A *Xenopus* DNA Microarray Approach to Identify Novel Direct BMP Target Genes Involved in Early Embryonic Development

Daniel A. Peiffer,[1] Andreas Von Bubnoff,[1] Yongchol Shin,[1] Atsushi Kitayama,[2] Makoto Mochii,[2†] Naoto Ueno,[2] and Ken W.Y. Cho[1*]

Bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor-beta (TGF-β) superfamily, were originally isolated from bone on the basis of their ability to induce ectopic bone development. Although BMPs are involved in a wide range of developmental and physiological functions, very few vertebrate target genes in this pathway have been identified. To identify target genes regulated by the BMP growth factor family in *Xenopus*, large-scale microarray analyses were conducted to discover genes directly activated by this factor in dissociated animal cap tissues treated with a combination of the protein synthesis inhibitor cycloheximide and BMP2. Consequent expression patterns and behaviors of the most highly induced genes were analyzed by in situ and reverse transcriptase-polymerase chain reaction analyses. Here, we describe two sets of the most highly induced direct BMP target genes identified using microarrays prepared from two different stages of early *Xenopus* development. A wide variety of genes are induced by BMP2, ranging from cell cycle controllers, enzymes, signal transduction cascade components, and components of the blood and vascular system. The finding reinforces the notion that BMP signals play important roles in a variety of biological processes. *Developmental Dynamics 232:445–456, 2005.*

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INTRODUCTION

Decades of research in both invertebrates and vertebrates have now shown that bone morphogenetic proteins (BMPs) are involved in a broad assortment of developmental and physiological functions in many different types of tissues and organ systems. For instance, BMPs are required for axis specification during embryonic development as well as growth and tissue patterning in a wide variety of organs in a range of organisms, including flies, frogs, mice, and humans. They also play roles in apoptosis/programmed cell death, cell cycle progression, and blood formation (Hogan, 1996; Huber and Zon, 1998; Dale and Jones, 1999). Defects in BMP signaling are known to cause human diseases (e.g., familial primary pulmonary hypertension and bone cancer) as well as birth defects (Gobbi et al., 2002; Zhang et al., 2002). Accordingly, a comprehensive understanding of the mechanisms of BMP signaling is fundamental to revealing exactly how mutations of BMPs and their downstream signaling components lead to developmental defects. Furthermore, subsequent identification of target genes regulated by these growth factors possibly may provide novel diagnostic tools to detect changes from normal to abnormal cell or tissue states as well as further un-

[1]Department of Developmental and Cell Biology, University of California, Irvine, California
[2]Department of Developmental Biology, National Institute for Basic Biology, Okazaki, Aichi, Japan
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[†]Dr. Mochii's present address is Graduate School of Life Science, University of Hyogo, Kamigori, Hyogo, Japan.
***Correspondence to: Ken W.Y. Cho, Department of Developmental and Biology, University of California, Irvine, California. E-mail: kwcho@uci.edu**

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derstanding of the basic developmental process regulated by BMPs and potential molecular links to other signal transduction cascades.

In likeness to other TGF-β signals, the transduction of BMP signals begins with the interaction between type I and II transmembrane serine/threonine receptors (Shi and Massague, 2003). After the formation of this receptor complex with the BMP ligand, the type II receptor phosphorylates the type I receptor, which then further transmits intracellular signals by phosphorylating the BMP pathway-specific receptor-regulated Smads, Smad 1, 5, and 8 (R-smads). These R-Smads then dissociate from the receptors, bind to Co-Smad (Smad4), and translocate to the nucleus, where heteromeric R-Smad/Smad4 complexes regulate the transcription of specific target genes, most probably by associating with other DNA-binding coactivators (von Bubnoff and Cho, 2001). Despite the multitude of cellular processes that BMPs play roles in (including development and cellular homeostasis), only a small number of direct BMP target genes have been identified using CHX as a criterion. Some of these genes include *Xvent2* and *Xvent2B*, *msx-1*, *msx-2*, *Id1*, *Id2*, *Id3*, *GATA2*, *Dlx-5*, and *Tob* (Ladher et al., 1996; Hollnagel et al., 1999; Miyama et al., 1999; Rastegar et al., 1999; Yoshida et al., 2000; Friedle and Knochel, 2002; Korchynskyi and ten Dijke, 2002; Hussein et al., 2003).

