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Phylogenetic footprinting and genome scanning identify vertebrate BMP response elements and new target genes

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Abstract

The complex gene regulatory networks governed by growth factor signaling are still poorly understood. In order to accelerate the rate of progress in uncovering these networks, we explored the usefulness of interspecies sequence comparison (phylogenetic footprinting) to identify conserved growth factor response elements. The promoter regions of two direct target genes of Bone Morphogenetic Protein (BMP) signaling in *Xenopus*, *Xvent2* and *XId3*, were compared with the corresponding human and/or mouse counterparts to identify conserved sequences. A comparison between the *Xenopus* and human *Vent2* promoter sequences revealed a highly conserved 21 bp sequence that overlaps the previously reported *Xvent2* BMP response element (BRE). Reporter gene assays using *Xenopus* animal pole ectodermal explants (animal caps) revealed that this conserved 21 bp BRE is both necessary and sufficient for BMP responsiveness. We combine the same phylogenetic footprinting approach with luciferase assays to identify a highly conserved 49 bp BMP responsive region in the *Xenopus Id3* promoter. GFP reporters containing multimers of either the *Xvent2* or *XId3* BREs appear to recapitulate endogenous BMP signaling activity in transgenic *Xenopus* embryos. Comparison of the *Xvent2* and the *XId3* BRE revealed core sequence features that are both necessary and sufficient for BMP responsiveness: a Smad binding element (SBE) and a GC-rich element resembling an OAZ binding site. Based on these findings, we have implemented genome scanning to identify over 100 additional putative target genes containing 2 or more BRE-like sequences which are conserved between human and mouse. RT-PCR and in situ analyses revealed that this in silico approach can effectively be used to identify potential BMP target genes.

Keywords: *VENTX2*; *Id3*; *Xenopus*; TGFβ; BMP; Phylogenetic footprinting; Genome scanning

Introduction

As biology enters the post-genome sequencing era, vast amounts of genome-based information are becoming available, enabling comparisons between whole genomes of different species to identify conserved sequences (phylogenetic footprinting). *Xenopus* is a well-suited system for the use of phylogenetic footprinting for several reasons. First, since its evolutionary distance from mammals such as human and mouse is large (350 million years) (Muller et al., 2002), sequence conservation between *Xenopus* and human or mouse is likely to yield biologically meaningful information. Second, the sequencing of the *Xenopus tropicalis* genome based on 7.5× coverage has been completed (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html). Third, while phylogenetic footprinting is a valuable tool to identify candidate sequences that may represent important *cis*-acting regulatory elements, it is essential to experimentally verify the biological importance of putative regulatory elements identified by this approach, preferably in vivo. In this regard, *Xenopus* embryos are an experimentally amenable system for the study of gene

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regulation, as promoter activity can be easily examined in transgenic embryos (Gottgens et al., 2000; Kroll and Amaya, 1996; Offield et al., 2000).

In this study, we combine phylogenetic footprinting and reporter gene assays to identify conserved Bone Morphogenetic Protein (BMP) responsive elements located within the promoters of two direct BMP target genes. BMPs are members of the TGFβ super family of secreted polypeptide growth and differentiation factors. During *Xenopus* embryonic development, *BMP2*, *4*, and *7* are expressed dynamically, sometimes in overlapping regions, but at other times are expressed in different regions, suggesting that BMPs have overlapping yet distinct roles in development. For instance, *BMP2*, *4*, and *7* are all expressed in overlapping regions of the embryonic mesoderm and ectoderm to regulate dorsal–ventral patterning of the mesoderm and specification of epidermis (Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1992; Suzuki et al., 1994). On the other hand, *BMP7* is expressed in the organizer, while neither *BMP2* nor *BMP4* are expressed in this region (Hawley et al., 1995).

A number of direct BMP target genes have been identified in recent years by treating cells or embryonic tissues with BMP in the presence of the protein synthesis inhibitor cycloheximide (CHX), among them *Xvent2* and *Xvent2B*, *msx1*, *msx2*, *Id1*, *Id2*, *Id3*, *GATA2*, *Dlx5*, *Tob*, and *FGFR2* (Friedle and Knochel, 2002; Hollnagel et al., 1999; Hussein et al., 2003; Korchynskyi and ten Dijke, 2002; Ladher et al., 1996; Miyama et al., 1999; Peiffer et al., 2005; Rastegar et al., 1999; Yoshida et al., 2000). The promoters of some of these genes, such as *Xvent2*, *Xvent2B*, and *Id1*, were shown to have a Smad binding element (SBE; [C]AGAC or its reverse complement, GTCT[G]) crucial for BMP responsiveness (Hata et al., 2000; Henningfeld et al., 2000). Interestingly, while the SBE is thought to represent a binding site for both R-Smads and the Co-Smad (Smad4) (Shi and Massague, 2003; Shi et al., 1998), this sequence has been shown to preferentially bind Smad4 in the context of the *Xvent2B* and *Id1* promoters (Henningfeld et al., 2000; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002).

While reporter genes with multimerized SBEs can respond to BMP signaling, Smad binding to DNA alone is generally believed to be too weak for Smads to function alone as effective and highly specific DNA binding proteins in vivo (Kusanagi et al., 2000; Shi et al., 1998). Thus, additional DNA-binding partners are thought to be required for efficient DNA binding and specific selection of BMP target genes. While numerous such cofactors have been identified for activin/TGFβ responsive genes (among others FAST1, mixer/milk, c-jun/c-fos, TFE3, and WBSCR11) (Chen et al., 1996; Germain et al., 2000; Hua et al., 1999; Ring et al., 2002; Zhang et al., 1998), only few candidate factors have been identified for BMP target genes, one of which is the zinc finger protein OAZ (Hata et al., 2000). OAZ is thought to be involved in activating the *Xvent2* gene upon BMP stimulation and has been suggested to bind to the sequence TGGAGC. Both this sequence and the SBE are required for BMP responsiveness of the *Xvent2* gene (Hata et al., 2000). Other studies show that sequences other than SBEs are important for BMP responsiveness, for example GCAT in the *Xvent2B* promoter (Henningfeld et al., 2000), and GC-rich sequences in the *Id1* and *Smad6* promoters (Ishida et al., 2000; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). However, both the GCAT and the GC-rich sequences can bind Smad proteins (Henningfeld et al., 2000; Ishida et al., 2000; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). Thus, it is still unclear as to whether factors other than Smads are involved in the activation of these BMP target genes. It should be kept in mind that much of the currently available evidence supporting the role of these sequence motifs in vertebrate BMP signaling derive from overexpression studies, while there is little in vivo evidence to suggest that these BMP response elements (BREs) actually respond to endogenous levels of BMP signaling in developing tissues with the exception of a recent study examining the BMP4 synexpression group in *Xenopus* (Karaulanov et al., 2004).

In the current study, we compared the promoter sequences between *Xenopus laevis* and human *Vent2* to identify a 21 bp core region of the *Xvent2* BRE that is both necessary and sufficient for BMP responsiveness. Using a similar approach, a highly conserved BRE in the *Xenopus Id3* promoter was also identified. Further alignments of the *Xvent2* BRE and the *XId3* BRE revealed a conserved core BRE consisting of an SBE and an OAZ-binding-site-like sequence. This core sequence is similar to that recently identified in *Drosophila* (Pyrowolakis et al., 2004). Importantly, this core region is still sufficient for BMP responsiveness in vivo as transgenic embryos harboring BRE-GFP reporters drove GFP expression in areas of endogenous BMP activity in living embryos. Next, implementing an in silico approach, we have used the consensus BRE sequence motif to scan the conserved regions of both the human and mouse genomes to uncover potential BMP responsive genes. Though 1104 genes contain only one copy of the BRE, 138 genes contain 2 or more potential BRE sites including several known BMP target genes. Moreover, we identified 26 orthologues of these genes in *Xenopus* using reciprocal BLAST searches and analyzed the response of these genes toward BMP2 by in situ and RT-PCR analyses. Nearly 50% of these genes are induced directly by BMP2 in dissociated animal cap tissues, suggesting the usefulness of a genome scan approach together with a phylogenetic footprinting method.

Materials and methods

Bioinformatic tools and genomic sequences

Alignments in Figs. 1 and 3 used the Family Relations software (http://family.caltech.edu) (Brown et al., 2002) with a 20 bp window sliding in 1 bp increments. Under these conditions, any similarity above 70% is highly unlikely to be

random (C.T. Brown and E.H. Davidson, pers. comm.). Alignments in Figs. 3C and 4A used ClustalW 1.8 (http://www.searchlauncher.bcm.tmc.edu). We used the following genomic sequences for alignments: *Xenopus Vent2* (Candia et al., 1997); *Xenopus Id3* (−2790/+70), provided by Dr. Georges Spohr (University of Geneve). All other upstream gene sequences were retrieved with the following ensembl IDs (http://www.ensembl.org): human *VENTX2*: ENSG00000151650; human *Id3*: ENSG00000117318; mouse *Id3*: ENSMUSG00000007872. The *Xvent2* and *XId3* transcriptional start sites were determined previously (Lee et al., 2002; Wilson and Mohun, 1995).

Embryo manipulations, microinjections, and luciferase reporter assays

Embryos were obtained by in vitro fertilization, dejellied, and cultured in 0.1× Modified Barth's Saline (MBS) using standard techniques (Blitz et al., 2003; Cho et al., 1991). Dominant negative BMP receptor (DNBR) and constitutively active BMP receptor (CABR) RNA were prepared as described previously (Candia et al., 1997; Suzuki et al., 1994).

For luciferase reporter gene assays in *Xenopus* embryos, 0.16–0.32 ng of the indicated *luciferase* reporter constructs was microinjected into the animal pole of 2–4 cell embryos, alone or together with 2–4 ng of DNBR or CABR mRNA. At the blastula stage, animal caps were dissected, cultured until control embryos reached stage 10.5–11, and subjected to luciferase assays as previously described (Watabe et al., 1995). To account for the inherent variability in luciferase counts between different batches of eggs, each experiment used eggs from a single female, and each luciferase count represents the average of usually ten pooled animal caps. In addition, all reporter gene experiments were repeated a minimum of three times.

