Activin redux: specification of mesodermal pattern in *Xenopus* by graded concentrations of endogenous activin B

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Summary

Mesoderm formation in the amphibian embryo occurs through an inductive interaction in which cells of the vegetal hemisphere of the embryo act on overlying equatorial cells. The first candidate mesoderm-inducing factor to be identified was activin, a member of the transforming growth factor type β family, and it is now clear that members of this family are indeed involved in mesoderm and endoderm formation. In particular, *Derrière* and five *nodal-related* genes are all considered to be strong candidates for endogenous mesoderm-inducing agents. Here, we show that activin, the function of which in mesoderm induction has hitherto been unclear, also plays a role in mesoderm formation. Inhibition of activin function using antisense morpholino oligonucleotides interferes with mesoderm formation in a concentration-dependent manner and also changes the expression levels of other inducing agents such as *Xnr2* and *Derrière*. This work reinstates activin as a key player in mesodermal patterning. It also emphasises the importance of checking for polymorphisms in the 5′ untranslated region of the gene of interest when carrying out antisense morpholino experiments in *Xenopus laevis*.

Key words: *Xenopus*, Mesoderm induction, TGFβ family, Activin

Introduction

The mesoderm of the amphibian embryo is formed through an inductive interaction in which cells of the vegetal hemisphere of the embryo act on overlying equatorial cells. The first candidate mesoderm-inducing factor to be identified was activin (Asashima et al., 1990; Smith et al., 1990), a member of the transforming growth factor type β family. The significance of activin as an endogenous inducing agent has been emphasised by the facts that (1) use of a dominant-negative activin receptor disrupts mesoderm formation in *Xenopus* (Dyson and Gurdon, 1997; Hemmati-Brivanlou and Melton, 1992; New et al., 1997), (2) activin can exert long-range effects in embryonic tissue (Gurdon et al., 1994; Gurdon et al., 1995; Jones et al., 1996; McDowell et al., 1997) and (3) it can activate different genes at different concentrations (Green et al., 1990; Green et al., 1992; Green and Smith, 1990; Green et al., 1994; Gurdon et al., 1994; Gurdon et al., 1995; Papin and Smith, 2000). Together, these observations suggested that activin might act as a morphogen in the developing embryo.

The role of activin in the early embryo has, however, remained unclear because other candidate inducing factors have been isolated, including *Vg1* (Thomsen and Melton, 1993; Weeks and Melton, 1987), the *nodal-related* genes (Jones et al., 1995; Joseph and Melton, 1997; Onuma et al., 2002; Takahashi et al., 2000) and *Derrière* (Sun et al., 1999; White et al., 2002), and because attempts to inhibit its function in a specific manner have produced contradictory results. It is not clear, for example, whether the activin-binding protein follistatin does (Marchant et al., 1998) or does not (Schulte-Merker et al., 1994) inhibit mesoderm formation. The most recent view on the role of activin in early *Xenopus* development has been articulated by Green, who says ‘although activin was and still is an excellent model for morphogen action, it may not be important in early vertebrate patterning’ (Green, 2002).

In this paper, we first reinvestigate the temporal expression pattern of activin B and demonstrate that zygotic expression of *activin B* precedes that of one of its putative target genes, *Xbra* (Smith et al., 1991). We then use antisense morpholino oligonucleotides to inhibit the function of activin B in the early *Xenopus* embryo. Our results indicate that activin B is required for normal mesoderm formation in *Xenopus* in a concentration-dependent manner. We also demonstrate, serendipitously, that in performing antisense experiments of this sort in *Xenopus* one must beware of polymorphisms in the 5′ untranslated region of the gene(s) of interest.

Materials and methods

Xenopus embryos and microinjection

Xenopus laevis embryos were obtained by artificial fertilisation and maintained in 10% MMR. They were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Embryos at the one- or two-cell stage were injected with RNA dissolved in water or with morpholino oligonucleotides dissolved in 10% MMR. Embryos were cultured in 10% MMR and isolated animal pole regions were cultured in 100% MMR. Partially purified activin was used at a concentration

Table 1. Primers and conditions used for real-time RT-PCR

Gene	Reference	Primer sequences (upstream/downstream)	Melting temperature (°C)	Annealing temperature (°C)/time (seconds)	Extension temperature (°C)/time (seconds)	Acquisition temperatures (°C)/time (seconds)
Activin B	Kofron et al. (1999)	5′ CAACCTGTGGCTGTACCTGAAG 3′ 5′ GCACTCGAGGCCTCTCTTACGGA 3′	95	55/5	72/14	86/3
Cerberus	Darras et al. (1997)	5′ GCTTGCAAAACCTTGCCCTT 3′ 5′ CTGATGGAACAGAGATCTTG 3′	95	60/5	72/20	81/3
Chordin	Kofron et al. (1999)	5′ AACTGCCAGGACTGGATGGT 3′ 5′ GGCAGGATTTAGAGTTGCTTC 3′	95	55/5	72/12	81/3
Der	Sun et al. (1999)	5′ TGGCAGAGTTGTGGCTATCA 3′ 5′ CTATGGCTGCTATGGTTCCTT 3′	95	55/5	72/18	82/3
Gsc	This paper	5′ TGGCAAGGAGGGTTCATCTCAGAG 3′ 5′ ATCCAGCTATCCCAATGTGCAAGT 3′	95	58/5	72/8	78/3
ODC	Heasman et al. (2000)	5′ GCCATTGTGAAGACTCTCTCCAATC 3′ 5′ TTCGGGTGATTCCTTGCCAC 3′	95	55/5	72/12	82/3
Xbra	Kofron et al. (1999)	5′ TTCTGAAGGTGAGCATGTCG 3′ 5′ GTTTGACTTTGCTAAAAGAGACAGG 3′	95	55/5	72/8	75/3
Xhex	Chang and Hemmati-Brivanlou (2000)	5′ AACAGCGCATCTAATGGGAC 3′ 5′ CCTTTCCGCTTGTGCAGAGG 3′	95	60/5	72/13	87/3
Xnot	This paper	5′ ATACATGGTTGGCACTGA 3′ 5′ CTCCTACAGTTCCACATC 3′	95	50/5	72/8	72/3
Xnr1	Kofron et al. (1999)	5′ TGGCCAGATAGAGTAGAG 3′ 5′ TCCAACGGTTCTCACTTT 3′	95	55/5	72/12	81/3
Xnr2	Kofron et al. (1999)	5′ GTCTTCTATATCCAGCAGCAAT 3′ 5′ TTGATGGAGATAATACTGGAGC 3′	95	55/5	72/11	81/3
Xnr4	Kofron et al. (1999)	5′ ACTTGGCTGCTCTACCTC 3′ 5′ CAGCAAGTTGATGTTCTTCC 3′	95	55/5	72/12	82/3
Xnr5	This paper	5′ TCCATTGTTACTCCAGGTTCC 3′ 5′ AAGCCGCTTCTTATGATGC 3′	95	55/5	72/12	81/3
Xnr6	This paper	5′ CAATGAGTTGAATTTGGCTGAG 3′ 5′ GTTTGTCTTTAGCGAACACCAC 3′	95	55/5	72/12	81/3
Xvent1	This paper	5′ TGGTTCAACAGGGATTCTC 3′ 5′ CTGCTAAGGAAGGATTTGC 3′	95	54/5	72/8	80/3
Xwnt8	Ding et al. (1998)	5′ CTGATGCCTTCAGTTCTGTGG 3′ 5′ CTACCTGTTTGCATTGCTCGC 3′	95	58/6	72/14	85/3