The expression patterns of the various BMPs have been well characterized during early *Xenopus* development, and the tissues and organs that express BMPs are known. During gastrulation, *BMP2* is expressed at a low level throughout the ectoderm and marginal zone (Hemmati-Brivanlou and Thomsen, 1995). *BMP4* transcripts are expressed in all three germ layers at the beginning of gastrulation and are cleared from the dorsal ectoderm and mesoderm by late gastrulation (Dale et al., 1992). Similar patterns of BMP activity in the gastrulating embryo are seen when observing the distribution of phosphorylated Smad1 (Faure et al., 2000). At later stages, *BMP2* and *BMP4* transcripts are associated with a variety of mesodermal structures, including the pharyngeal pouches, heart, and blood island (Clement et al., 1995). At tailbud stages, *BMP2* is expressed in neural tissues, including the neural tube and brain, as well as the heart, somites, eye, and otic vesicle (Nishimatsu et al., 1992). At tailbud stages, *BMP4* is expressed in some similar locations including the eye, otic vesicle, and heart but is not expressed in the somites as is *BMP2*.

To better understand BMP signaling processes, we have implemented DNA microarray technology to assist in the identification of novel direct BMP target genes using the *Xenopus* animal cap assay system. In addition to previously well-characterized BMP target genes, we have identified over 20 novel promising direct BMP target genes and analyzed the expression patterns of these genes. Analysis of these expression patterns shows that some of these genes fall into a BMP synexpression group, i.e., at the tailbud stage, expression of many of these genes can be found in areas where *BMP2* is expressed, including the brain, heart, somites, eye, and otic vesicle. Furthermore, some of these newly identified genes bear no homology to what is represented in existing databases.

RESULTS

Isolation of BMP Targets Using Microarrays

Animal cap explants were removed from a mixture of early gastrula stage (stage 8–9) *Xenopus* embryos obtained from three different sets of eggs each fertilized in vitro, and the explants were divided into two populations. Because intact animal cap explants secrete endogenous BMPs, dissociation of the animal caps into single cells is necessary before treatment with BMP2. By first dispersing the explant cells in Ca^{2+}/Mg^{2+}-free Barth's saline solution, the endogenous BMPs are diluted and the cellular response becomes dependent on the amount of exogenously added BMPs (Wilson and Hemmati-Brivanlou, 1995). The two populations of dissociated animal cap cells were then soaked in medium containing the protein synthesis inhibitor cycloheximide (CHX) at 5 μg/ml plus BMP2 protein at a final concentration of 3 ng/ml, or cycloheximide only. By doing so, we are able to induce target genes that do not require any additional protein synthesis; therefore labeled "direct" target genes. To ensure that cells remained dissociated for the duration of the treatment, the medium was disturbed every 15 min by vigorous pipetting. Here, we hope to use cycloheximide as "background" as it is included in both experimental channels. Although treatment of animal cap cells with cycloheximide alone is known to induce some genes (e.g., *Xmsx-1*, see Fig. 1B), thus masking the effect of BMPs, we were more interested in selectively obtaining bona fide target genes rather than identifying a mixture of direct and indirect target genes.

During gastrula stages (stage 10.5–11), total RNA samples (50 μg) were extracted from the aforementioned cultured animal cap explants (CHX, CHX+BMP2; Fig. 1A). From the resulting RNA, cDNA was synthesized and labeled with Cy3 (control; CHX, green) and Cy5 (experimental; CHX+BMP2, red), respectively. This cDNA was used as probes in hybridization experiments on an arrayed neurula and tailbud stage cDNA library prepared from two stages of development; a mixture of cDNAs from stage 15 and stage 25 are printed onto Set1 slides and cDNAs from stage 25 are printed onto Set2 slides (for specific procedures, please refer to Shin et al., 2005).