Whole-mount in situ hybridization

Hybridizations were performed essentially as described (Blitz and Cho, 1995; Harland, 1991) except for the use of BM purple (Boehringer–Mannheim Biochemicals) as colorimetric substrate. *XId3* and *XBMP4* probes were made from clones XL013b11 and XL109p12, respectively (http://Xenopus.nibb.ac.jp), using T7 RNA polymerase, after linearization with *Pst*I (*XId3*) or *Not*I (*XBMP4*). Clones used for in situ hybridization of the *Xenopus* BRE orthologues are listed in Table 1 and were all linearized using *Not*I. *GFP* probe was generated from pCARGFP2 (Kroll and Amaya, 1996) using T7 polymerase after linearization with *Hin*dIII.

Promoter constructs for luciferase- and GFP-reporter experiments

Xvent2 luciferase reporter genes (−252/+29/*Luc*; −167/+29/*Luc*) were generated by cloning between the *Bam*HI/*Hin*dIII sites of the promoterless luciferase vector pOLuc (de Wet et al., 1987) using PCR. To generate the 5x(−173/−151)/−150/*Luc* reporter construct, we first generated 5-mers of the *Xvent2* promoter region −173/−151. Pairs of 2x(−173/−151) oligos were subjected to PCR, generating 5x(−173/−151) concatamers, which were subsequently cloned into pCR2.1-TOPO to generate 5x(−173/−151)/pCR2.1. The 5-mer insert was then subcloned into pOLuc to generate 5x(−173/−151)/*Luc*. The *Xvent2* minimal promoter (−150/+29/*Xvent2*) was subcloned into this construct, resulting in 5x(−173/−151)/−150/*Luc*.

An 8x(−173/−151)/−150 construct with a 5 bp spacer (AAGAG) between the repeats, which also responded well to BMP signaling in luciferase assays (data not shown), was used for our transgenic GFP reporter experiments. This concatamer was generated similarly to the 5x(−173/−151) concatamer and then subcloned in front of the *Xvent2* minimal promoter in pCXGFP3 (−151/+29/GFP), resulting in 8x (−173/−151)/−150/GFP.

Full length *XId3* promoter/*Luc* reporter, −2790/+70/*Luc*, was generated by subcloning −2790/+70/*XId3* from pBLCAT3 (Zhang et al., 1995) into *Bam*HI cut pOLuc. The resulting −2790/+70/*Luc* construct was used to generate *XId3* promoter deletion constructs in pOLuc using the reverse primer 5′-TTTAAGCTTGAATTCGGAGATCAGGTTCG-3′ and the appropriate forward primers for −763/+70, for −724/+70, and for −201/+70. The resulting PCR products were digested with *Bam*HI/*Hin*dIII and ligated into pOLuc. *XId3*-BRE 7x(−718/−673) concatamers were generated as described previously (Kadonaga and Tjian, 1986; Watabe et al., 1995) using oligos encoding the −718/−673 region (sense: 5′-GATCCTCTGGTCACAGGATAATAATCCYGACGCCAGAAAGTCTGGAGGTCA-3′; antisense: GATCTGACCTCCAGACTTTCTGGCGTCAGGATTATTATCCTGTTGACCAGAG-3′). Resulting multimers were ligated into the *Id3* minimal promoter/*Luc* reporter construct (−201/+70/*Luc*) to generate 7x(−718/−673)/−201/*Luc*. To generate the 7x(−718/−673)/−201/GFP reporter construct, 7x(−718/−673)/−201 was subcloned into the *Bam*HI/*Hin*dIII sites of pCXGFP3.

To generate *Luc*-reporter constructs containing small pieces of the *XId3* BRE [(−718/−697)/−201/*Luc* and (−704/−680)/−201/*Luc*], oligos were phosphorylated, mixed, and ligated into the *Bam*HI site of −201/+70/*Luc*. The (−685/−673)/−201/*Luc* reporter construct was generated using PCR with −201/+70/*Luc* as template and a primer encoding the −685/−673 sequence. The PCR product was then subcloned into pOLuc.

Xenopus transgenesis

Transgenic embryos were generated as described previously (Kroll and Amaya, 1996) except that egg extracts were heated at 80°C for 5 min and centrifuged. Eggs were injected using a Drummond NANOJECT II and GFP fluorescence was observed with a Leica MZ FLIII

microscope. Only healthy embryos without mosaic expression were analyzed.

BMP treatment of animal caps and RT-PCR

Animal caps were cut at blastula stages 8–9 and left intact or dissociated in Ca^{2+}/Mg^{2+} free 1× MBS medium and incubated in 5 μg/ml of the protein synthesis inhibitor cycloheximide (CHX) ±15 ng/ml BMP2 protein. Because intact animal cap explants secrete endogenous BMPs, dissociation of the animal caps into single cells is necessary before treatment with BMP2. The protocol for RT-PCR was as previously described (Blitz and Cho, 1995), except that the reaction products were visualized on ethidium bromide stained agarose gels. Total RNA was extracted when control sibling embryos reached gastrula stage 10.5. PCRs were performed at 21 cycles (*Histone H4*) or 28 cycles (*Xld3* and *Xvent2*; 94°C, 5 min; 28 times (94°C, 30 s; 55°C, 30 s; 72°C, 30 s); 72°C, 10 min) with the following primer pairs for *Xld3*: 5′-TGCGGTCC-ATGTCAAGC-3′ and 5′-CAGAGAGGTTAGAACGGC-TCAG-3′. *Xvent2* and *Histone H4* primers were as described previously (Blitz et al., 2003). RT-PCR analysis on the *Xenopus* orthologues obtained from the BRE genome scanning was performed similarly. RT-PCR oligos for these orthologues are shown in Supplementary Table 3 and specific PCR cycle numbers for each gene are shown in Table 1.

In silico identification of human genes with conserved BRE-like sequences

Part of the core BRE sequence (5′-CCANNNNGTCTG-3′) conserved between *Vent2* and *Id3* was used for the genome-scanning analysis (see Fig. 5A). Using the human and mouse conserved regions from the UCSC web site located at http://genome.cse.ucsc.edu/goldenPath/hg16/vsMm4 (based on NCBI human Build 34, Jun 2003, and NCBI mouse Build 32, Oct 2003), we found 2000 conserved BRE sites in 1933 conserved sequence segments. After excluding BMP sites located in the coding regions of the human genome (based on results from NCBI blastx against all human proteins downloaded from NCBI), 1319 sites were mapped to the non-coding regions of 1104 genes, shown in Supplementary Table 1 (based on NCBI Human Build 33). Of these sites, 316 sites were located within introns, 157 sites were within 0 to 5 kb from the start of each gene, 362 sites were within 5 to 100 kb, and the remainder of the sites were located more than 100 kb away. Since transcription regulatory elements can be located both upstream or downstream of a coding region, the following guidelines were implemented to assign conserved BRE sites to genes: first, a BRE site in the 5′ upstream region of a gene is always assigned to that gene, therefore, if a site is upstream of two genes, then the site is assigned to both genes. Secondly, a BRE site downstream of a gene is assigned to that gene if the site is within 500 bp of the 3′ end of the gene. Lastly, if a BRE site is downstream of two genes and both genes are more than 500 bp away, then that BRE site is assigned to the nearest gene among the two. Based on this scheme, we found a total of 113 genes with 2 or more BREs, and the remainder of the genes each has only one BRE site. Supplementary Table 2 shows the 113 genes with 2 or more BREs and genes with BREs in intronic regions (138 total genes; 80 of which are annotated). Using reciprocal BLAST searches beginning with each annotated human gene with 2 or more BRE sites, we identified 26 *Xenopus* orthologues. Of these orthologues, we were able to analyze the RT-PCR behavior of 23 genes and the expression pattern of 19 genes, which could be retrieved from the *Xenopus* NIBB library (http://Xenopus.nibb.ac.jp). Supplementary Fig. 1 shows the nucleotide alignment of the BRE sites found in 13 bona fide BMP targets (Table 1), along with a Logo representation of the consensus sequences.

Results

Phylogenetic footprinting identifies a highly conserved BMP response element in the Xenopus Vent2 promoter

Xvent2 is one of the best known direct BMP target genes in vertebrates, and its promoter contains a well-characterized BMP responsive region (Candia et al., 1997; Hata et al., 2000). To examine the effectiveness of the phylogenetic footprinting approach, we aligned the *Xenopus Vent2* promoter with the promoter of its putative human orthologue, *VENTX2* (Fig. 1) (Moretti et al., 2001). A search for regions that are at least 75% similar within a sliding 20 bp window revealed two most highly conserved regions (Fig. 1A). The first, a 21 bp-region (−173/−153) of the *Xvent2* promoter, overlaps with the 53 bp BMP responsive region reported previously (Hata et al., 2000). A similar potential BMP responsive region also appears to be present in the *Xvent2B* promoter (data not shown). The second conserved region, −85/−57, has not been characterized previously and lies within the part of the promoter (−150/+29) that does not respond to BMP signaling by itself (Fig. 1B) and so it was not analyzed any further.

To test whether region −173/−151 is sufficient for BMP responsiveness, it was multimerized and placed in front of the minimal (−150/+29) *Xvent2* promoter (Fig. 1B). Concatamerization was sufficient to respond to BMP signaling (Fig. 1B). The core BRE includes both the SBE and the 3′ flanking sequence previously suggested to be an OAZ binding site (Fig. 1B) (Hata et al., 2000). Taken together, our results show that phylogenetic footprinting can successfully identify a conserved BMP response element and this element contains an SBE as well as an OAZ-binding-site-like sequence.