of 16 U ml^{-1} (Cooke et al., 1987) in the presence of 0.1 mg ml^{-1} bovine serum albumin.

Antisense morpholino oligonucleotides

These were purchased from GeneTools. Sequences were as follows: MO1, 5′-GCAGAGGCAGTAACAGGAGAGCCAT-3′; mMO1, 5′-GCAGACGCACTAACATGAGAACCAT-3′; MO2, 5′-CCCGGCG-AGGGTCTCCGAGCGGAAA-3′; MO3; 5′-CGAGGGTCTCCAAG-CGGAGAGAGAA-3′.

The gross phenotypes obtained with these antisense morpholino oligonucleotides, described in Figs 3A-C and 6A-D, were observed in experiments involving at least 100 embryos for each morpholino.

Expression constructs and transcription

Expression constructs were generated by subcloning cDNAs between the *Eco*RI and *Xba*I sites of pCS2+ (Rupp et al., 1994; Turner and Weintraub, 1994). Standard techniques were employed and C-terminal HA tags were introduced into all constructs via the downstream PCR primer. *Xenopus activin B* was cloned using the primers 5′-AGCTGAATTCCGGACCCAGCGCCGCTCC-3′ and 5′-ACGTTCTAGATCAAGCGTAATCCGGGACATCGTACGGGTATG-CACAGCCGCACTCGTCC-3′. *Xenopus Follistatin* (Hemmati-Brivanlou et al., 1994) was cloned using the primers 5′-AGC-TGAATTCCCCACTCCCAGCACTGAGG-3′ and 5′-ACGTTCTAG-ATCAAGCGTAATCCGGGACATCGTACGGGTACTTACAGTTGC-AAGATCCA-3′. Site-directed mutagenesis of the pCS2+.*Xenopus activin B* construct (see Results) used the primer 5′-CCCAGCG-CCGCTCCATGGTTCTCATGTTAGTGCGTCTGCTTCTGGCCGG-ACTC-3′ and its reverse complement. A construct comprising the Smad-binding domain (SBD) and the FYVE domain of SARA (Tsukazaki et al., 1998), together with an HA tag, was cloned into pCS2+ and used as a loading control in Fig. 2C. This construct was created using the primers 5′-GTGACTAGTTGTAGTTACGTTTG-CATTATCTG-3′ and 5′-ACCTCTAGACTAGGGGAAGTGGCTC-CAGTCTG-3′.

Transcription of mRNA was carried out as described (Smith, 1993). Plasmids were linearised with *Not*I.

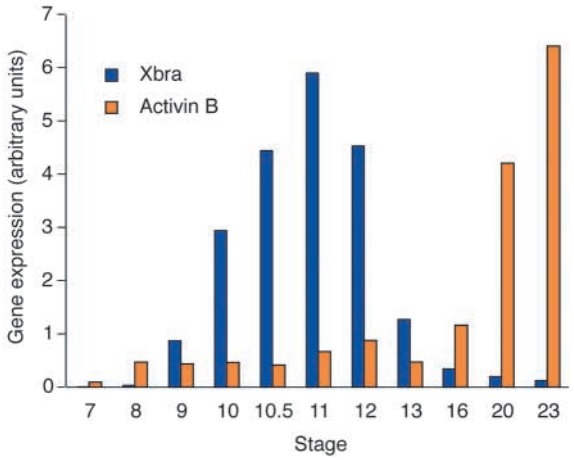

Fig. 1. The temporal expression patterns of *activin B* and *Xbra* studied by real-time RT-PCR. Levels of gene expression are normalised to those of *ornithine decarboxylase*. Activation of *activin B* precedes that of *Xbra*.