Results, in triplicate, were compiled to form a list of genes up-regulated upon the growth factor treatment along with statistical *P* values. *P* values and fold-induction levels were calculated using the *Cyber-T* program with Bayesian statistical analysis to determine confidence levels for each spot value in the replicates (http://visitor.ics.uci.edu/genex/cybert/index.shtml; Baldi and Long, 2001; Long et al., 2001). The clones with the highest induction levels were selected from our cDNA library. For a schematic diagram of this aforementioned microarray process, refer to Figure 1A.

For reasons that are unclear, the fold-induction levels between each set were different, one being much higher than the other. As it is difficult to directly compare the results from both sets, despite finding control genes (e.g., *Xvent2*, *XId3*) appropriately in-

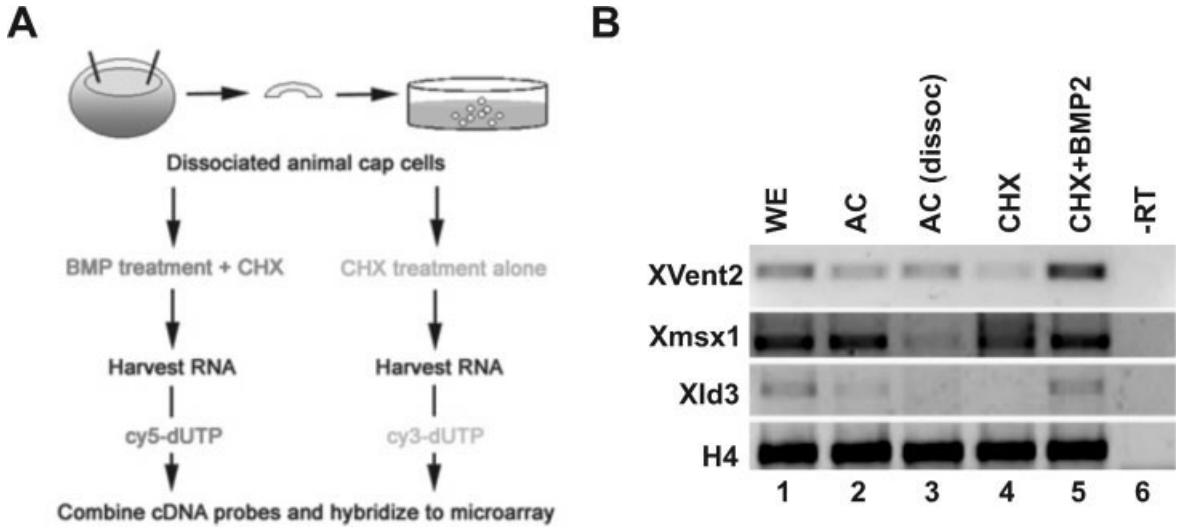

Fig. 1. Microarray procedures used to identify direct bone morphogenetic protein (BMP) target genes. **A:** Animal caps were cut at blastula stages 8–9, left intact or cultured in Ca^{2+}/Mg^{2+}-free Barth's saline solution to dissociate the cells, and incubated in 5 μg/ml cycloheximide (CHX) with or without 3 ng/μl BMP2 protein. During gastrula stages (stage 10.5–11), 50 μg of total RNA was harvested, and cDNA was synthesized and labeled with Cy3 (control; CHX, green) and Cy5 (experimental; CHX+BMP2, red), respectively, and used in microarray hybridizations on an arrayed neurula and tailbud stage cDNA library. **B:** RT-PCR analysis using cDNA from A. Lane 1, whole embryo (WE) cDNA is from uninjected embryos. Lane 2, intact animal caps that are untreated (AC). Lane 3, untreated animal caps that have been dissociated into single cells (AC dissoc). Lane 4, dissociated animal cap cells treated with CHX only (CHX). Lane 5, dissociated animal cap cells treated with CHX plus BMP2 (CHX+BMP2). Lane 6, reverse transcriptase (-RT) control. Here, we have used *Xvent2*, *Xmsx-1*, and *Xld3* as marker genes to confirm our microarray approach. Histone (H4) is used as a loading control.

duced in each set, we treated these data independently and will refer to them as Set1 and Set2. For reasons of simplicity, a cutoff value of two-fold was used for genes induced in Set1 and 3.5 for genes included in Set2 as the fold induction for the latter set was much greater. Among the induced genes in each set, bona fide BMP targets such as *XId3*, *Xvent2*, and *Xmsx-1* were present, and here we show the 12 most highly induced genes from each set of experiments.