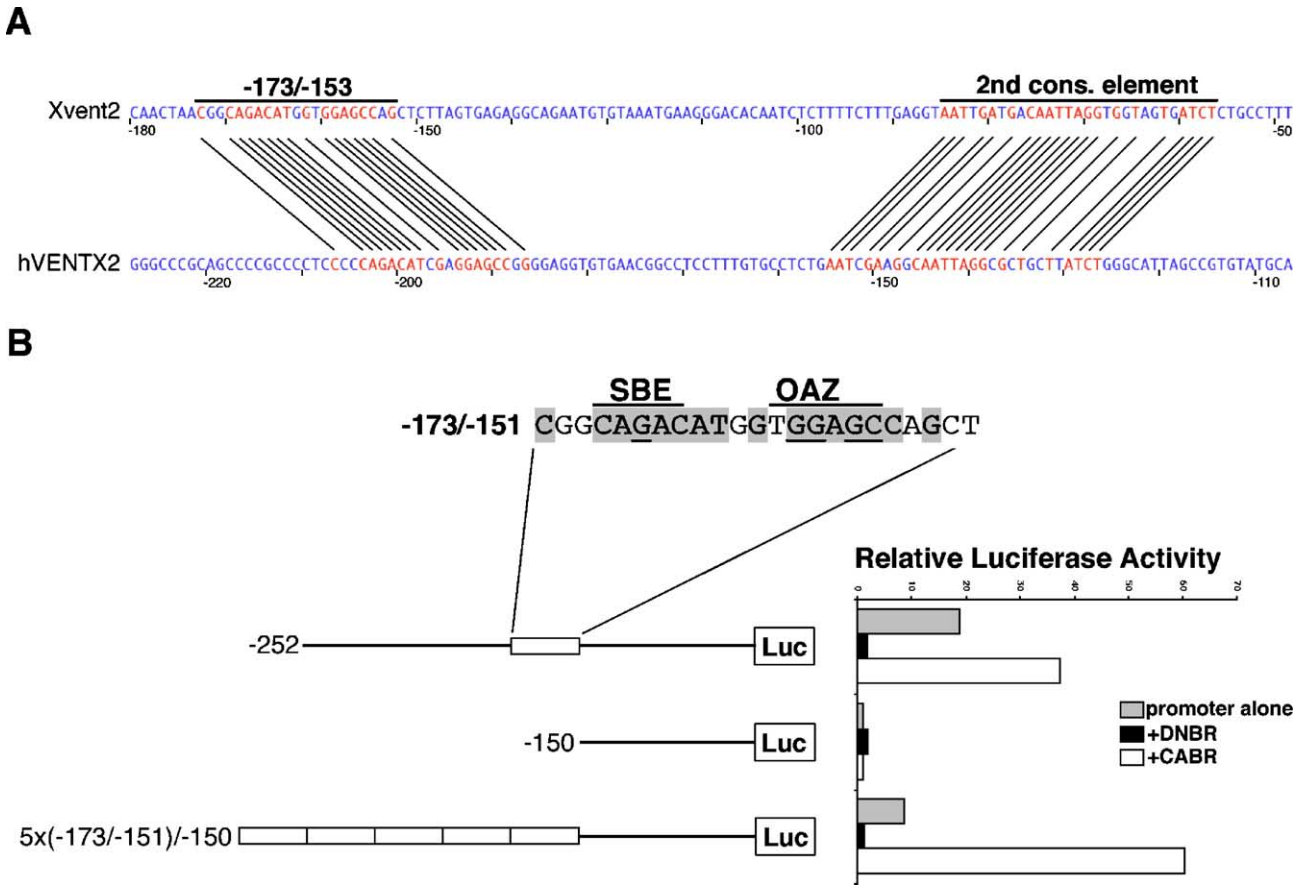

Fig. 1. Identification of a BMP response element (BRE) in the *Xvent2* promoter. *VENTX2* is the human orthologue of *Xvent2* and shares a similar exon/intron organization to *Xvent2B* (Moretti et al., 2001; Rastegar et al., 1999). *Xvent2B* represents a second genomic copy of *Xvent2*, likely due to the pseudotetraploid nature of the *Xenopus laevis* genome (Henningfeld et al., 2002). The *VENTX2* homeobox is most similar to the *Xvent2* and *2B* homeoboxes at the amino acid level (63% and 65%, respectively) (Moretti et al., 2001). (A) Detailed view of the two most highly conserved sequences in the *Xvent2* and *VENTX2* promoters at a setting of 75% identity cutoff. −173/−153 in *Xvent2* overlaps with the *Xvent2* BRE described previously (Hata et al., 2000), while the second conserved region (−85/−57) has not been characterized before. Note that, upon resequencing the *Xvent2* promoter, we found a C at position −155, whereas Hata et al. (2000) reported a gap here. The positions of nucleotides in the *Xvent2* promoter are in relation to the transcriptional start site as previously determined (Lee et al., 2002), and the positions in the human *VENTX2* promoter are relative to the transcriptional start site according to the ensembl genome browser (http://www.ensembl.org). (B) Nucleotide sequence of the *Xvent2* BRE that is sufficient for BMP inducibility (−173/−151). Nucleotides that are conserved between the *Xenopus* and the human *Vent2* promoter are shaded in gray. Note that the underlined positions where mutations lead to a loss of BMP responsiveness (Hata et al., 2000) are all conserved between *Xenopus* and human, and −173/−151 contains all of these conserved bases. Concatamerization of this BRE (5x(−173/−151)/−150) was sufficient to respond to BMP signaling in reporter gene assays.

Id3 is a direct BMP target gene in Xenopus embryos

We next wished to examine whether this phylogenetic footprinting approach can be used to identify a novel BRE in another direct BMP target gene. We chose *XId3* (*Xenopus Inhibitor of differentiation 3*) since it was consistently identified as one of the most strongly induced direct BMP targets in our microarray experiments (Peiffer et al., 2005). RT-PCR analysis confirmed that *XId3* is a direct target gene regulated by BMP2 protein (Fig. 2, lane 6), consistent with previous observations in mouse embryonic stem cells (Hollnagel et al., 1999). *XId3* expression begins at blastula stages (stage 9; data not shown), around the same stage when *Xvent2* is first expressed (Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996), and just after BMP signaling is first detectable in the early *Xenopus* embryo (Faure et al., 2000).

Phylogenetic footprinting identifies two highly conserved elements in the Id3 promoter that are similar to each other

A comparison between the *Xenopus* and human *Id3* promoter sequences revealed two regions that are most highly conserved (at least 90% similar over a sliding 20 bp window): an 18 bp region located distally (−2340/−2323) and a 32 bp region located proximally (−701/−670). A comparison of these two elements revealed that they are similar to each other and also include a conserved potential Smad binding element (SBE) (Fig. 5A). There is also a highly conserved 17 bp region immediately upstream of the −701/−670 region (Fig. 3A), which is not detected at a 90% similarity cutoff, since the Family Relations software does not take gaps into account (C.T. Brown, pers. comm.). However, a ClustalW alignment, which creates gaps to improve alignment results, revealed the entire 49 bp region (−718/−670) as conserved (Fig.

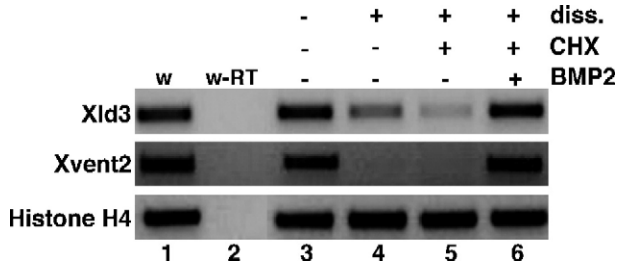

Fig. 2. RT-PCR analysis demonstrates that *XId3* and *Xvent2* are direct BMP target genes. RT-PCR analysis of *Xenopus* embryonic tissue untreated or treated with cycloheximide (CHX) with or without BMP2 protein. Animal caps were cut at stages 8–9, left intact or cultured in Ca^{2+}/Mg^{2+} free media to dissociate the cells, and incubated in 5 μg/ml CHX with or without 15 ng/ml BMP2 protein. Total RNA was extracted at stage 10.5 of controls. *Histone H4*, loading control; w, whole embryo control at stage 10.5–11; w-RT, whole embryo control stage 10.5 without reverse transcriptase (RT). Lanes 3–6 represent cDNA from animal cap tissue, undissociated (lane 3) or dissociated (diss.; lanes 4–6).

3C). An mVISTA alignment (Mayor et al., 2000) (data not shown) of the *Xenopus Id3* promoter sequence with its mouse and human counterparts confirmed that the distal and proximal regions are the most highly conserved regions in the three promoter sequences.

The conserved proximal element in the XId3 promoter contains a BRE

To determine which part of the *XId3* promoter is a functional BRE, we performed a deletion analysis of the *XId3* promoter. Deletion of the distal parts of the *XId3* promoter, including the distal conserved −2340/−2323 element (Fig. 3L), reduced, but did not abolish, the response to BMP signaling (Fig. 4B; data not shown), while removal of the region between −724 and −673 completely abolished the response to BMP signaling. This region is almost identical to the proximal highly conserved region between −718 and −670 identified by phylogenetic footprinting (Fig. 3).

To examine whether this proximal conserved region is also sufficient for BMP responsiveness, we subcloned either a single copy or seven copies of this region in front of a −201/+70 *XId3* minimal promoter/*Luc* reporter gene and performed luciferase assays in animal caps (Fig. 4C). Even one copy of the element showed clear induction by BMP signaling, and the 7-mer led to a much stronger induction.

To determine whether the *XId3* BRE was in any way similar to the *Xvent2* BRE, we aligned them and found that the *XId3* BRE is similar to the reverse complement of the *Xvent2* BRE. Both BREs consist of a fully conserved SBE (GTCTG) and a four nucleotide spacer followed by a sequence element similar to the putative OAZ binding site (or 3′ flanking box) previously identified in the *Xvent2* promoter (GCTCCA; Fig. 5A) (Hata et al., 2000). Interestingly, the distal conserved element in the *XId3* promoter also shares the same sequence motifs, suggesting that it, too, is a BRE.