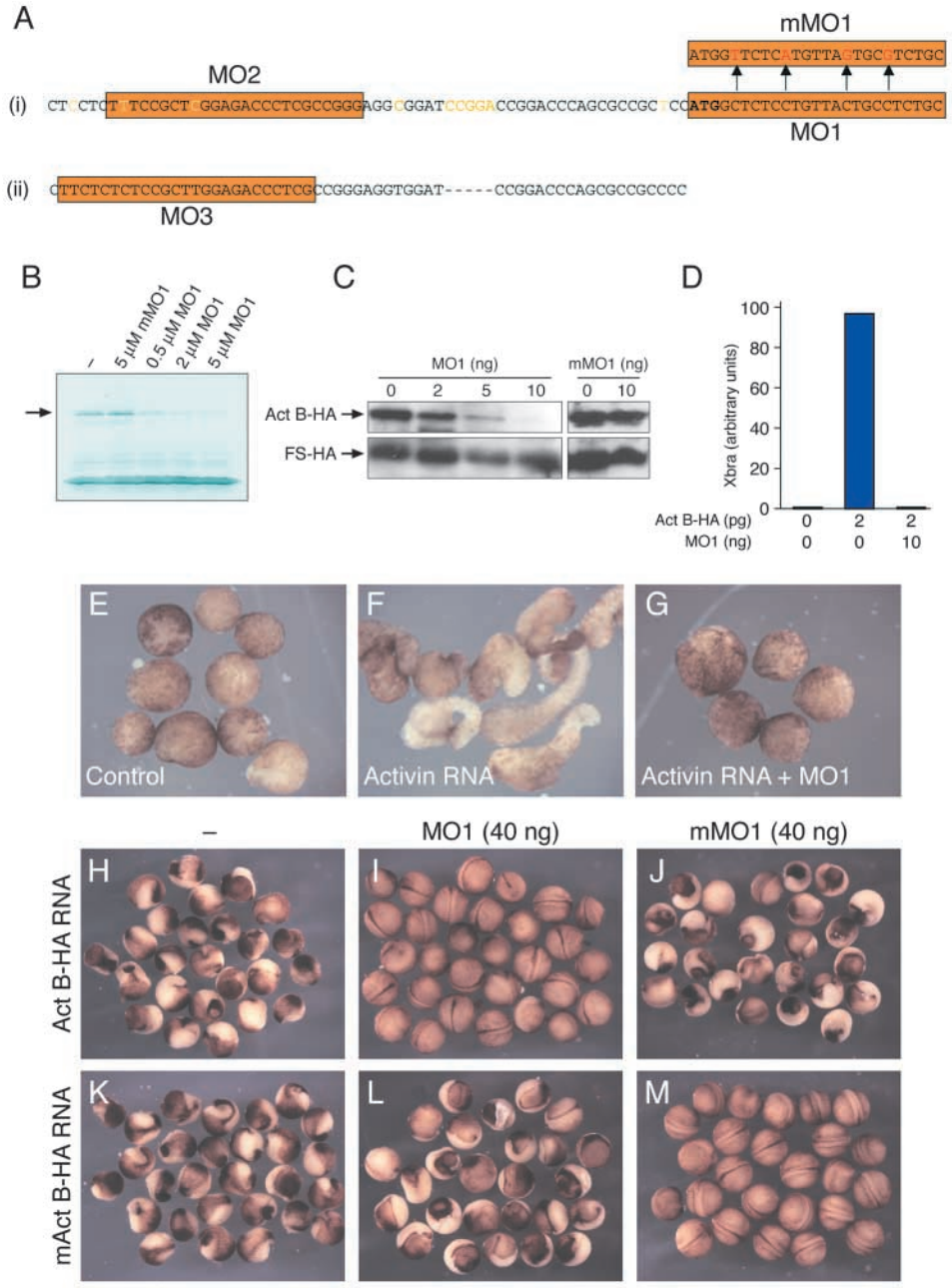

Fig. 2. Design and verification of activin B antisense morpholino oligonucleotides. (A) Sequence of the 5′ untranslated region of *activin B* derived from GenBank (i) and derived from the *Xenopus* colony at the Wellcome Trust/Cancer Research UK Gurdon Institute (ii). Differences between the two sequences are highlighted and the sequences targeted by the antisense morpholino oligonucleotides used in this paper are boxed. (B) MO1 inhibits in vitro translation of activin B. Arrow indicates activin B. mMO1 was included in one reaction at a final concentration of 5 μM and MO1 was included at 0.5, 2.0 and 5.0 μM. – indicates no addition of morpholino oligonucleotide. (C) MO1 inhibits, in a dose-dependent fashion, translation of RNA encoding HA-tagged activin B following injection into *Xenopus* embryos. RNA encoding activin B-HA, together with RNA encoding an HA-tagged version of the FYVE and SBD domains of SARA (see Materials and methods), was injected into *Xenopus* embryos at the one-cell stage in the absence of MO1 or at the indicated concentrations of morpholino. Embryos were cultured to early gastrula stage 10 and subjected to western blotting using an anti-HA antibody. Activin B-HA and FYVE/SBD-HA are indicated by arrows. Inhibition of activin B translation is not observed with mMO1. (D) MO1 prevents activin-induced expression of *Xbra* in *Xenopus* animal caps. *Xenopus* embryos were injected with the indicated combinations of activin and MO1. They were cultured to early gastrula stage 10.5 and assayed for expression of *Xbra* by real-time RT-PCR. (E-G) MO1 inhibits activin-induced elongation of animal caps. Animal pole regions were derived from uninjected embryos (E) or embryos injected with RNA (5 pg) encoding activin B in the absence (F) or the presence (G) of 40 ng MO1. MO1 inhibits the elongation of animal pole regions (G). (H-M) MO1, but not mMO1, inhibits the function of exogenous activin in intact *Xenopus* embryos; mMO1 but not MO1 inhibits the function of a mutated form of activin in which the sequence has been mutated to match that of mMO1. Embryos were injected with wild-type activin (H-J; Activin B-HA; 10 pg) or mutated activin (K-M; mActivin B-HA; 10 pg) in the absence of morpholino oligonucleotides or in the presence of MO1 (40 ng) or mMO1 (40 ng).

Whole-mount antibody staining

Whole-mount antibody staining using the monoclonal antibodies MZ15 (Smith and Watt, 1985) and 12/101 (Kintner and Brockes, 1984), specific for notochord and muscle respectively, was carried out as described (Smith, 1993).

Whole-mount in situ hybridisation

In situ hybridisation was carried out essentially as described (Harland, 1994), except that BM purple was used as a substrate. Probes used were for *Chordin* (Sasai et al., 1994), *Derrière* (Sun et al., 1999), *Goosecoid* (Cho et al., 1991), *Xbra* (Smith et al., 1991), *Xnot* (von Dassow et al., 1993), *Xnr2* (Jones et al., 1995), *Xvent-1* (Gawantka et al., 1995) and *Xwnt-8* (Christian et al., 1991; Smith and Harland, 1991). The open reading frame of Xnr2 was amplified using the polymerase chain reaction and the primers 5′-TCTGAATTC-ATGGCAAGCCTAGGAGTCATC-3′ and 5′-ATTTCTAGAGTTAC-ATCCACACTCATCCAC-3′. It was cloned into pCS2+, linearised with *Eco*RI and transcribed with T7 RNA polymerase. Each experiment shown was carried at least twice, with at least 20 embryos per treatment.

RNA isolation and real-time RT-PCR

Total RNA was prepared from five pooled embryos using the TriPure reagent (Roche), followed by DNAseI digestion, proteinaseK

treatment, phenol/chloroform extraction and ethanol precipitation. RNA was dissolved in water and used as a template for real-time RT-PCR. Reactions were performed in the LightCycler instrument (Roche) using a SYBR GreenI-based one-step RNA amplification kit (Roche). A standard curve was generated from diluted RNA derived from control embryos and experimental results were quantified accordingly. PCR primers are listed in Table 1. Each experiment was carried out at least twice and usually three times.

Cloning the 5′ untranslated region of activin B

The 5′ untranslated region of *Xenopus activin B* was cloned by reverse transcription of total RNA isolated from five stage 12 sibling embryos followed by PCR amplification. The primers used were 5′-CGACACTGGCAGCACCTTC-3′ and 5′-GGCAGTAACAGGAGAGCCATG-3′.