Prospective Direct BMP Target Genes Identified by Microarray Analysis

We have subjected these BMP target genes to whole-mount in situ hybridization analysis to determine both their spatial and temporal expression patterns during early development. In addition, we have applied reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to all of the genes listed in Table 1 (excluding XL087j15 as PCR primer pairs did not work), including several of the known BMP target genes to confirm our array data (*Xvent2*, *Xmsx-1*, and *XId3*, Fig. 1B). Here, we will describe the genes identified from each set of experiments beginning with the most highly induced, including a brief description of its expression pattern at gastrula, neurula, and tailbud stages of early *Xenopus* development.

We began by examining genes induced from Set1 (stage 15 and stage 25; neurula stage and tailbud stage) slide. In this experiment, the known BMP target genes *XId3* and *Xmsx-1* were induced by BMP2 in the presence of cycloheximide at fold-induction levels of 9.0 and 2.0, respectively. The induction level for *XId3* was the highest of any gene for this set of experiments. For this set of genes, we then decided to use 2.0 as a cutoff value, as this was an arbitrary cutoff value often used in array experiments and *Xmsx-1* was induced at this level. These genes are shown in Table 1, part A.

As described before, the induction levels for experiments done on Set2 (st25; tailbud stage) were higher. The highest induced gene was XL088k01, fibroblast growth factor receptor 2 at 13.7-fold. In this set of genes, a well-known BMP target gene, *Xvent2* was induced at 4.4-fold and *Xpo* was induced at 3.7-fold. To make this data set comparable in size to that chosen for Set1, 3.5-fold was set as the cutoff value and these genes are shown in Table 1, part B.

Genes Up-regulated in Set1 Experiments

As mentioned above, the gene with the highest induction level in experiments performed on Set1 slide was *XId3* (XL013b11; 9.0-fold). *Xenopus* Inhibitor of Differentiation 3 has frequently been identified as one of the most strongly induced direct BMP target genes in our microarray experiments. Recent work including ours showed that *XId3* expression begins at blastula stages (Wilson and Mohun, 1995), around the same stage when other direct BMP target genes, such as *Xvent2* are first expressed (Onichtchouk et al., 1996; Candia et al., 1997) and when BMP signaling becomes detectable in the early *Xenopus* embryo (Faure et al., 2000). At the gastrula stage, *XId3* is expressed in the animal pole as well as the marginal zone (von Bubnoff et al., manuscript submitted for publication; Liu and Harland, 2003). At later stages, the expression of XId3 is well characterized (Wilson and Mohun, 1995; Zhang et al., 1995; Reynaud-Deonauth et al., 2002). The expression pattern of *XId3* very closely mimics that of *BMP4*.

Propionyl Coenzyme A Carboxylase was the second highest induced gene in experiments performed on Set1

