of *Xnr6* by VegT in animal cap explants. We have also concluded that VegT prevents the induction of *Xnr3* by wnt signalling, and that this effect is mediated by the proximal promoter region of *Xnr3*. Our conclusions about regulation of the individual *Xnr* genes by combinations of factors are summarised in Table 1. *Xnr3* is induced by wnt signalling in the absence of VegT. The five mesoderm-inducing *Xnr* genes comprise two groups with broadly similar (but not identical) patterns of inducting factors. *Xnrs 1,* 2, and *4*, form one group induced by VegT, or activin synergised by β-catenin, and the other group comprises *Xnrs 5* and *6*, induced by VegT in the presence of β-catenin signalling. While these results are fundamental to understanding *Xnr* gene induction in early embryos, there are many remaining questions. For example the differences in expression patterns of *Xnrs 5* and 6 at gastrulation are unexplained.

In interpreting the role of activin-like signalling in early embryos, it is important to note that it has recently been shown that Smad 2 activation first becomes detectable at stage 9. The activation is first apparent on the dorsal side and then spreads as a wave across the embryo towards the ventral region (Lee *et al*., 2001). It therefore seems likely that the earliest phase of transcription in the embryo, immediately following the mid-blastula transition, occurs in the absence of activin-like signalling which then soon, by the late blastula stage, becomes active dorsally.

Wnt/β-catenin Signalling is Permissive for Xnr5 *and* Xnr6 *Induction by VegT*

In animal caps, we have shown that activation of the wnt signalling pathway occurs in response to VegT expression and that the induction of *Xnrs 5* and *6* in caps is dependent upon both VegT and the induced activation of β-catenin. The results of experiments on whole embryos in which β-catenin mRNA translation or TCF function were perturbed have led us to conclude that wnt is required but not sufficient for *Xnr5* and *6* induction by VegT, extending the previous suggestion that wnt signalling potentiates the induction by VegT (Takahashi *et al*., 2000). There is a growing list of examples of the permissive role for wnt signalling. Examples include *even skipped* and *lethal of scute* expression in the *Drosophila* mesoderm (Carmena *et al*., 1998). It has been suggested that the LEF1/Tcf effectors of wnt signalling play a role similar to other HMG box-containing proteins in establishing a chromatin structure that permits instructive transcription factors to function effectively and stably (reviewed by Sharpe *et al*., 2001).

Immunolocalisation has shown that VegT protein is found in nuclei throughout the vegetal region in early to late blastula embryos (Stennard *et al*., 1999). The requirement for wnt signalling is the probable mechanism for limiting early expression of *Xnr5* and *6* to the dorsal side of the vegetal region of the early embryo.

VegT Inhibits wnt-Mediated Induction of Xnr3

We have found that VegT inhibits the ability of wnt signalling to induce *Xnr3* in animal caps at stage 9, the effect being substantial but less strong at stage 10. It is likely that this effect explains the inability of VegT to induce *Xnr3* in animal caps despite the concomitant activation of the wnt pathway in the caps. It is tempting to speculate that this negative effect of VegT has a natural role in refining the spatial pattern of expression of *Xnr3.* Consistent with this role for VegT, *in situ* hybridisation experiments have shown that at stage 9 *Xnr3* transcripts are located in the dorsal marginal region, including deep cells of this region, but are absent from the vegetal region (Takahashi *et al*., 2000). By stage 10.25 *Xnr3* expression extends

into the dorsal vegetal region close to the vegetal pole (Darras *et al.*, 1997), possibly partly as a result of relaxation of the inhibitory effect of VegT at this stage. What is the mechanism by which VegT effects and wnt-signalling converge upon *Xnr3* transcription? A general effect of VegT upon wnt signalling is ruled out because of the positive synergistic interaction between them for induction of *Xnr5* and *6*, simultaneously with their negative interaction for *Xnr3* induction. For *Xnr3* induction it is known that LEF-1/Tcf effectors are involved (McKendry *et al.*, 1997), and we have shown, by use of a dominant negative Tcf3 construct, that Tcf function is also required for *Xnr5* and *6* induction. Hence the *Xnrs 3*, *5* and *6* genes are induced by the same arm of the wnt signalling pathways and we therefore can exclude a mechanism requiring a differential effect of VegT upon different arms of wnt signalling pathways. We have also excluded an effect upon *siamois* expression. Whatever the mechanism, the effect can be mediated at a 294 bp *Xnr3* promoter region, which has been characterised in some detail previously (McKendry *et al.*, 1997). It is possible that DNA elements that respond to a repressive signal are associated with the gene, in which case they must lie within this proximal promoter region. This is open to further investigation.