Next, we subcloned various parts of the *XId3* BRE in front of the *XId3* minimal promoter/*Luc* reporter gene and performed luciferase assays (Fig. 5). Only the central region containing the OAZ-binding-site-like sequence and the SBE (−704/−680) responded to BMP stimulation (Fig. 5B). In contrast, we did not observe any BMP response with a construct lacking both the SBE and the OAZ-binding-site-like element (−718/−697) or a construct containing only the SBE (−685/−673; results not shown). Our results are consistent with the notion that both the SBE and the OAZ-binding-site-like element are necessary and sufficient for BMP responsiveness of the *XId3* BRE and the *Xvent2* BRE (Fig. 1) (Hata et al., 2000), while the SBE or the OAZ-binding-site-like element alone is not sufficient for a BMP response. Note that an *Xvent2/Luc* reporter construct that had only the OAZ-binding-site-like element (−167/+29/*Luc*) did not respond to BMP signaling (results not shown).

The Xvent2 and XId3 BREs recapitulate endogenous BMP activity in transgenic embryos

In order to examine how the *Xvent2* BRE responds to endogenous BMP signaling in embryos, we compared the expression patterns of the BRE driving a GFP reporter to that of *BMP4* (Figs. 6A–C). At the gastrula stage, *Xenopus* embryos harboring eight copies of the −173/−151 core BRE drove GFP expression only in the ventral region in most (85%) of the embryos (n = 13; Fig. 6G). At the tailbud stage (Fig. 6H), most of the embryos (n = 10) showed expression in the proctodeum (100%), the ventral branchial arches/heart region (100%), the eyes (100%; 80% of these showed GFP expression in the dorsal eye), and the otic vesicle (70%). These are the regions where *BMP4* is normally expressed (Fig. 6C; compare Fig. 6H). GFP fluorescence in living embryos was observed in similar regions (Fig. 6I). In addition, most embryos expressed GFP in the brain and in the somites.

We also created transgenic embryos containing seven copies of the −718/−673 *XId3* BRE driving a GFP reporter and compared the expression patterns to those of *XId3* (Figs. 6D–F, J–Q). At the gastrula stage, this construct drove reporter gene expression in the ventral mesoderm and ectoderm region of embryos as analyzed both by GFP fluorescence microscopy (Fig. 6J) and by in situ hybridization (100%; n = 10; Figs. 6K, L). Interestingly, most of the gastrula embryos with strong expression also showed expression in the yolk plug (Fig. 6L). At the neurula stage, GFP fluorescence (Fig. 6M) and transcripts (100%; n = 9; Figs. 6N, O) were excluded from the neural plate and observed in the ectoderm. This is consistent with the notion that neural induction occurs in the absence of BMP signaling (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Xu et al., 1995) and with the localization of phosphorylated Smad1 at this stage (Schohl and Fagotto, 2002). At the tailbud stage (Fig. 6P), *GFP* transcripts were detected in the proctodeum (100%; n = 16), the ventral branchial arch/heart region (81%), the eyes (100%), the brain (100%), and the somites (63%). GFP fluorescence was observed in similar regions (Fig. 6Q) and again, these are regions where *BMP4* is

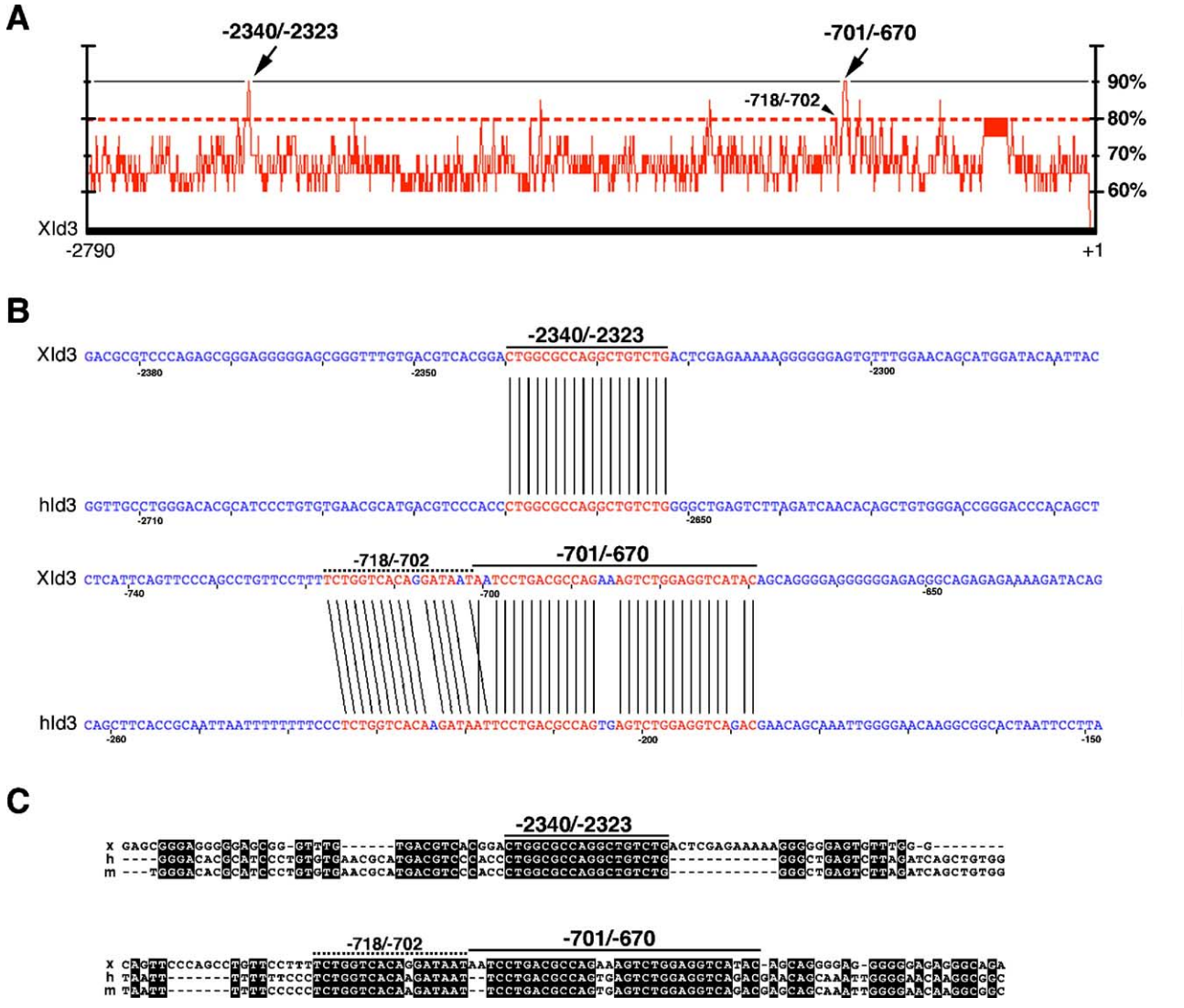

Fig. 3. Identification of highly conserved regions in the *XId3* promoter. (A) Similarity histogram using Family Relations comparing the *Id3* promoters of *Xenopus* (−2790/+1; base sequence shown at *x* axis) and human (−5000/+1). The histogram shows percent similarity over a 20 bp window sliding across the sequences in 1 bp increments. The arrows point to the most highly conserved regions (at 90% similarity cutoff, i.e. at least 90% similar over 20 bp; 90% = thin black line): a distal 18 bp region (−2340/−2323) and a proximal 32 bp region (−701/−670). Note that the Family Relations sequence alignment software does not take into account a gap in the human sequence at −700/−701 of the *Xenopus* sequence, which is why another conserved region immediately adjacent to the −701/−670 region is detected as a separate region 80% similar to the human sequence (arrowhead; 80% = dotted line). (B) Detailed alignment of the conserved sequences identified in panel (A) showing the distal −2340/−2323 region at 90% similarity cutoff and the proximal −718/−670 region at 80% similarity cutoff. (C) Detailed alignment of the *Xenopus*, human, and mouse *Id3* promoter sequences around the two conserved regions using the ClustalW alignment algorithm.

expressed (Fig. 6C). Taken together, these results demonstrate that both the *Xvent2* and the *XId3* BRE drive GFP reporter gene expression in similar regions to where BMP signaling is active in vivo, whereas transgenic embryos created with multimerized SBE or OAZ elements alone driving GFP reporters were unable to recapitulate endogenous BMP activity (results not shown).

In silico human genome scanning identifies prospective BMP responsive genes

As this BRE is conserved among diverse vertebrate species including human and mouse, we attempted to use a computational approach to identify potential BRE containing target genes. This approach may enable us to uncover novel, as yet unidentified BMP targets. To identify additional BRE-like sites in both the human and mouse genomes and potential novel BMP target genes, we scanned the entire human and mouse genome sequences with the core BRE sequence (5′-CCANNN-NGTCTG-3′). 1104 genes were shown to have conserved BREs between species (Supplementary Table 1). Among them, 138 genes contain 2 or more BRE sites (including intronic sites; see Supplementary Table 2). This strict restriction was imposed as a single site comparison between mouse and human genomic sequences may yield BRE