TABLE 1. Prospective Direct BMP Target Genes Identified by Microarray Analysis

				Expression pattern										
				Gastrula				Tailbud						
ID	Description	Fold	RT-PCR	ap	de	ve	mz	he	so	e	ov	ba	cns	Reference (*Xenopus*)
A. Top induced genes from Set I slide														
XL013b11	XId3 [*Xenopus laevis*]	9.0	+	+	+	+	+	−	+	+	+	+	+	Wilson and Mohun, 1995
XL022f20	Propionyl Coenzyme A carboxylase [*Mus musculus*]	5.3	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	This paper
XL008i07	No hits	4.2	+	+/−	+/−	+/−	−	−	−	+	−	+	+	This paper
XL051i05	Ubiquitin carrier protein [*Homo sapiens*]	3.9	+	+	+	+	+	−	−	+	−	+	+	This paper
XL029c21	Nucleoporin-like protein Rab [*Homo sapiens*]	3.0	+	+	+	+	−	−	+/−	+	+	+	+	This paper
XL015o18	Murin endogenous retrovirus MuERV [*Mus musculus*]	2.8	+/−	−	−	−	−	+	+	−	−	−	+/−	This paper
XL051b09	Red cell anion exchanger [*Xenopus laevis*]	2.4	+	−	−	−	−	−	−	−	−	−	−	This paper
XL049i08	CG6618 gene product [*Drosophilia melanogaster*]	2.4	+	+	+/−	+/−	−	−	−	−	−	−	+/−	This paper
XL053b17	Ribosomal protein L15 [*Homo sapiens*]	2.4	+	+/−	−	−	−	−	−	+	+/−	+	+	This paper
XL006p11	RACK1 [*Xenopus laevis*]	2.1	+	+	+/−	+/−	+	−	+	−	−	−	+	Kwon et al., 2001
XL025i18	Xmsx-1 [*Xenopus laevis*]	2.0	+	+	−	+	+	−	+	+	+	−	+	Suzuki et al., 1997
XL019c19	Unknown protein [*Homo Sapiens*]	2.0	+/−	+/−	+/−	+/−	+/−	−	−	−	+	−	+/−	This paper
B. Top induced genes from Set 2 slide														
XL088k01	Fgf receptor-2 [*Xenopus laevis*]	13.7	+	+	+	+	+	−	−	+	+	−	+	Friesel and Brown, 1992
XL106m02	Angiomoton-like 2 [*Mus musculus*]	12.2	+	+	+/−	+	+	+	+	+	−	+	+	This paper
XL096a24	No hits	11.9	+	+	+/−	+	+	−	−	+	−	−	+	This paper
XL087j15	Phosphoethanolamine methyltransferase [*Caenorhabditis elegans*]	10.8	nd	+	+	+	+	−	+/−	+	+	+/−	+	This paper
XL068a16	Lodestar protein [*Homo sapiens*]	8.1	+	+	+	+	−	−	−	+	+/−	+	+	This paper
XL098n19	NipSnap1 [*Mus musculus*]	7.9	+	+	+	+	+	−	+	+	+	+	+	This paper
XL104e22	No hits	7.4	+/−	+	+	+	+	+	+	+	+	+	+	This paper
XL063e22	Oracle 2 protein [*Mus musculus*]	7.0	+	+	+	+	+	+	−	+	−	−	−	This paper
XL085p07	NADH dehydrogenase [*Mus musculus*]	7.0	+/−	+	+	+	+	−	+	+	+	+	+	This paper
XL072k08	DNA helicase homolog [*Homo sapiens*]	6.9	+	+/−	+	+	+/−	−	+	+	+	+	−	This paper
XL106b07	Xvent2 [*Xenopus laevis*]	4.4	+	+	−	+	+	−	−	+	+	+	−	Onichtchouk et al., 1996
XL077m12	Xpo [*Xenopus laevis*]	3.7	+	+	+	+	+	−	−	−	−	−	+	Sato and Sargent, 1991
For comparison	BMP2 [*Xenopus laevis*]			+	+	+	+	+	+	+	+	+	+	Nishimatsu et al., 1992

[a]Genes from microarray experiments performed of both sets of slides were sorted by average fold induction (Fold). All of the genes (including known BMP target genes, which are shown in Figure 2B) were chosen for RT-PCR analysis. All but one clone (XL087j15) were successfully PCR amplified. Induction by BMP2 in the presence of CHX was scored (+, induction; +/−, no change/induction; nd, no data). Using densitometry analysis, a 1.2-fold (or greater) difference between CHX and CHX+BMP2 was used as criteria for positive RT-PCR results. *In situ* hybridization patterns at gastrula (st. 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). Expression in tissues/organs where BMPs are expressed was scored at the tailbud stage. Abbreviations are as follows: he, heart; so, somites; e, eye; ov, otic vesicle; ba, branchial arches; 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 in *Xenopus,* its citation is noted (Reference). The expression pattern of *Xenopus BMP2* is used for reference. RT-PCR, reverse transcriptase-polymerase chain reaction; nd, no data; CHX, cycloheximide.