A deduction from this negative interaction between VegT and wnt signalling is that *Xnr3* expression should be increased in embryos lacking maternal VegT, in which mesoderm forms ectopically from the vegetal area (Zhang *et al.*, 1998). Kofron *et al.* (1999) have already examined this and found that, contrary to our simple deduction, the amount of *Xnr3* transcript was reduced to about 20% or 60% of normal control values, at stage 10, in VegT mRNA-depleted embryos. Intriguingly, however, the embryos injected with 6ng of antisense VegT oligonucleotide contained the higher amount of *Xnr3* transcript (60% of normal), whereas embryos injected with 5ng of antisense oligonucleotide had the lower amount (20% of normal). It seems likely that the effects of depleting VegT so profoundly disrupt the embryo that it is difficult to interpret quantitative effects upon some downstream genes such as *Xnr3*.

Understanding Xnr *Gene Induction at the DNA Sequence Level*

We would like to be able to relate the regulation of the six *Xnr* genes to their genomic DNA sequences. Limited functional analysis of regulatory elements has been performed previously for VegT regulation of *Xnr1* (Kofron *et al.*, 1999; Hyde and Old 2000), and for TGFβ family members controlling the asymmetric expression of *Xnr1* in post-gastrula embryos (Osada *et al.*, 2000). In the case of *Xnr3* wnt-responsive elements have been identified by functional assays (McKendry *et al.*, 1997). In order to take this further, we have determined sequences from the *Xnr2, 4, 5,* and *6* loci, and extended the known sequences for *Xnr1* (Kofron *et al.*, 1999, Hyde and Old 2000, Osada *et al.*, 2000). These sequences have been submitted to Genbank (Accession numbers: *Xnr1* AF410903, AF410904; *Xnr2* AF410801; *Xnr4* AF410800; *Xnr5* AY050647; *Xnr6* AY050648). Inspection of the sequences shows, perhaps surprisingly, that the obvious similarities between *Xnr1* and *Xnr3* in their proximal promoter regions (Hyde and Old 2000), do not extend simply to these other *Xnr* genes. Further analysis of these promoter regions in the light of conclusions reported here should be revealing.

The Xnr *Gene Family: Different Roles and Regulation*

The six members of the *Xnr* gene family have different expression patterns, and there are some well-documented differences in their roles. Despite this it is not clear why *X. laevis* should have 5 distinct mesoderm inducing *Xnrs* (ignoring pseudotetraploid, A and B copies), especially in view of the single *nodal* gene in the mouse. Experiments reported here using cycloheximide to inhibit translation support the emerging view that among the *Xnr* genes, *Xnrs 4, 5,* and 6 are 'primary' *Xnr* genes whose expression in mid to late blastula embryos is controlled solely by proteins present in the embryo before the mid-blastula transition. By contrast *Xnrs 1* and *2* are substantially, but not completely, controlled by proteins synthesised after the mid-blastula transition. Among the proteins contributing to *Xnr1* and *Xnr2* induction are nodal-related proteins themselves and other TGFβ family members (Clements *et al.*, 1999; Yasuo and Lemaire, 1999). Hence *Xnr1* and *Xnr2* can be termed 'secondary' *Xnr* genes.

Materials and Methods

Embryos, Treatments and Injections

Embryos were cultured and dissected by standard methods, and staged according to Nieuwkoop and Faber (1967). Synthetic mRNA injections were performed at the 1-cell stage using a Drummond microinjector to inject 13.8 nl. For animal cap explants, injection was into the animal pole. During injection, and for subsequent incubation, embryos were maintained in 6% Ficoll in 0.1x Barth's saline and cultured at 13°C or 18°C. Animal caps were dissected at stage 8.5. Where indicated, embryos were treated continuously with cycloheximide at 10 μg/ml in from stage 7 onwards and analysed at stage 9.