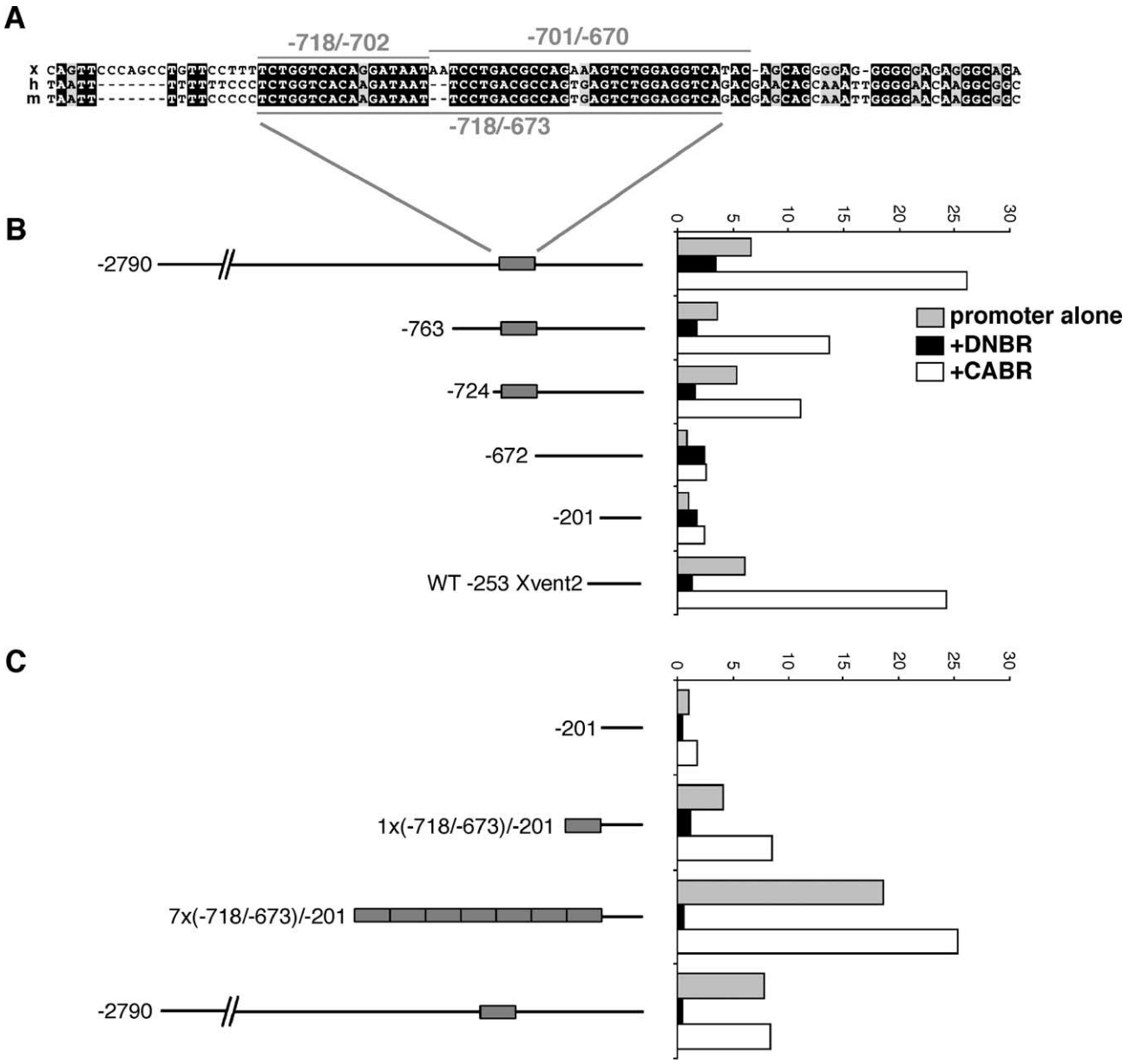

Fig. 4. Identification of a BMP response element in the *XId3* promoter. (A) ClustalW alignment of the *Xenopus*, human, and mouse *Id3* promoter sequences showing the conserved region identified in this figure (lines on top) and the part of the sequence that was used as a concatamer in the reporter gene assays (−718/−673; line underneath; gray box in panels B–C). (B–C) Identification of an *XId3* BMP response element using luciferase assays. (B) Inducibility by BMP signaling decreases after deletion of the most distal part of the promoter (−2790/−763) and is lost completely after deleting the −724/−673 region, which contains the conserved sequence (gray box). Wild type *Xvent2* −253 promoter was injected as a positive control. (C) The conserved −718/−673 region was placed in front of an *XId3* minimal promoter (−201/+70) as 1-mer, 1x(−718/−673)/−201, or as 7-mer, 7x(−718/−673)/−201. Both the 1-mer and the 7-mer restore response to BMP signaling, the 7-mer much stronger than the 1-mer. Note that these luciferase experiments were not performed in the context of the full *XId3* promoter.

sequences by chance due to the extensive regions of homology between these two species.

This in silico search for genes with 2 or more BRE-like motifs identified *Id3* itself in addition to several other genes shown to be upregulated by BMPs including: *HOXA13*, *Dlx5*, *PKC*, *MEF2C*, *sal-like 1 (Drosophila spalt)*, and *PPAR gamma* (peroxisome proliferative activated receptor, gamma), (Feledy et al., 1999; Hay et al., 2001; Knosp et al., 2004; Luo et al., 2001; Nellen et al., 1996; Wang et al., 1999; Zehentner et al., 2000). Interestingly, 6 copies of the BRE were found in *PPAR gamma*, which has recently been identified in a screen for novel BMP targets induced by the overexpression of the BMP Type I receptor in C2C12 cells (Korchynskyi et al., 2003). Note that the human version of *Vent2* (*VENTX2*) was not identified in this search because of a single base pair mismatch in the *VENTX2* BRE sequence when compared to that of the *Xenopus Xvent2* BRE (see Fig.

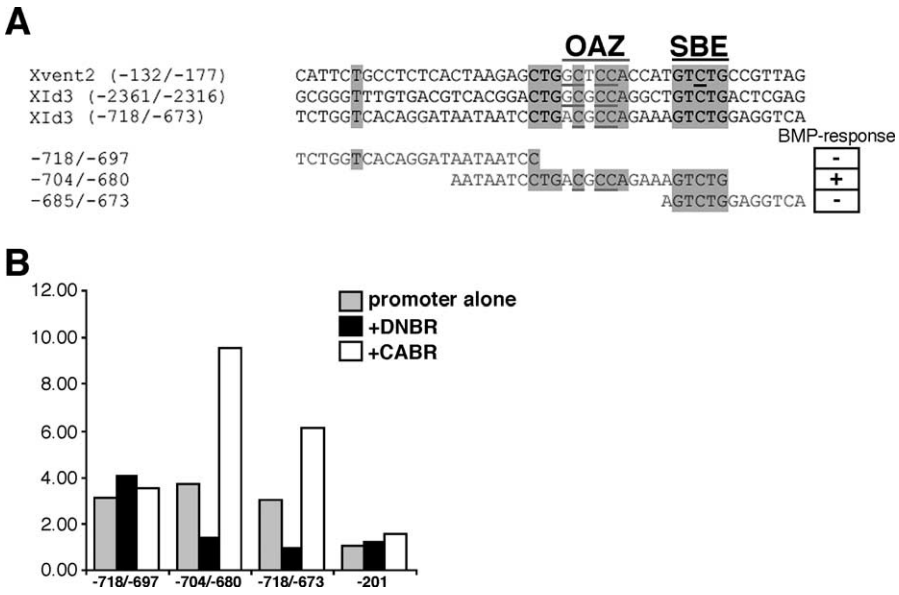

Fig. 5. The *Xvent2* and *XId3* core BREs are similar. (A) Alignment of the opposite strand of the *Xvent2* promoter with the distal (−2361/−2316) and proximal (−718/−673) BRE-like elements in the *Id3* promoter. Note that, in addition to the Smad binding element (SBE), parts of the OAZ binding element (or "3′ flanking box") are also conserved (shaded gray). Underlined positions are where mutations lead to a loss of BMP responsiveness (Hata et al., 2000). Fragments −718/−697, −704/680, and −685/−673, each containing a portion of the *XId3* BRE (−718/−673), were tested in luciferase assays. (B) Luciferase assays in animal caps using some of the constructs shown in panel (A). Only −704/−680, which contains both the conserved SBE and the OAZ binding element, is sufficient for BMP response. In four experiments, there was no response above base line levels for construct −685/−673 alone (data not shown).

1A), thereby excluding it from the results of the genome scanning. Several other genes were found in the single site search, including *Dlx3*, *Id2*, *Insulin-like growth factor 1*, *myosin light chain 2*, *Smad6*, and *FGFR2*, which have all been demonstrated to be genes upregulated by BMPs (Hollnagel et al., 1999; Ishida et al., 2000; Luo et al., 2001; Peiffer et al., 2005).

Analysis of the Xenopus orthologues identified from in silico BRE scanning of the human and mouse genomes

We wished to examine whether the genes identified in silico are in fact BMP inducible in dissociated animal cap tissues. Of the 138 genes with 2 to 6 BRE-like sites, we were able to identify 26 representative *Xenopus* orthologues from the 80 genes with available annotation (Table 1) by reciprocal BLAST searches and the expression patterns of 19 genes of those were analyzed by whole mount in situ hybridization (see "Expression pattern" Table 1, and Fig. 7). Of these 26 genes, 23 samples were successfully PCR amplified (88%) using cDNA prepared from whole embryos at stage 10.5 and of these genes, 18 (69%) were expressed in the animal cap at stage 10.5. We further determined if these genes are inducible by BMP2 in the presence of cycloheximide at the gastrula stage in a similar fashion to the analysis done for *XId3* and *Xvent2*. Out of the entire set of analyzed genes (n = 26), 13 (50%) are direct BMP targets. 5 genes (19%) were inducible by CHX alone and did not show a significant change after BMP treatment, and 5 genes (19%) were neither expressed in animal caps nor induced by BMP. It is unclear whether these 11 genes represent BMP targets at later stages in development or perhaps may be regulated by other factors.