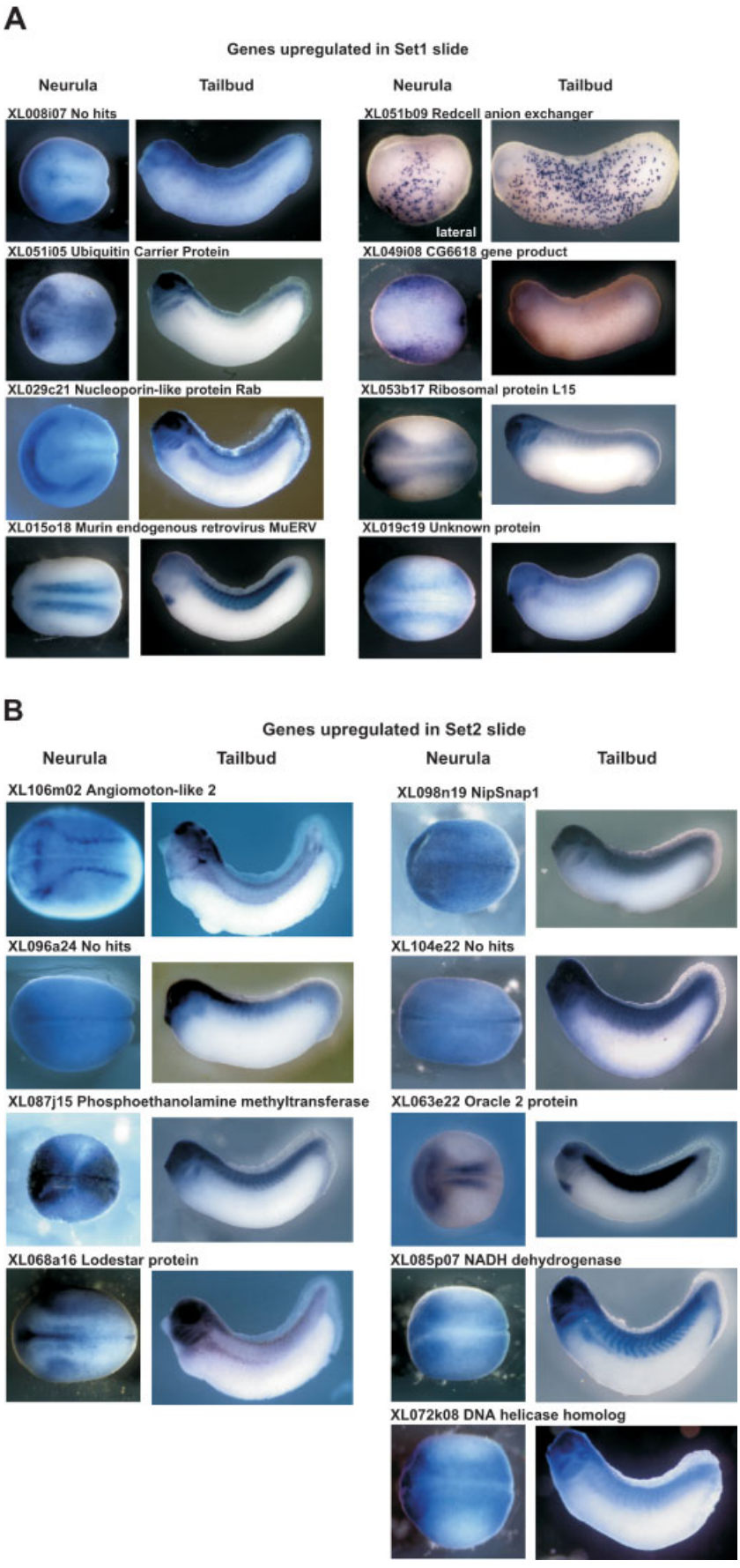

Fig. 2.

slide (5.3-fold). In bacteria, work has suggested that the propionyl-CoA carboxylase is responsible for the carboxylation of acetyl-CoA to malonyl-CoA used for the synthesis of long-chain fatty acids during development (Kimura et al., 1998). We were unable to recover XL022f20 from the bacterial plate and were therefore not able to examine its expression pattern.

XL008i07 (no homology) was induced at a level of 4.2-fold. XL008i07 is weakly expressed in the animal pole of a gastrula stage embryo. Of interest, at the neurula stage, expression is detected exclusively in the epidermis surrounding the neural tissue. At tailbud stages, XL008i07 transcripts are present in the brain and the neural tube (Fig. 2A).