Results

Expression of activin in the *Xenopus* embryo

If activin were to play a role in mesoderm induction in *Xenopus*, its expression should precede that of genes such as *Xbra*, which is expressed in presumptive mesoderm and which can be activated in animal pole tissue in response to mesoderm induction (Smith et al., 1991). To investigate this issue, RNA was extracted from *Xenopus* embryos between stage 7 (early blastula) and stage 23 (by which time the neural tube has closed). Samples were assayed by real-time RT-PCR for expression of *activin B* and *Xbra* and, as a reference gene, *ornithine decarboxylase*. Zygotic expression of activin, which is known to occur shortly after the mid-blastula transition (Clements et al., 1999; Dohrmann et al., 1993; Thomsen et al., 1990), preceded that of *Xbra*, consistent with the idea that *activin B* functions as an endogenous mesoderm-inducing agent (Fig. 1).

Inhibition of activin B translation

To investigate the role of activin B in early *Xenopus* development, we designed an antisense morpholino oligonucleotide that recognises the first 25 nucleotides of the open reading frame (MO1; Fig. 2A). A control morpholino oligonucleotide (mMO1) contained four evenly spaced base changes. The ability of the specific morpholino oligonucleotide to inhibit activin B translation in a dose-dependent manner was confirmed in an in vitro translation reaction, in which the mutated oligonucleotide had no effect (Fig. 2B). The specific morpholino oligonucleotide also inhibited translation in a dose-dependent manner of a tagged form of activin B following injection of mRNA into *Xenopus* embryos (Fig. 2C), and it inhibited activin-induced expression of *Xbra* in animal caps (Fig. 2D). The specific morpholino oligonucleotide also inhibited animal cap elongation in response to activin B (Fig. 2E-G) and it prevented the disruption of development that is caused by widespread expression of activin B in the embryo (Fig. 2H,I), in which assay the mutated oligonucleotide mMO1 had no effect (Fig. 2J).

The results that follow indicate that the mutated morpholino oligonucleotide mMO1 has no effect on *Xenopus* development. To confirm that the oligonucleotide is capable, given the opportunity, of inhibiting translation, we changed four nucleotides in the open reading frame of activin B to match the sequence of the mutated morpholino oligonucleotide. Mis-expression of the mutated activin B was still capable of

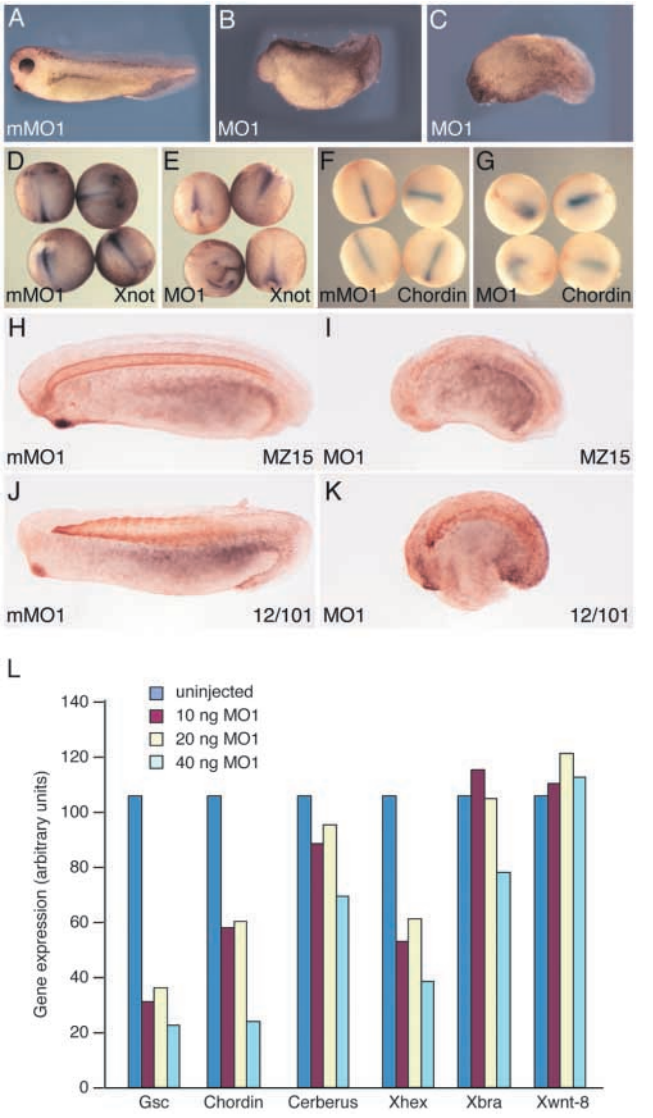

Fig. 3. MO1 disrupts axial development in *Xenopus* and exerts dose-dependent effects on gene expression in the early gastrula. (A-C) *Xenopus* embryos were injected at the one-cell stage with mMO1 (A; 40 ng) or MO1 (B,C; 40 ng) and allowed to develop to tadpole stage 33. MO1 causes axial defects and a disruption of anterior development. (D-G) The phenotype illustrated in B,C is presaged by disruption of expression of *Xnot* (D,E) and *Chordin* (F,G). *Xnot* expression persists around the blastopore of embryos injected with MO1. (E) Expression of *Chordin* in embryos injected with MO1 (F) is more diffuse than that in embryos injected with mMO1 (G). (H-K) Although axial morphogenesis is disrupted in embryos injected with antisense morpholino oligonucleotide MO1, notochord and muscle do nevertheless form in such embryos. (H,J) Embryos injected with mMO1 stained with monoclonal antibody MZ15 (H) or 12/101 (J). (I,K) Embryos injected with MO1 stained with MZ15 (I) or 12/101 (K). (L) Embryos were injected with the indicated amounts of MO1 and allowed to develop to early gastrula stage 10.5, when gene expression was assessed by real-time RT-PCR. Levels of gene expression are normalised to those of *ornithine decarboxylase*. Increasing concentrations of MO1 cause the downregulation first of dorsally expressed genes such as *Goosecoid* and *chordin* and then the downregulation of *Xbra*, which is expressed throughout the marginal zone. Expression of *Xwnt8*, which occurs in lateral and ventral tissue, is little affected.