Additionally, we have also attempted to determine how these genes performed in our recent microarray experiments (Peiffer et al., 2005) where dissociated animal cap cells are treated with a combination of BMP2 (3 ng/ml) and CHX to identify novel direct BMP targets. It was in these screens where we identified both *Xvent2* and *XId3*. Although direct comparison between the microarray data and the data presented here is somewhat difficult as different concentrations of BMP are used (RT-PCR data shown in Table 1 utilize a much higher concentration of BMP2; 15 ng/ml), for the reason that known BMP target genes such as *Xvent2* and *XId3* respond at an elevated level (see Fig. 2), we do find a correlation between the in silico data and the microarray results. Unfortunately, a majority of the genes analyzed here were not available for analysis using our microarray system, as they are components of a gastrula library not spotted onto our current microarray slides. Additionally, cutoff values (2 fold and higher) used previously have removed some of these genes from the analysis. Nevertheless, genes such as *Id3* (9.0 fold), *Protein kinase C-like-2* (1.8 fold), and *Protein kinase C* (1.9 fold) did show induction in these microarray experiments. Again, the induction level of *BMP2* may be low in this case when 3 ng/ml of BMP2 is used while the RTPCR analyses shown here utilize 15 ng/ml. We have previously found that some BMP target genes respond differently depending on BMP concentrations. On the whole, genes that are not affected by BMPs were by and

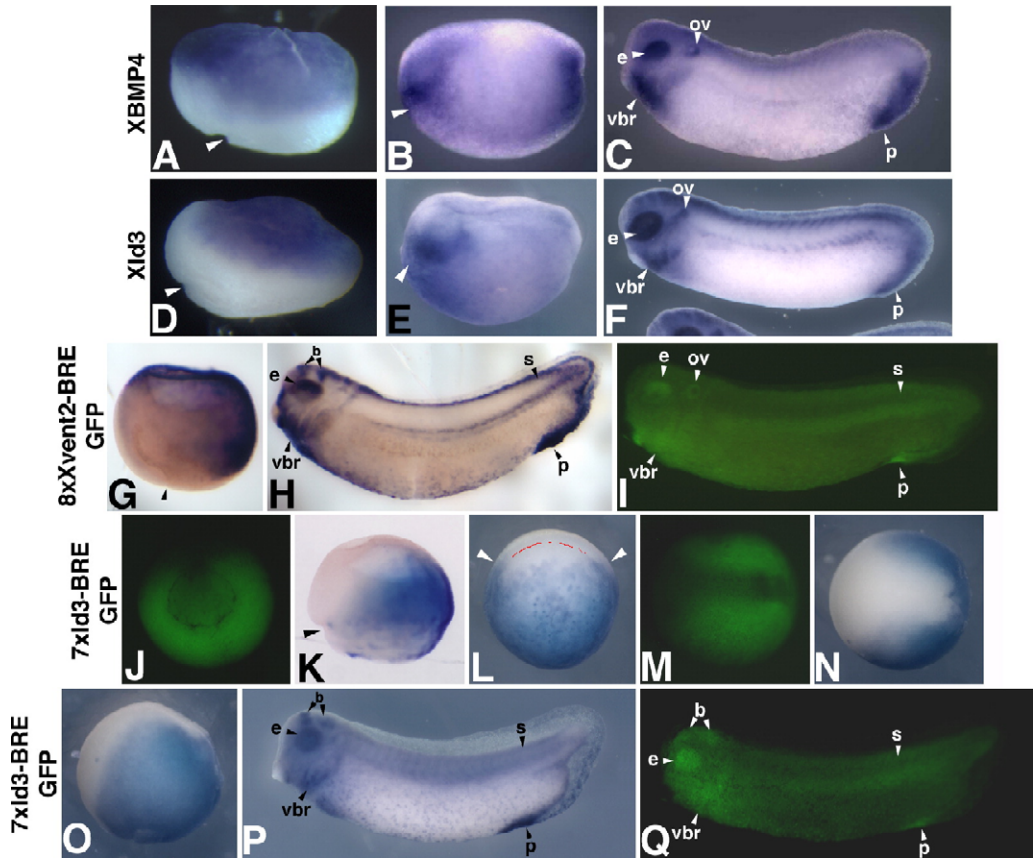

Fig. 6. The *Xvent2* and *XId3* BREs direct expression of a GFP reporter gene to regions of BMP activity in transgenic frogs. Expression of *XBMP4* compared to *XId3* expression in gastrula, neurula, and tailbud stage embryos is shown in panels (A–C) and (D–F), respectively. Unless otherwise indicated, for gastrula stages, dorsal is to the left and the animal pole up. For neurula stages, anterior is to the left and dorsal is up. For tailbud stages, lateral views are shown. (A, D) At the gastrula stage (10.5), both genes are expressed in the animal cap and excluded from part of the area above the dorsal blastopore lip (arrowheads). (B, E) At the neurula stage (14–15), both *XId3* and *XBMP4* are expressed in the future cement gland (arrowhead) and the ventral region. (C, F) At tailbud stages (27–28), both genes are expressed in the proctodeum (p), the ventral branchial arch/heart region (vbr), the eyes (e), and the otic vesicle (ov). (G–I) Expression of the 8x*Xvent2*-BRE/GFP-reporter gene analyzed in transgenic embryos. (G) Gastrula stage embryo (stage 10.5–11); arrowhead, dorsal blastopore lip. *GFP* mRNA is expressed on the ventral side only, including the ventral marginal zone and the ventral ectoderm. (H) Stage 32 embryo; stained with antisense GFP mRNA. (I) Stage 32 embryo, showing GFP protein fluorescence of the eye (e), ventral branchial arch/heart region (vbr), otic vesicle (ov), somites (s) and proctodeum (p). (J–Q) Expression of the 7x*Id3* BRE/GFP reporter gene in transgenic embryos; (J, M, Q) fluorescence of GFP protein; (K, L, N, O, P) expression of GFP mRNA. (J) Stage 11 embryo, vegetal view, dorsal is up. GFP protein is expressed ventrally and laterally but not dorsally. Note that there is also fluorescence in the yolk plug. (K) Stage 10.5 embryo; arrowhead, dorsal blastopore lip. GFP mRNA expression is excluded from the dorsal side. (L) Stage 10.5 embryo, uncleared, vegetal view, dorsal is up; white arrowheads show the limits of the ventral expression; red line, dorsal blastopore lip. Expression is observed only on the ventral side and in the yolk plug. (M) Neurula stage embryo (stage 14), dorsal view, anterior is to the left. GFP protein fluorescence is excluded from the neural plate. (N) Same embryo as in panel (M), dorsal view, anterior is to the left. GFP mRNA expression is excluded from the neural plate. GFP mRNA appears to be expressed in a slightly smaller domain than that of the GFP protein shown in panel (M) likely to due the fact that some of the mRNA disappears by this stage. (O) Same embryo as in panels (M, N), lateral view. GFP mRNA expression is observed only ventrally and is excluded from the neural plate. (P) Stage 32 embryo, stained with antisense GFP mRNA. (Q) Same embryo as in panel (P), but showing GFP protein expression.

large not induced by BMP in our microarray experiments. Overall, we were able to identify potential BMP targets using in silico approaches and provide in vivo data to verify the extent of such analyses.

Discussion

In this study, we have used a combination of interspecies sequence comparison (phylogenetic footprinting), luciferase assays, and transgenic GFP-reporter gene experiments to identify and characterize BMP response elements of *Vent2* and *Id3* in vivo. The phylogenetic footprinting approach has already been successfully used, for example to identify a conserved SBE essential for BMP-dependent cardiac expression of *Nkx2-5* (Lien et al., 2002) and to identify enhancers of the chicken *Sox2* (Uchikawa et al., 2003) and human *DACH* genes (Nobrega et al., 2003). However, to our knowledge, the current study is one of only a few where phylogenetic footprinting is used successfully to predict and then experimentally test the role of growth factor response elements in developing vertebrate embryos and further

Table 1
Xenopus orthologues to human genes with 2 or more BRE-like sequences

Gene description	Human accession no	NIBB clone ID	MA fold	BRE *n*	RT-PCR analysis					Expression pattern												Available reference (expression)
						WE	AC	CHX Only	CHX BMP2		Gastrula				Tailbud								
					cat					cyc	ap	de	ve	mz	he	so	e	ov	ba	pn	nc	cns	
Id3	NP_002158	XL013b11	9.0	2	strong					28	+	+	+	+	+/-	+	+	+	+	-	-	+	Wilson and Mohun, 1995
Zinc Finger homeobox 1b (SIP1)	NP_055610	XL207j23	na	4						30	+/-	-	-	-	-	-	+	+	+	+	+	-	Nitta et al., 2004
Hypothetical protein FLJ20364	NP_060255	XL211p03	na	2						30	+	+	+	+/-	-	-	+	+	-	-	-	+	This paper
STK 3 (STE20 homolog, yeast)	NP_006272	XL198p03	na	2						28	+	+	+	+	+/-	+	+	+	+	+/-	-	+	Hemmati-Brivanlou et al., 1992
snRPE	NP_003085	XL155f08	na	2						28	+/-	-	-	-	-	-	+	+	+	+	+	+	This paper
AMMECR1	NP_056180	XL210o15	na	2						28	+	+/-	+/-	+/-	-	+/-	+	+	+	+/-	-	+	This paper
PTPN3	NP_002820	XL087f05	nd	2	weak					30	-	-	-	-	-	-	-	-	-	-	-	-	This paper
Protein kinase C-like-2	NP_006247	XL052b21	1.8	3						30	+	-	-	-	-	-	+/-	+/-	-	-	-	+	This paper
BMP2	NP_001191	XL096l22	1.1	2						30	+	+	+	+	+	+	+	+	+	+	+	+	Nishimatsu et al., 1992
Distal-less homeo box 5 (DLX5)	NP_005212	XL159o04	na	2						32	+/-	-	-	-	-	-	-	+	+	-	-	+	Luo et al., 2001
Lim domain kinase 2 (LIMK2)	NP_057952	no hits	na	2						30			ne				not examined					+	Takahashi et al., 2001
Hypothetical protein FLJ20436	NP_060292	XL216a01	na	2						32	+	+/-	+/-	-	-	-	+	+	-	-	-	+	This paper
Protein kinase C mu	NP_002733	XL020g13	1.9	3						32	+	-	-	-	-	+/-	+	+	-	+	-	+	This paper
Chromosome 11 ORF 8	NP_001575	no hits	na	3	no change					32			na				na						none
Casein kinase 2 alpha 1	NP_001886	XL034d13	1.2	2						28	+	+/-	+/-	-	-	+/-	+	+/-	+	+/-	-	+	This paper
Alpha aminotransferase (AADA)	NP_057312	XL092n04	1.2	2						30	+	+	+	+	-	+	-	-	+	-	-	+	This paper
Olfactomedin 4 (OLFM4)	NP_006409	XL041l03	-1.3	2						30	-	-	-	-	-	-	-	-	-	-	-	-	This paper
TRPS1	NP_054831	no hits	na	3						30			na				na						none
Sal-like 1 (Drosophilia spalt)	NP_002959	XL217i18	na	2	no data					32	+/-	-	-	-	-	-	-	+	+	-	-	+	Hollemann et al., 1996
FLRT3	NP_037413	XL050c15	nd	2						32	-	-	-	+	-	-	-	-	+	-	-	+	Bottcher et al., 2004
HOXA13	NP_000513	no hits	na	2						32			ne				not examined						Endo et al., 2000
KIAA1497 protein	NP_065924	XL105d03	1.2	2						32	+	+	+	+/-	-	-	+	-	-	+	+	+	This paper
Follistatin	NP_006341	XL052g09	-0.2	2						32	+/-	-	-	-	-	-	+	-	-	+	+	+	Fainsod et al., 1997
Wnt11	NP_004617	XL084o21	-1.0	3			no data				+/-	-	-	-	+	-	-	-	-	-	-	+	Tada and Smith, 2000
Dachshund (DACH1)	NP_542937	XL070p11	1.0	2			no data				+/-	-	-	-	-	-	+	-	-	-	-	+	Wu et al., 2003
MEF2C	NP_002388	XL108m05	na	2			no data						ne		+		not examined						Dodou et al., 2004