Ubiquitin carrier protein (XL051i05) was induced at a fold level of 3.9. Proteolysis by means of the ubiquitin system plays important roles in a variety of basic cellular processes, such as regulation of the cell cycle and division, modulation of the immune and inflammatory responses, as well as development and differentiation of cells (Ciechanover et al., 2000). Recent studies have shown that degradation of smad proteins is regulated by an ubiquitin-dependent pathway (Wang, 2003). Interestingly, we have found that ubiquitin carrier protein is strongly expressed in the animal pole of gastrula stage embryos, where BMP signaling is active. A dorsal view of neurula stage embryos shows expression in the developing nervous system with strong expression in the anterior neuroectoderm. Expression

Fig. 2. The expression pattern of novel bone morphogenetic protein (BMP) target genes. **A,B:** Whole-mount in situ hybridization was performed to confirm localization of transcripts in whole embryos from genes induced in Set1 experiments (A) and Set2 experiments (B). Gene expression in the embryos is shown and scored for each gene at the neurula stage (stage 15–18) and the tailbud stage (stage 30–32). For neurula stages, anterior is to the left and dorsal is pointing upward. In some cases, a lateral view is shown in which anterior is to the left and dorsal is up. For all tailbud stages, anterior is to the left and dorsal is up. The expression pattern of each gene at the gastrula stage is referred to in Table 1. Each expression pattern was analyzed in at least two different batches of embryos.

of ubiquitin carrier protein in the tailbud stage is strong in the head region, specifically, the brain, eye, and branchial arches. Ubiquitin carrier protein appears to be excluded from the cement gland (Fig. 2A).

Nucleoporin-like protein Rab (XL029c21) was induced 3.0-fold. A recent screen in *Caenorhabditis elegans* has shown that of 20 assigned *C. elegans* nucleoporin genes, 17 were found to be essential for embryonic development either alone or in combination (Galy et al., 2003). Expression of Nucleoporin-like protein Rab at the gastrula stage is strong in the animal pole. A dorsal-anterior view of a neurula stage embryo shows expression in the dorsal-most region of the embryo, as well as expression along the border of the neural plate and epidermis. Tailbud expression of Nucleoporin-like protein Rab shows that this gene is most strongly expressed in the cement gland, eye, otic vesicle, brain, branchial arches, as well as weak expression in the pronephros (Fig. 2A). Note the similarities in the expression pattern of Nucleoporin-like protein Rab with that of ubiquitin carrier protein (XL051i05) at the tailbud stage.

Murin endogenous retrovirus MuERV (XL015o18) was induced 2.8-fold. The expression pattern of MuERV is quite unique in that it does not appear to be expressed in the animal pole at the gastrula stage, but at the neurula stage, it is only expressed in the somites. Additionally, RT-PCR analysis was not able to confirm this gene as a direct BMP target. At the tailbud stage, MuERV transcripts are strongly detected in the dorsal somites, the heart anlage, and the brain. Although this expression pattern does not seem to resemble that of BMP target genes, such as *Xvent2* or *XId3*, it is known that *BMP2* is expressed in the brain and somites at the tailbud stage (Nishimatsu et al., 1992; Fig. 2A).

Red cell anion exchanger (XL051b09) was induced at a level of 2.4-fold. We find that Red cell anion exchanger is not expressed in the animal pole of the gastrula stage embryo and its expression can only be detected from the neurula stage onward. At the neurula stage, Red cell anion exchanger appears to be restricted to the epidermis in an extremely unique punctate pattern, in which, it is possible to visualize each individual cell expressing transcripts. The same holds true for tailbud stages, in which similar punctate epidermal staining is seen. At these later stages, the punctate staining seems to be excluded from the head region and is mostly present in the ventrolateral (trunk) region of the developing embryo. Interestingly, the expression pattern of Red cell anion exchanger does not match that of gene expected to be involved in blood maintenance, such as *GATA2* (Walmsley et al., 2002), as it appears to be strictly localized to the epidermis (Fig. 2A). It may be true that Red cell anion exchanger is a direct BMP target gene perhaps normally activated at a later stage in development (e.g., during organogenesis). However, when animal caps are stimulated with a high amount of BMPs, this gene may become fortuitously activated and as a consequence; RT-PCR and microarray analyses show up-regulation of this gene, although it is not normally expressed in the gastrula.