Plasmids and Transcriptions for Injection

Capped mRNA was synthesised using the mMessage mMachine (Ambion) kits with templates prepared as described by suppliers. The VegT and activin cDNA plasmids were as described (Clements *et al.,* 1999), and were the kind gift of Dr D. Clements. The Xwnt8 plasmid (Smith and Harland, 1991), and the dominant negative activin receptor ActRIIB (Hemmati-Brivanlou and Melton, 1992) were as described. The dominant negative Tcf3 construct, pcΔNXTcf3, was the gift of Dr. M. Molenaar, (Molenaar *et al.*, 1997).

RT-PCR Analysis

Total RNA was prepared from embryos and explants as described previously (Hyde and Old, 2000). 0.5-1 μg of total RNA was reverse transcribed using Superscript RT (Gibco BRL). All PCRs were carried out with an annealing temperature of 55°C, except for *Xnr*s *3, 5* and *6,* which were carried out at 58°C.

PCR primers used with number of cycles used in brackets:

EF1α	F CAG ATT GGT GCT GGA TAT GC and	
	R CAC TGC CTT GAT GAC TCC TA	(25 cycles);
Xnr1	F GAG AGG CTC AGG TAT GAG and	
	R CTA CTA GCT TTC TCT ATG TC	(31 cycles);
Xnr2	F GAA AAG CAG CTA AGA TCC and	
	R CGA TTG CCC ACT ACA ACA C	(32 cycles);
Xnr3	F GTG CAG TTC CAC AGA ATG AG and	
	R CCA TGG ATC GGC ACA ACA GA	(30 cycles);
Xnr4	F ACT TGG CTG CTC TAC CTC and	
	R CAG CAA GTT GAT GTT CTT CC	(31 cycles);
Xnr5	F TCA CAA TCC TTT CAC TAG GG and	
	R GGA ACC TCT GAA AGG AAG C	(32 cycles);
Xnr6	F TCC AGT ATG ATC CAT CTG TTG C and	
	R TTC TCT GTT CCT CTT GTG CCT T	(32 cycles);
siamois	F ACC CCA CCA GGA TAA ATC TG and	
	R GGT ACT GGT GGC TGG AGA A	(35 cycles).

Luciferase Reporter Gene Assay

The 294 bp region of the *Xnr3* promoter as described (McKendry *et al.*, 1997) was obtained by PCR from genomic *X. laevis* DNA and cloned into the luciferase reporter vector, pGL3Basic (Promega). DNA sequence analysis showed the cloned fragment to be identical in sequence to that published previously. To test for promoter activity one-cell embryos were

injected with 30 pg of *Xnr3* promoter plasmid and 30 pg of pRL-TK (*Renilla* luciferase control promoter-reporter plasmid; Promega). Where indicated embryos were also injected with 1 ng of *Xwnt8* mRNA as a source of Xwnt8 ligand, and with 2 ng of VegT mRNA. Embryos were harvested at stage 11 for luciferase assay as previously described (Hyde and Old, 2000). In each experiment approximately 10 embryos were used to make the cell extract and 3 embryo equivalents were used in each assay. The assays were carried out in duplicate. The amount of *Renilla* luciferase was used to normalise the luciferase values in each experiment.

Morpholino Oligonucleotide

The antisense morpholino oligonucleotide, directed against β-catenin mRNA, was identical to that used previously (Heasman *et al.*, 2000), having the sequence TTT CAA CCG TTT CCA AAG AAC CAG G, and was obtained from Gene Tools Inc.

Acknowledgements

This work was supported by a Biotechnology and Biological Sciences Research Council award to E.H., and by the Wellcome Trust. We are grateful to Hugh Woodland, Debbie Clements, and M. Molenaar for donating plasmids.

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Received: November 2001
Reviewed by Referees: January 2002
Modified by Authors and Accepted for Publication: February 2002