utilize these consensus sequences to identify additional elements in the mammal, potentially uncovering novel target genes and links to other signaling pathways and networks.

On the utility of Xenopus to identify gene regulatory elements

Comparison of *Xenopus laevis* with human and/or mouse genome sequences identified two similar BREs in the *Id3* promoter and another BRE-like sequence in the *Vent2* promoter. Intriguingly, all three elements were among the sequences most highly conserved between *Xenopus* and human or mouse. We also find that the sequence similarities between the promoter regions of mouse and human were too extensive to identify BRE-like sequences in the *Id3* promoter (Fig. 3C; data not shown) likely due to the relatively close evolutionary distance between human and mouse. While interspecies sequence comparison, such as between *Xenopus* and human is quite useful, we note that in some cases, a human/mouse alignment has successfully uncovered conserved regulatory regions, due to different nucleotide divergence rates along different genomic loci (Hardison, 2000; Hardison et al., 2003; Waterston et al., 2002).

It should be emphasized that the outcome of phylogenetic footprinting is significantly influenced by the choice of search parameters such as window size and minimum percentage similarity. Since the invariant core sequences of most transcription factor binding sites are quite short (4–8 bp) (Pennacchio and Rubin, 2001), it would be ideal to perform phylogenetic footprinting analysis using similarly small window sizes (e.g. 10 bp). However, window sizes shorter than 20 bp are unlikely to yield meaningful results because, even with a 100% similarity cutoff, a comparison of two random sequences will probably yield many (random) hits, i.e. false positives (C.T. Brown, pers. comm.). Therefore, we chose to use a window size of 20 bp with relatively high percentage similarity cutoffs of 75–90%. With this window size and cutoff value, the identified conserved elements represent more than single 4–8 bp transcription factor binding sites as the identified element needs to be of at least 15 bp in length in order to survive the analysis. In case of the *Id3* promoter, this approach identified a core BRE and additional conserved sequence that surrounds the BRE. While it is unclear at present what the surrounding sequence represents, it may indicate an intricate regulatory network involved in BMP signaling.

The Vent2 and Id3 BREs are similar

Comparisons between the *Xenopus* and human or mouse *Id3* promoter sequences reveal two most highly (at least 90% over 20 bp) conserved regions of 18 bp (−2340/−2323) and 49 bp (−718/−670) length that are similar to each other and to the *Xvent2* BRE. The common sequence represents a core BRE with an SBE and a GC-rich sequence similar to the OAZ binding site (Fig. 5A). This suggests that BMP induction of the *Id3* and *Vent2* genes may involve similar DNA binding factors, Smads and OAZ. Alternatively, the OAZ binding site could be recognized by a factor other than OAZ. At the *Xenopus* tailbud stage, the expression of OAZ (Hata et al., 2000) does not overlap that of *XId3*, *Xvent2*, or *BMP4*, while expression of these latter three genes are very similar to each other in the embryo (a synexpression group). These observations suggest that BMP-mediated induction of *XId3* and *Xvent2* is regulated by a factor other than OAZ in the tailbud stage embryo.

The *Id3* and *Vent2* BREs share similarities with a BRE found in the *Id1* promoter (Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). The *Id1* BRE also has an SBE and a GC-rich palindromic sequence, GGCGCC, both of which are essential for the response of the *Id1* promoter to BMPs. This palindromic sequence is also found in the *Id3* BRE (GNCGCC; Fig. 5A), where it largely overlaps with the conserved OAZ-binding-site-like sequence. In the *Id1* promoter, the GC-rich palindromic sequence can bind Smad4 and 5 (Korchynskyi and ten Dijke, 2002), and the SBE has been shown to preferentially bind Smad4 in the

Notes to Table 1:

Using reciprocal BLAST searches, 26 *Xenopus* orthologues were found in available NCBI sequence databases. Each gene is shown (Gene description) with its respective human accession number (Human accession number). If available, each representative *Xenopus* cDNA clone was selected from (http://www.Xenopus.nibb.ac.jp) and its behavior in recent microarray experiments is noted (MA fold). The number of BRE sites found in the human sequence for each gene as a result of the genome searching is shown (BRE *n*). RT-PCR analysis was performed to assess if each gene is expressed in the animal cap of a gastrula (stage 10.5) embryo as well as for inducibility by BMP2 in the presence of CHX. Shown are PCR results like that shown in Fig. 2. The PCR cycle number for each gene is shown (cyc). The RT-PCR results have been divided into 4 categories based on available data shown here and are denoted by arrows; strong, strong induction by BMP2 in the presence of CHX; weak, weak induction; no change, no discernible difference, and no data; no data are available in dissociated animal cap tissues (6 genes, representing 23% of the total genes were classified as “strong”, 7 genes, representing 27% of the total were classified as “weak”, 5 genes (19%) did not show a change and there were no data available for 5 genes, 19% of the total). If available, *in situ* hybridization patterns at gastrula (stage 10.5) were scored specifically for expression solely in the animal pole ectoderm (ap), a site with high BMP activity, the dorsal ectoderm (de), the ventral ectoderm (ve), and the marginal zone cells (mz) (−, no specific expression; +/−, weak/moderate expression; +, strong expression) if such data were available for analysis. Expression in tissues/organs was scored at the tailbud stage (Fig. 7). Abbreviations are as follows: he, heart; so, somites; e, eye; ov, otic vesicle; ba, branchial arches; pn, pronephros; nc, neural crest, and cns, components of the central nervous system (−, no specific expression; +/−, weak/moderate expression; +, strong expression). Finally, if the expression pattern of a gene has been published previously, its citation is noted (Available reference). na; not applicable, nd; no data, and ne; the expression pattern has been studied but not examined in this tissue or organ. Expression patterns of several genes have been studied only in components of the central nervous system (cns) and/or not in other regions of early development.

Nitta et al. (2004); Hemmati-Brivanlou et al. (1992); Takahashi et al. (2001); Holleman et al. (1996); Bottcher et al. (2004); Endo et al. (2000); Fainsod et al. (1997); Tada and Smith (2000); Wu et al. (2003); Dodou et al. (2004)

