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Identification of a BMP inhibitor-responsive promoter module required for expression of the early neural gene *zic1*

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Abstract

Expression of the transcription factor *zic1* at the onset of gastrulation is one of the earliest molecular indicators of neural fate determination in *Xenopus*. Inhibition of bone morphogenetic protein (BMP) signaling is critical for activation of *zic1* expression and fundamental for establishing neural identity in both vertebrates and invertebrates. The mechanism by which interruption of BMP signaling activates neural-specific gene expression is not understood. Here, we report identification of a 215 bp genomic module that is both necessary and sufficient to activate *Xenopus zic1* transcription upon interruption of BMP signaling. Transgenic analyses demonstrate that this BMP inhibitory response module (BIRM) is required for expression in the whole embryo. Multiple consensus binding sites for specific transcription factor families within the BIRM are required for its activity and some of these regions are phylogenetically conserved between orthologous vertebrate *zic1* genes. These data suggest that interruption of BMP signaling facilitates neural determination via a complex mechanism, involving multiple regulatory factors that cooperate to control *zic1* expression.

Keywords: zic1; Neural induction; Neurectoderm; BMP inhibition; Noggin; *Xenopus*; Promoter; Transgenics

Introduction

Bone morphogenetic proteins (BMPs) are essential for patterning the primary dorsoventral body axis in both vertebrates and invertebrates. BMPs act within both mesoderm and ectoderm to specify ventral regions, and as they are initially ubiquitously expressed, BMP signaling must be inhibited to allow dorsal determination. Within the embryonic ectoderm, inhibition of BMP signaling is a crucial step in neural cell fate determination in all vertebrates (Sasai and De Robertis, 1997; Munoz-Sanjuan and Brivanlou, 2002).

BMP signaling can be inhibited by several mechanisms, with one major mechanism involving extracellular BMP antagonists, including Noggin, Chordin and Follistatin that bind to and sequester BMP ligands (Sasai, 2001). In *Xenopus*, expression of these antagonists is activated prior to gastrulation on the dorsal side of the blastula stage embryo through a β-Catenin-dependent mechanism (Wessely et al., 2001), consistent with a requirement for Wnt signaling in *Xenopus* dorsal determination (Yamaguchi, 2001). Additional signaling pathways may regulate BMP RNA levels (Bainter et al., 2001).

During gastrulation in *Xenopus*, BMP expression is repressed on the dorsal side of the embryo, partly though interrupting a positive feedback loop (Jones et al, 1992). Wnt signaling can repress *bmp4* expression (Baker et al., 1999), in part, through the Xiro1 homeodomain protein that functions as a transcriptional co-repressor and also activates expression of neural-specific genes (Gomez-Skarmeta et al., 2001). In zebrafish, ectopic expression of the homeodomain protein Bozozok, which is a downstream effector of Wnt signaling (Driever et al., 1996; Solnica-Krezel et al., 1996; Solnica-Krezel and Driever, 2001) can suppress *bmp2b* and *bmp4* expression (Koos and Ho, 1999; Fekany-Lee et al., 2000). Interestingly, genes whose expression is activated by inhibition

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of BMP signaling, such as *otx2*, can also suppress expression of *bmp4* in *Xenopus* (Gammill and Sive, 2001), suggesting that a feedback mechanism may maintain neural gene expression through sustained repression of BMP gene transcription.

In *Xenopus*, activation of BMP inhibitor expression in the dorsal mesendoderm (organizer) at or before the onset of gastrulation is closely associated with the onset of expression of very early neural marker genes, including *zic1/opl* (Kuo et al., 1998; Mizuseki et al., 1998a), *soxD* (Mizuseki et al., 1998b) and *geminin* (Kroll et al., 1998). Consistent with a central role for BMP inhibition in neural determination, expression of *zic1* and *soxD* is activated by Noggin, Chordin or a dominant-negative form of BMP4 in ectodermal explants (Mizuseki et al., 1998a,b; Gamse and Sive, 2001).

The mechanism by which inhibition of BMP signaling leads to neural determination involves a connection with MAP kinase signaling. In *Xenopus*, activation of neural gene expression by BMP antagonists can be blocked by a truncated fibroblast growth factor (FGF) receptor construct or FGF inhibitor, indicating a connection between FGF and BMP signaling (Launay et al., 1996; Gamse and Sive, 2001; Delaune et al., 2005). Furthermore, Smad1 phosphorylation by FGF-dependent MAP kinase signaling inhibits BMP signaling and facilitates neural induction in *Xenopus* (Pera et al., 2003; Kuroda et al., 2005). In early chick embryos, part of the function of FGF signaling is apparently to suppress BMP expression in the epiblast and potentiate neural fate determination (Wilson et al., 2001; Streit et al., 2000). Consistent with this, the early neural gene *otx* in the primitive chordate *Ciona* requires FGF signaling that is mediated by both Ets and GATA transcriptional effectors (Bertrand et al., 2003).

The mechanism by which BMP inhibition leads to transcriptional activation of early neural genes is unknown. In order to understand this mechanism, we have analyzed the promoter of the *Xenopus zic1* gene that is activated by BMP inhibition. We report identification of a 215 bp genomic module that is both necessary and sufficient to mediate activation of *zic1* transcription upon interruption of BMP signaling. Within this module, we identify several putative transcription factor-binding elements that are together required to activate *zic1* transcription in the absence of BMP signaling.