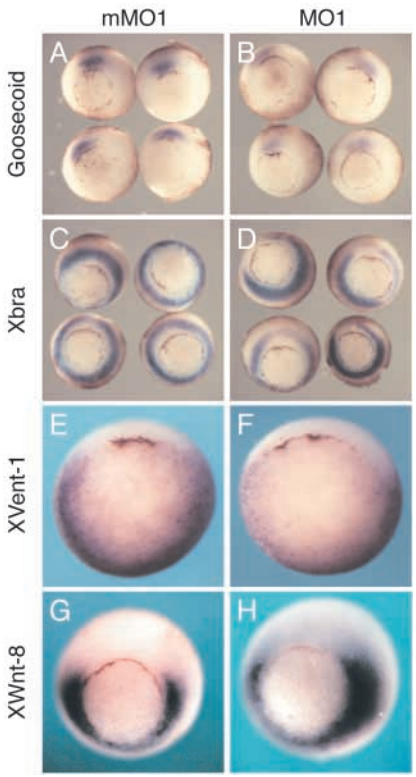

Fig. 4. Gene expression patterns in embryos injected with MO1. (A,B) Expression of *Goosecoid* is downregulated in response to MO1 (B), but its expression domain is unaltered. (C,D) Downregulation of *Xbra* in response to MO1 (D). See text for details. Embryos in A-D were fixed at similar morphological stages rather than at different times after fertilisation to compensate for slight delays in development. (E-H) The expression domains of *Xvent-1* (E,F) and *Xwnt-8* (G,H) are unaffected by MO1, even though their expression levels are very slightly (*Xwnt-8*; Fig. 3) or significantly (*Xvent-1*; Fig. 6) elevated. Embryos were injected at the two-cell stage, with 20 ng MO1 into each blastomere.

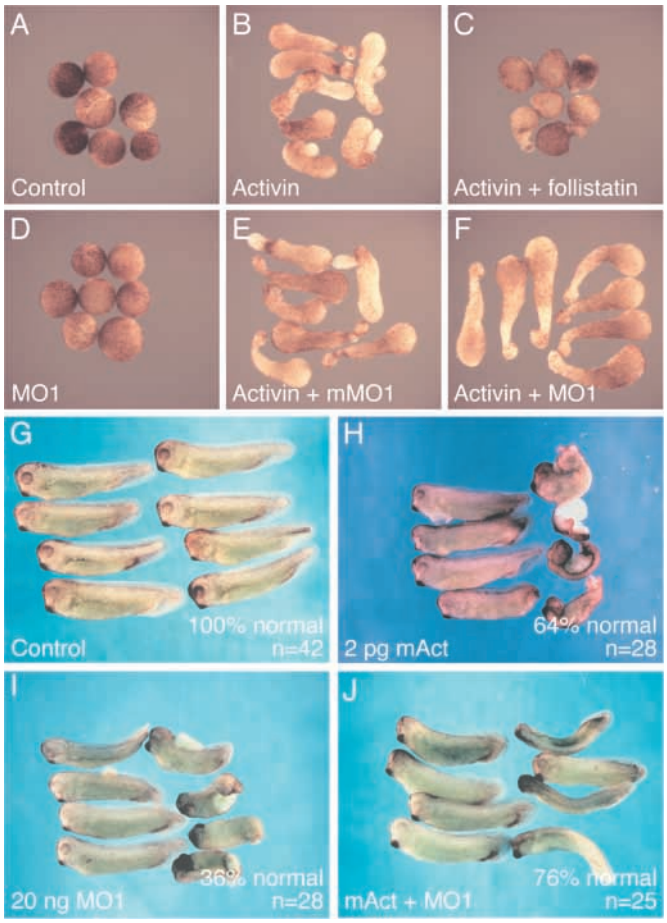

Fig. 5. Antisense morpholino oligonucleotide MO1 does not affect the activin signal transduction pathway (A-F). Exogenous activin B can 'rescue' the effects of MO1 (G-J). (A-F) Animal pole regions derived from uninjected *Xenopus* embryos or from those injected with MO1 (40 ng) form spheres (A,D), while those treated with activin (16 U ml^{-1}) elongate (B). Elongation is substantially inhibited in animal caps derived from embryos injected with RNA (500 pg) encoding *Xenopus* follistatin (C) but not by 40 ng mMO1 (E) or MO1 (F). (G-H) Thirty-six percent of embryos injected into one cell at the four-cell stage with 2 pg RNA encoding mutated activin B-HA suffer defects in early development (H; compare with normal embryos in G), while 64% of embryos injected with 20 ng MO1 display a 'knockdown' phenotype (I). Co-injection of mutated activin B-HA and MO1 'rescues' development, such that only 24% are abnormal (J).

disrupting *Xenopus* development (Fig. 2K), and this disruption was prevented by the mutated oligonucleotide mMO1 (Fig. 2M) but not by the original version (Fig. 2L). These observations show that the mutated antisense morpholino oligonucleotide is stable and functional.

An antisense morpholino oligonucleotide directed against activin B disrupts *Xenopus* development in a dose-dependent fashion

Xenopus embryos were injected with increasing concentrations of specific or mutated antisense morpholino oligonucleotide and then allowed to develop to tadpole stage 32. The mutated oligonucleotide had no effect on development, but the specific oligonucleotide MO1 caused severe disruption to dorsal axial development, with both head and tail being affected (Fig. 3A-C). Disruption to the dorsal axis was presaged by slow passage through gastrulation (see Fig. 5G-I and Fig. 7H,I), and was confirmed by in situ hybridisation, which showed that expression of *Xnot* (Fig. 3D,E) persists around the closing blastopore and that expression of *chordin* is more diffuse than in control embryos (Fig. 3F,G). These observations suggest that disruption of activin B function may cause a disruption of convergent extension (see Discussion).

Use of the monoclonal antibodies MZ15, which is specific for notochord, and 12/101, which is specific for muscle, demonstrated that these tissues are disrupted in embryos in which activin function is inhibited, although notochord and muscle cells are present (Fig. 3H-K).

To examine the concentration-dependent effects of the antisense morpholino oligonucleotide, and to investigate its effects on early development, we assayed the expression of a panel of genes expressed in mesoderm and mesendoderm using RT-PCR (Fig. 3L). Dorsally expressed genes such as *Goosecoid* (Cho et al., 1991), *chordin* (Sasai et al., 1994) and

Xhex (Jones et al., 1999; Newman et al., 1997) were most severely affected by low concentrations of oligonucleotide, while expression of the pan-mesodermal marker *Xbra* was reduced only by the highest concentration. These observations are consistent with the suggestion that patterning of the mesoderm in the *Xenopus* embryo occurs in response to different concentrations of activin (Green et al., 1992).