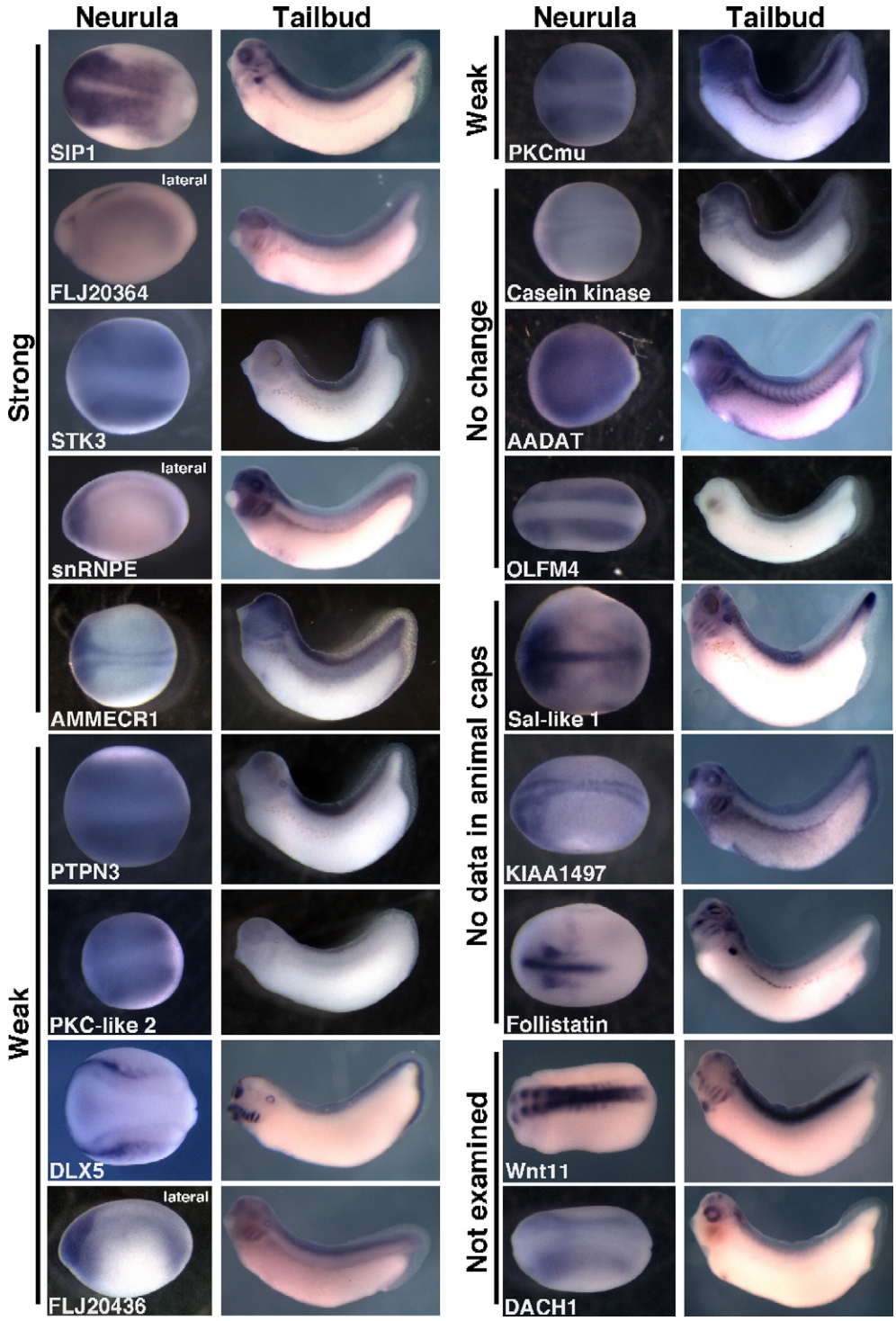

Fig. 7. Expression patterns of *Xenopus* orthologues to human genes with multiple BREs. Of the 26 representative orthologues from the 80 genes with available annotation in *Xenopus*, the expression patterns of 19 genes were analyzed by whole mount in situ hybridization. Expression patterns are organized based on the behavior of each gene in the RT-PCR experiments shown in Table 1 (strong, weak, no change, no data, not examined). Not shown are *Id3* (Fig. 3) and *BMP2* (Nishimatsu et al., 1992). Shown are the patterns at the neurula and tailbud stages for each gene; refer to Table 1 for a description of each pattern at the gastrula stage. Unless otherwise indicated, for neurula stages, anterior is to the left and dorsal is up. For tailbud stages, lateral views are shown.

context of the *Xvent2B* and *Id1* promoters (Henningfeld et al., 2000; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). Thus, an alternative model consistent with the currently available data is that Smad4 binds to the SBE of the *Id3* BRE and BMP-specific R-Smads bind to the GC-rich OAZ-binding-site-like sequence. This notion is consistent with the recent report in *Drosophila* suggesting that a Dpp-dependent silencer element interacts with the Smads

Mad and Medea to recruit the repressor protein Schnurri to confer BMP-induced repression (Pyrowolakis et al., 2004). According to this model, the OAZ binding site is required for BMP-responsiveness although this site could interact with Smads. Further analysis is required to address this issue and the in vivo relevance of the existing Smad binding data to these BREs.

The palindromic GC-rich motif (GGCGCC) found in the *Id1* BRE, which overlaps with the OAZ-binding-site-like sequence in the BREs identified in this study, has been predicted to be a common feature of all BREs (Korchynskyi and ten Dijke, 2002). While our work was in progress, another study (Karaulanov et al., 2004) also identified BREs with this motif in the *Smad7* and *BAMBI* promoters, consistent with the notion that this motif may indeed be a common feature of all BREs. Using this motif, these authors predicted a BRE in the human and mouse *Id3* promoters corresponding to the upstream BRE (−2340/−2323) in the *Xenopus Id3* promoter identified in this study. However, the downstream *Id3* BRE (−704/−680) escaped detection, presumably because the GC-rich motif in the downstream *Id3* BRE (−704/−680; GACGCC) is slightly different from the one found in the upstream BRE (−2340/−2323; GGCGCC). Our results suggest that not all nucleotides of the GGCGCC sequence are required for a BRE to function, as neither the *XId3* BRE nor the *Xvent2* BRE characterized in this study contain a perfect copy of this motif (Fig. 5) while still being sufficient for BMP response.

The number of BREs may affect the sensitivity to BMP signaling

We have shown that a single copy of the *XId3* BRE is sufficient for a clear response to BMP signaling when placed in front of a minimal promoter (Figs. 4C, 5B). Moreover, a 7-mer of the *Id3* BRE leads to a much stronger response than a 1-mer (Fig. 4C). Similarly, the −2790/+70/*Luc* reporter construct, which contains both the distal and proximal BREs, shows a stronger BMP response than the −724/+70/*Luc* part of the promoter, which contains only the proximal BRE (Fig. 4B). Together, these results suggest that the number of BREs can control the sensitivity of a gene to BMP signaling. Since, in our microarray experiments, *XId3* was consistently one of the most strongly induced BMP target genes, it is conceivable that genes such as *Id3* are more sensitive to BMP signaling due to the presence of multiple BREs in the promoter and/or in other regions (e.g. intronic; there is a third conserved BRE-like sequence in intron 2 of the human, mouse, and zebrafish *Id3* genes; not shown).

The Vent2 and Id3 BREs direct GFP reporter gene expression to regions of endogenous BMP signaling

The *Xvent2* and *XId3* BREs drive similar GFP expression patterns, demonstrating that the sequence similarity between these elements reflects a common regulatory function. In addition, both BREs drive GFP in areas of endogenous BMP signaling in the embryo, suggesting that these elements function as BREs in vivo (Fig. 6). In gastrula embryos, both BREs direct GFP expression to ventral regions (Figs. 6G, J, K, L), which is where endogenous BMP signaling is active, as visualized by the distribution of phosphorylated Smad1 (Faure et al., 2000; Schohl and Fagotto, 2002). Interestingly, most of the transgenic *XId3*-BRE/GFP gastrula embryos showed expression in the yolk plug. This is consistent with the previously reported distribution patterns of phosphorylated Smad1 (Schohl and Fagotto, 2002) and the observation that *BMP2*, *4*, and *7* are expressed maternally (Dale et al., 1992; Hawley et al., 1995; Nishimatsu et al., 1992). We do not find endodermal GFP expression in the *Xvent2*-BRE/GFP transgenic embryos, although staining this region is difficult and in this case may be too weak to detect.

At the neurula stage, the *XId3* BRE directs GFP expression to ventral regions, while expression is absent from the dorsal side (Figs. 6M–O). This is consistent with the distribution of phosphorylated Smad1 at a similar stage (Schohl and Fagotto, 2002), but it is different from *BMP4* expression, which is most strongly expressed in an antero-ventral and postero-ventral domain (Fig. 6B). This discrepancy may suggest that *XId3*-BRE/GFP responds not only to signaling activated by BMP4, but also by BMP2 and/or 7 or a combination of these signals. At tailbud stages, both the *Xvent2* and *Xld3* BREs direct GFP reporter gene expression to the eyes, the proctodeum, and the ventral branchial arch/heart region (Figs. 6H, I, P, Q), which is where *BMP4* is expressed (Fig. 6C) and where phosphorylated Smad1 has been observed (Schohl and Fagotto, 2002). Some embryos also showed expression in the otic vesicle and in the brain, where *BMP4* expression is observed (Fig. 6C) (Christian and Nakayama, 1999; Nakayama et al., 1998). However, most embryos showed expression in the somites (Figs. 6H, I, P, Q), where *BMP4* is not expressed. Since phosphorylated Smad1 and *BMP2* are expressed in the somites (Hemmati-Brivanlou and Thomsen, 1995; Schohl and Fagotto, 2002), this may reflect the fact that both the *Xvent2* and *XId3* BREs respond to endogenous BMP2 signaling in the somites. Alternatively, it is possible that these isolated BREs do not exactly recapitulate all aspects of BMP expression, and the ectopic expression in the somites could be a nonspecific effect, perhaps due to a position effect or to the multimeric nature of the BRE. Similar ectopic expression was also observed by Karaulanov et al. (2004) in transgenic embryos harboring a 53 bp BRE of the *Xvent2* promoter published by Hata et al. (2000). We also note that our transgenic expression patterns appear to be less variable than the ones observed by Karaulanov et al. (2004). One possible explanation for this discrepancy is that, in our study, we have narrowed down the *Xvent2*-BRE to a 23 bp sequence that is still sufficient for BMP responsiveness and also conserved, whereas Karaulanov et al. use the longer 53 bp sequence (Hata et

al., 2000) which may include additional sequence not related to BMP responsiveness.

Our data are consistent with the notion that a subset of BMP target genes that share similar expression patterns, known as synexpression group genes (Niehrs and Pollet, 1999), share a common BRE regulatory motif to coordinate the expression. Here, we show that, using a specific BRE reporter, one cannot only measure, but also visualize the transcriptional response of cells toward BMPs in the embryo. Since the major features of BREs appear to be conserved between both mammals and *Xenopus*, we anticipate that the BRE reporter gene constructs we describe in this study will give researchers an important tool in the future to study BMP signaling in many different systems, ranging from live embryos to mammalian cell lines.

Numerous additional BRE-like sequences are present in the human genome

We have also shown that it can be effective to use our short BRE consensus sequence to scan the human and mouse genomes to identify prospective BMP target genes (Table 1, Supplementary Table 1). Though a similar approach was recently implemented in *Drosophila* (Pyrowolakis et al., 2004), whose genome size is approximately 1/16 that of the human, we believe this to be the first study to demonstrate the effectiveness of using whole genome scanning to identify growth factor response elements in mammals. It should be noted that the assays described here were preformed at only one stage of development (st. 10.5, gastrula) and a number of the *Xenopus* orthologues examined here do not show expression in any embryonic region at this stage. Perhaps some of the genes that we were not able to verify using these techniques are BMP targets at a much later stage in development, beyond the current stage of analysis and other in vivo confirmation will be needed to verify the role of each candidate BRE. It should also be noted that many of the BMP targets (80%) we previously identified by microarrays (Peiffer et al., 2005) do not have the typical BRE we identified here (Supplementary Table 1), possibly due to the stringency of the search used. Alternatively, those genes could represent a subset of target genes that may be regulated by different mechanisms. With further refinement, this type of short nucleotide search could be combined with microarray data to extract more meaningful information when linked together with in vivo verification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.02.014.

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