Materials and methods

Growth, microinjection, dissection and culture of embryos and explants

Xenopus laevis eggs were collected, fertilized and cultured as previously described (Sive et al., 2000) and staged according to (Nieuwkoop and Faber, 1967). Embryos were dejellied in 2% cysteine (Sigma, pH 8.0) 30 min after fertilization and rinsed extensively in 0.1× MBS. At the two-cell stage, embryos were placed in 0.1× MBS + 2% Ficoll (Sigma) and one blastomere was microinjected at the animal pole region with a solution of plasmid reporter construct DNA and mRNA encoding Noggin, β-Globin or BMP4 in a final volume of 10 nl per injection. Standardized volumes of DNA/RNA solutions for injection were confirmed by measuring the volume of solution for each needle before injecting and by monitoring the size of a characteristic pigment dispersion 'halo' that is left behind after superficial injections into the heavily pigmented blastomeres. Relative luciferase activity was normalized to the same number of caps used per group per experiment. Concentrations of DNA and mRNA are as indicated. Embryos were allowed to develop at 25°C in 0.1× MBS + 2% Ficoll. For ectoderm (animal cap) dissections, late blastula stage embryos (stage 9–9.5) were manually devitellinated and animal hemisphere ectoderm was dissected and incubated until stage 16 equivalent in 1× MBS alone or with other reagents as indicated.

Cloning the Xenopus laevis zic1 core promoter and 5′ upstream regulatory region

Standard molecular techniques were used unless otherwise indicated. Enzymes were obtained from Stratagene or New England Biolabs. 1×10^6 plaques of amplified *Xenopus laevis* EMBL4 lambda phage genomic library (a kind gift from T. Sargent) were screened using a probe made from *zic1* (*opl*) cDNA (Kuo et al., 1998). Four positive clones were obtained and Southern blots revealed that one clone (2b2) contained approximately 6 kb of genomic sequence between a 5′ *Bam*HI site and a 3′ *Not*I site. This fragment was excised from the phage and subcloned into pBluescript KS (Stratagene) and sequenced. The genomic fragment was found to contain 5.272 kb of sequence upstream of the 5′ end of the *zic1* cDNA, between the upstream *Bam*HI site and a downstream *Bss*HII site (see Fig. 1A). To definitively identify the transcriptional start site of *zic1*, 5′ rapid amplification of cDNA ends (5′RACE) was performed. To generate a luciferase reporter construct containing the *zic1* upstream genomic fragment (assigned as full length), pBS–2b2 was cut with *Bss*HII and *Eco*RV (just upstream of *Bam*HI), the overhang generated by *Bss*HII was filled in with the large fragment of DNA polymerase I (Klenow fragment), and this blunt ended fragment was cloned into the *Sma*I site of pGL3 Basic luciferase reporter vector (Promega).

Mutagenesis mapping

ExoIII deletions of the full-length *zic1* 5′ genomic regulatory region (construct 5045) were generated using the Exo/Mung Bean Deletion kit (Stratagene) following the manufacturer's instructions. Nested deletions of the full-length construct (5045d1–d4) were generated by inverse PCR using the Expand Long Template PCR System (Roche) to ensure for highly accurate amplified DNA and using 5′-phosphorylated, sequence-specific primers (MWG Biotech). PCR conditions were as follows: 4 min at 94°C, followed by 16 cycles of 1 min at 94°C, 1 min at 55°C, 8 min at 68°C, followed by a final extension of 10 min at 72°C. Products were treated with 1.75 U of *Pfu* at 72°C for 30 min and phenol/chloroform extracted. The correct size of amplified product was confirmed on a 1% agarose gel. Amplified DNA was ligated with T4 DNA ligase and template DNA removed by DpnI digestion. After a second phenol/chloroform extraction, DNA was resuspended in 10 μl water and 5 μl was used to transform TOP10 chemically competent bacteria (Invitrogen). Clones with the correct deletion were confirmed by sequence analysis. To generate the luciferase reporter construct containing a heterologous core promoter, the adenovirus major late promoter (MLP) was excised from pPolyA-MLP-PTCAT as a *Hin*dIII–*Bam*HI fragment, end filled with Klenow and cloned into the *Hin*dIII linearized and end filled pGL3 vector. The resultant construct (pGL3-MLP) contained a unique *Bgl*II cloning site upstream of MLP that was subsequently used to generate reporter constructs. The 588, 293, 215, 115, 100, 108, 100(×2) and 108(×2) reporter constructs were generated by PCR using sequence-specific primers anchored with *Bgl*II restriction sites and 5045 as a template. The amplified product was subcloned into pCR2.1 TOPO-TA cloning vector (Invitrogen). Insert DNA from positive transformants was retrieved by *Bgl*II digestion, column purified (Qiagen) from a 1% agarose gel and subloned into *Bgl*II linearized pGL3-MLP. Correct sequence and orientation of all constructs was subsequently confirmed. Deletions of 588 (588d1–d5) were generated using inverse PCR as above. Linkerscan mutagenesis of the 215-reporter construct was performed with the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Each cloned linkerscan construct (LS1–13) was sequenced to confirm mutation. To generate multimerized cassette reporter constructs, 5′-phosphorylated, sequence-specific sense and anti-sense oligonucleotides containing anchored *Bgl*II overhangs (MWG Biotech) were annealed and subcloned into the *Bgl*II digested pGL3-MLP construct. All constructs (m1–7) were sequenced to confirm orientation and insert number. All primer sequences used in the mutagenesis mapping are available upon request.