In situ hybridisation confirmed that expression of *Goosecoid* and *Xbra* is decreased in embryos in which activin B function is inhibited. We note that downregulation of *Goosecoid* is not accompanied by a significant restriction of its expression domain but by a general decrease in levels of transcription (Fig. 4A,B), whereas downregulation of *Xbra* tends to occur not throughout the marginal zone but in a more restricted domain (Fig. 4C,D). This may correspond to the dorsal marginal zone, but it is also possible that it reflects the region where the concentration of MO1 is highest (Fig. 4C,D). The expression domains of other genes, including *Xwnt8* and *Xvent1*, which are expressed laterally and ventrally, are, like that of *Goosecoid*, little affected by the antisense morpholino oligonucleotide (Fig. 4E-H), suggesting that the upregulation of *Xvent1* observed in Fig. 6 is due to elevated levels of transcription within its normal expression domain.

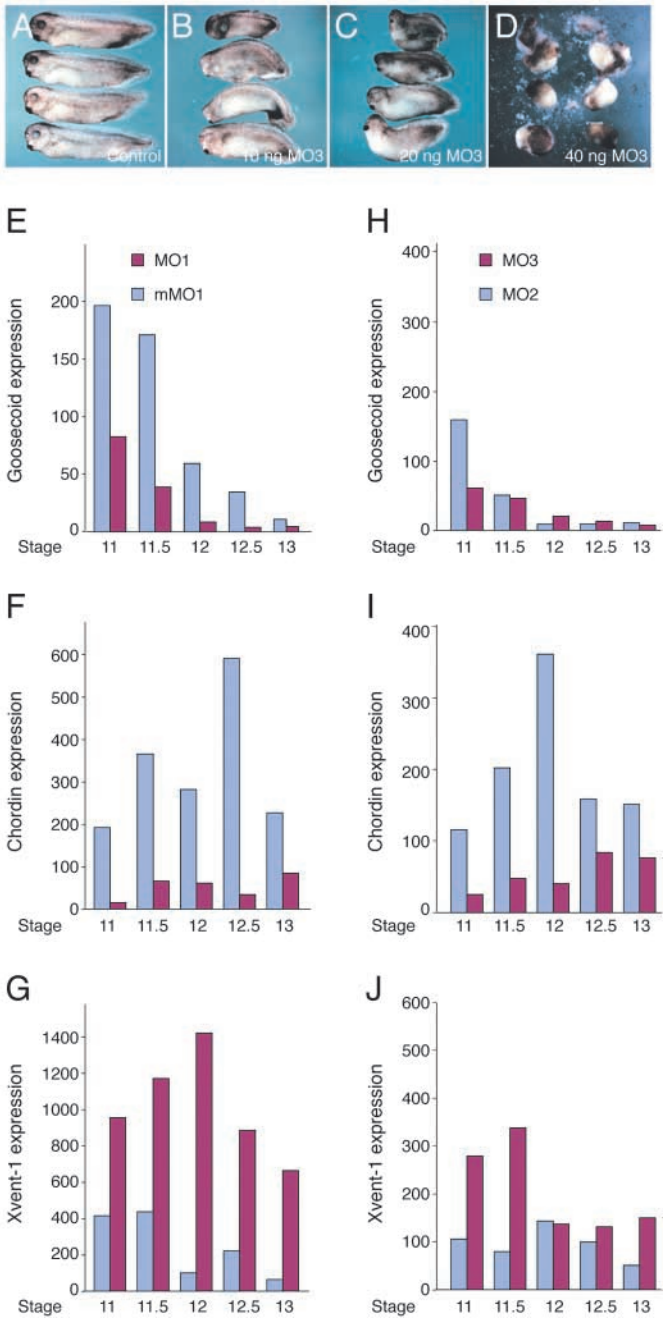

Fig. 6. MO3 causes a phenotype similar to that caused by MO1. (A-D) Embryos injected with increasing concentrations of an alternative activin B antisense morpholino oligonucleotide termed MO3 (B-D) show a similar phenotype to that observed with MO1, although MO3 is effective at lower concentrations (compare C with Fig. 3B). (E-J) Expression of *Goosecoid* and *Chordin* is reduced by both MO1 and MO3, and expression of *Xvent1* is elevated. For MO1, mMO1 was used as a control, and for MO3, MO2 was used as a control. Embryos were analysed at the indicated stages. In this experiment, inhibition of *Goosecoid* expression by MO3 was most marked at stage 11 (H).

Further controls

The results described above suggest that activin B plays a role in the development of the mesoderm in *Xenopus*. Experiments using antisense technology, however, require careful controls; we first asked whether the activin B antisense morpholino oligonucleotide functions, as we would predict, by interfering with activin translation or whether it interferes with the activity of some component of the activin signal transduction pathway. To address this point, animal caps were dissected from control embryos or from embryos injected with the specific morpholino oligonucleotide or the mutated version. The caps were treated with activin protein and induction was assessed by observing their elongation (Symes and Smith, 1987). Activin caused animal caps to elongate (Fig. 5A,B), and this elongation was inhibited by the activin-binding protein follistatin (Fig. 5C). No inhibition was observed with the activin B antisense morpholino oligonucleotide MO1 (Fig. 5F), indicating that the effects of the oligonucleotide on early development are likely to be due to inhibition of activin B translation and not to interference with the activin signal transduction pathway.

We next attempted to ‘rescue’ the MO1 phenotype by co-injecting the mutated form of activin B RNA that is not affected by the antisense oligonucleotide (see Fig. 2K-M). One difficulty with experiments of this sort is that injected RNA diffuses less well than the morpholino itself, so that the distribution of the two will differ (Nutt et al., 2001; Saka and Smith, 2004). Another is that activin causes a severe phenotype on its own, so that one has to inject enough activin RNA to rescue, but not so much so as to cause defects that are due to overexpression. In our attempts to address these concerns, we injected constructs into one cell of the four cell stage embryo rather than into the newly-fertilised egg, and we varied the concentration of injected RNA in an effort to find a dose that would rescue the MO1 phenotype but not cause defects through overexpression. The best results were obtained in an experiment in which 2 pg of mutated activin B-HA RNA was used in conjunction with 20 ng MO1 (Fig. 5G-J). In this experiment, 2 pg activin RNA caused abnormal development in 36% of embryos (n=28; Fig. 5H), and a MO1 phenotype was observed in 64% of embryos (n=28; Fig. 5I).

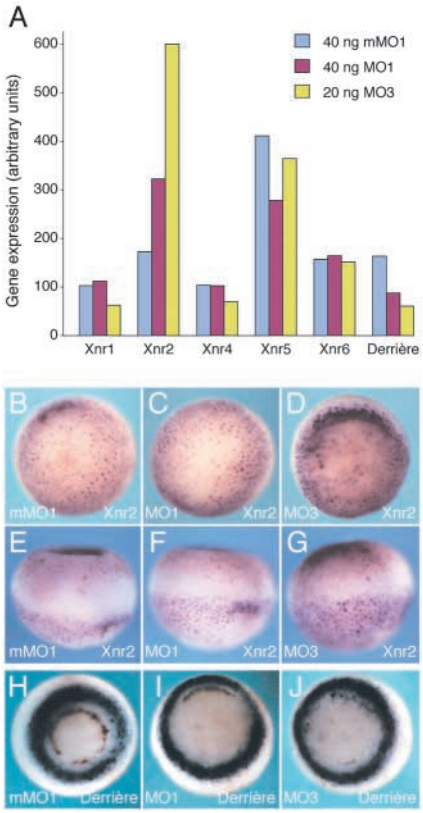

Fig. 7. Inhibition of activin function causes an upregulation of *Xnr2* expression and a downregulation of *Derrière*. (A) Embryos were injected with the indicated antisense morpholino oligonucleotides and allowed to develop to early gastrula stage 10.5, when they were assayed for expression of *nodal-related* genes and *Derrière* by real-time RT PCR. (B-G) Upregulation of *Xnr2* expression is not accompanied by an expansion of its expression domain. Embryos were subjected to in situ hybridisation to detect *Xnr2* RNA and viewed from the vegetal pole (B-D) or from the side (E-G). Transcription of *Xnr2* in embryos injected with MO1 (C,D) or MO3 (F,G) is not observed beyond its normal expression domain. (H-J) Downregulation of *Derrière* is not accompanied by a restriction in its normal expression domain. All embryos were injected at the two-cell stage, with 20 ng MO1 or 15 ng MO3 into each blastomere.

Injection of both mutated activin B-HA RNA and MO1 reduced the incidence of abnormal embryos to 24% (n=25; Fig. 5I). χ^2 analysis indicates that the observed rescue is significant at P<0.01.

As a final control, we designed an alternative antisense oligonucleotide positioned 5′ of the original target sequence (MO2; Fig. 2A). To our surprise this oligonucleotide proved to have little or no effect on development (see Fig. 6). To explore this observation, we isolated and sequenced the 5′ untranslated region of activin B from members of our own colony of *Xenopus laevis* (see Materials and methods). The sequence of this region proved to differ from the published sequence, resulting in a two nucleotide mismatch with our second antisense oligonucleotide (Fig. 2A). We note that a four nucleotide mismatch is sufficient to render an oligonucleotide completely ineffective (see Fig. 2A), and indeed a single mismatch can lead to a significant reduction in potency (Khokha et al., 2002), so it is likely that the efficacy of this alternative antisense morpholino oligonucleotide will be significantly compromised.

A third antisense morpholino oligonucleotide, MO3, was therefore designed to hybridise with the 5′ untranslated region of activin B derived from our own colony of *Xenopus laevis*. This reagent proved to have similar effects to our original activin B morpholino, but to be even more effective (Fig. 6A-D).

These conclusions were confirmed at the molecular level by comparing the expression of *Goosecoid* (Cho et al., 1991), *chordin* (Sasai et al., 1994) and *Xvent1* (Gawantka et al., 1995) in embryos injected with different antisense morpholino oligonucleotides. In this experiment, embryos were isolated at different stages to investigate any temporal effects of inhibition of activin function. MO1 and MO3 gave similar results, with a significant downregulation of *Goosecoid* (particularly at mid-gastrula stage 11 for MO3) and of *chordin*, and a strong upregulation of *Xvent1* (Fig. 6E-J), indicating that in the absence of activin function embryos acquire more ventral characteristics.

Depletion of activin B changes the expression of other inducing factors

The effects of activin depletion might be exacerbated if other mesoderm-inducing factors, such as the nodal-related proteins or Derrière, are downregulated, or they might be reduced if the expression of such factors is enhanced. To explore this issue, we investigated the expression of these inducing factors in embryos injected with mMO1, MO1 or MO3 at early gastrula stage 10.5. Expression of *Xnr1*, *Xnr4*, *Xnr5* and *Xnr6* was little affected by MO1 or MO3, but expression of *Xnr2* was substantially increased and expression of *Derrière* was reduced. As observed with the expression of genes such as *Goosecoid* and *Xvent1*, the change in expression levels of these genes was not accompanied by changes in their expression domains (Fig. 7B-J). This is discussed below.

Discussion

The work described in this paper shows that activin B plays a role in the early development of the *Xenopus* embryo, and particularly in the specification of the mesoderm. It thus reinstates activin as a potential inducing or patterning agent in the early amphibian embryo. *Xenopus* embryos lacking activin B function display defects in both anterior and posterior structures, and axial tissues such as notochord and muscle are present but severely disrupted (Fig. 3A-C,H-K). The expression of dorsally expressed genes such as *goosecoid* and *chordin* is downregulated (Fig. 3L), and that of *Xvent1* is upregulated (Fig. 6G,J), but as discussed below, the expression domains of these genes are unaffected. We note that embryos in which activin B function is inhibited suffer defects in convergent extension (Fig. 3D-F). This may be a direct consequence of the lack of activin B, or a result of the downregulation of genes such as *goosecoid* and *Xbra* (Fig. 3L, Fig. 4A-D).

The requirement for zygotic activin B function in normal mesoderm formation in *Xenopus* contrasts with a requirement for maternal activin in Medaka (Wittbrodt and Rosa, 1994) and with the absence of a requirement for zygotic *activin A* and

activin B expression in mesoderm formation in the mouse (Matzuk et al., 1995). It is clear, however, that in all these species members of the TGFβ family, and particularly the nodal proteins, play significant roles in mesoderm formation (Schier, 2003).

Other attempts to interfere with activin function

Our experiments do not represent the first attempt to investigate the role of activin in *Xenopus* development, but they may use the most specific tool to inhibit activin function. Dominant-negative activin receptors, for example, disrupt normal development (Hemmati-Brivanlou and Melton, 1992; New et al., 1997), but they are as likely to inhibit the functions of other members of the TGFβ family, including BMPs and nodal-related proteins, as they are to inhibit activin (Hawley et al., 1995; Schulte-Merker et al., 1994; Wilson and Hemmati-Brivanlou, 1995; Yamashita et al., 1995). Even a secreted version of the type II activin receptor, which displays significantly greater specificity for activin (Dyson and Gurdon, 1997), may also inhibit other members of the TGFβ family, although we note that the phenotype of embryos expressing such a construct resembles, at least superficially, the phenotypes of embryos injected with MO1 or MO3 (Dyson and Gurdon, 1997).

Other attempts to inhibit activin function have employed the activin-binding protein follistatin. Experiments by Schulte-Merker and colleagues were unable to demonstrate a role for activin following injection of RNA encoding rat follistatin (Schulte-Merker et al., 1994), although more recent experiments, using higher concentrations of RNA encoding the *Xenopus* protein, suggest that follistatin does inhibit mesoderm formation (Marchant et al., 1998). Interpretation of these experiments is further complicated by the observation that the inhibitory effects of follistatin are not restricted to activin, and that it also binds to members of the BMP family (Iemura et al., 1998).

A final approach has involved the use of dominant-negative 'cleavage mutants', where expression in the embryo of a TGFβ construct in which the proteolytic cleavage site is mutated prevents the release of active dimers (Lopez et al., 1992). In *Xenopus laevis*, however, activin cleavage mutants prove to have little effect on development (Hawley et al., 1995; Osada and Wright, 1999), and indeed similar results have been obtained in Medaka, although in this species it is maternal activin that appears to be required for proper mesoderm formation (Wittbrodt and Rosa, 1994). Potential pitfalls concerning the use of cleavage mutant constructs have been discussed by Eimon and Harland (Eimon and Harland, 2002), but explanations for inappropriate lack of activity of a construct are few. One possibility is that endogenous and exogenous *activin B* RNAs are processed in different compartments of the cell. Another is that endogenous activin can employ an alternative cleavage site; although activin is cleaved at a single cleavage site in oocyte expression studies (Hawley et al., 1995), an additional site may be employed after the mid-blastula transition. The inability of activin cleavage mutants to affect early *Xenopus* development requires further investigation.

Post-translational regulation of activin B

Analysis of the spatial expression pattern of activin B in the early *Xenopus* embryo is hampered by its low expression level, but dissection of embryos indicates that transcripts are distributed ubiquitously (Dohrmann et al., 1993). This observation suggests that there is translational or post-translational control of activin function, as also occurs with BMP family members and the nodal-related genes (Agius et al., 2000; Bouwmeester et al., 1996; Cheng et al., 2000; Dale and Wardle, 1999; Glinka et al., 1997; Jones and Smith, 1998; Smith, 1999). The spatial control of effective activin concentration is likely to be very complicated; for example, one known activin antagonist, Xantivin (Cheng et al., 2000), is more effective in marginal zone tissue than in the animal cap (Tanegashima et al., 2000), while experiments involving injection of activin into the blastocoels of *Xenopus* embryos suggest that there is in addition an intrablastocoelic inhibitor of activin function (Cooke et al., 1987).

The complicated regulation of activin function may help explain why it is so difficult to 'rescue' MO1- and MO3-injected embryos to normality by introducing activin B RNA. The problem is exacerbated by the facts that endogenous activin B expression levels are so low and that injected RNA diffuses less well in the embryo than does injected morpholino oligonucleotide (Nutt et al., 2001; Saka and Smith, 2004). These problems notwithstanding, we have achieved partial rescue of the phenotype caused by MO1 by injecting a quantity of activin B RNA that is just sufficient, in 36% of embryos, to cause defects through overexpression (Fig. 5G-J). These results confirm the specificity of the observed phenotype and reinforce the conclusion that activin B is required for normal development in *Xenopus*.

Loss of activin function is accompanied by an upregulation of Xnr2 and a downregulation of *Derrière*

Activin is but one of several mesoderm-inducing factors in the early *Xenopus* embryo; there are, in addition, five *nodal-related* genes (Takahashi et al., 2000; Thomas et al., 1997) as well as *Derrière* (Sun et al., 1999) and *Vg1* (Dale et al., 1993; Thomsen and Melton, 1993; Weeks and Melton, 1987). It is remarkable that the abolition of just one of these, activin, should cause such a dramatic phenotype, especially as the embryo seems to make some attempt to compensate for the loss of activin activity; although the inhibition of activin function is accompanied by a downregulation of *Derrière*, there is a marked upregulation of *Xnr2* (Fig. 7). The first of these results is consistent with the observation that a dominant-negative Derrière construct inhibits *Xnr2* expression in the *Xenopus* embryo, indicating that mesoderm-inducing factors might positively regulate their own expression (Eimon and Harland, 2002). The upregulation of *Xnr2* in embryos injected with MO1 or MO3 does not accord with this idea, however, and it may be necessary in the future to conduct a systematic analysis of the effects of ablating candidate inducing factors and to ask how they regulate each other's expression. We note that inhibition of all mesoderm-inducing *Xenopus* nodal-related genes, by expression of the C-terminal region of Cerberus (Bouwmeester et al., 1996), causes severe defects in mesoderm formation. Such embryos form just a small tail-like structure, and expression of α-actin and α-globin is severely reduced (Wessely et al., 2001).

Changes in gene expression levels caused by MO1 and MO3 are not associated with changes in expression domains

The downregulation of *Goosecoid* expression in response to MO1 and MO3, and the upregulation of *Xvent1*, appear to occur without significant changes in the expression domains of these genes (Figs 4, 7). This suggests that during normal development the spatial expression patterns of regionally expressed genes are defined by the combined effects of members of the TGFβ family, including the *nodal-related* genes and *derrière* as well as *activin B*. Loss of just one member of this network, such as activin, may not disrupt spatial expression patterns to a significant extent, but may affect expression levels such that development is severely perturbed.

Polymorphism and the design of antisense morpholino oligonucleotides

The final point to be made from the results described is that one should not rely solely on sequences derived from GenBank when designing antisense morpholino oligonucleotides. Polymorphisms, particularly in the 5′ untranslated regions of *Xenopus* mRNAs, are likely to be frequent, and even a single nucleotide difference between oligonucleotide and target RNA may produce a significant reduction in potency (Khokha et al., 2002). It may be impractical to confirm the sequence of the mRNA of interest in every experiment one does, but a failure to obtain a phenotype in one egg batch does not necessarily invalidate the rest of one's results.

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