XL049i08 (CG6618 unknown gene product) was induced at a level of 2.4-fold. The function of this gene is unknown, however, it does contain an ankyrin repeat, a domain found in over 400 proteins of different functions (Sedgwick et al., 1998). XL049i08 is weakly expressed in the animal pole ectoderm of the gastrula stage embryo. At the neurula stage embryo, XL049i08 is strongly expressed in the epidermis and is completely excluded from the neural plate. At this stage, the staining abutting the neural plate is slightly punctated. At the tailbud stage, XL049i08 is weakly expressed in the head region (Fig. 2A).

Ribosomal protein L15 (XL053b17) was induced at a level of 2.4-fold. We find that Ribosomal protein L15 is very weakly expressed in the animal pole ectoderm of the gastrula stage embryo and more strongly expressed in the developing nervous system of the neurula stage embryo. At tailbud stages, Ribosomal protein L15 transcripts can be detected in the eye, brain, cement gland, and branchial arches (Fig. 2A), somewhat similar to the expression pattern of ubiquitin carrier protein (XL051i05).

RACK1 (XL006p11) was induced at a level of 2.1-fold. RACK1 is the *Xenopus* homologue of the receptor for activated C-kinase 1. Previous in situ analyses have shown that *XRACK1* is a maternally expressed gene and is strongly expressed in the animal pole region (Kwon et al., 2001). Its zygotic expression is detected in the anterodorsal region and dorsal midline in the late neurula. Expression of *XRACK1* at later stages is referred to in Table 1.

Xenopus msx-1 (XL025i18) was induced at a level of 2.0-fold. The msx homeodomain protein was shown to be a downstream target of the BMP pathway (Suzuki et al., 1997). Overexpression of *Xmsx-1* in early *Xenopus* embryos leads to ventralization of embryos as described for *BMP4* and is sufficient to induce epidermis in dissociated ectodermal cells, which would otherwise differentiate into neural cells (Suzuki et al., 1997). *Xmsx-1* expression is strongly located in the animal pole region of the gastrula stage embryo and becomes localized in the lateral edge of the neural plate in the neurula stage (Suzuki et al., 1997). Expression of *Xmsx-1* at later stages is referred to in Table 1.

XL019c19, an unknown protein was induced at a level of 2.0-fold. XL019c19 is weakly expressed in the animal pole of a gastrula stage embryo and at the neurula stage expression is in the neuroectoderm along the border of the neural plate. Little expression is seen at the tailbud stage and transcripts can be detected in the otic vesicle, cement gland, and brain (Fig. 2A).

Genes Up-regulated in Set2 Experiments

The gene that was induced at the highest level in Set2 experiments was Fibroblast growth factor receptor 2 (*XFGFR-2*; XL088k01) at 13.7-fold. The fibroblast growth factors (FGFs) are involved in various developmental processes, including angiogenesis, proliferation, neuronal survival, and mesoderm induction of *Xenopus* embryonic ectoderm (Burgess et al., 1994). Whole-mount in situ hybridization experiments demonstrated that

XFGFR-2 expression begins at gastrulation, where it is expressed in the animal pole region (Friesel and Brown, 1992). Table 1 includes a description of *XFGFR-2* at later stages in development. Here, it is significant to mention that a study in the chick has shown that overexpression of constitutively active type I BMP receptor leads to the ectopic expression of *msx-1* and *FGFR-2* throughout the maxillary mesenchyme (Ashique et al., 2002), suggesting that in addition to *msx-1*, *FGFR-2* is a downstream target of BMPs and potential evidence for crosstalk between the BMP and FGF pathway.

XL106m02 exhibits weak homology to angiomotin-like 2 and was induced at a level of 12.2-fold. Angiomotin was previously identified in a yeast two-hybrid screen by its ability to bind to angiostatin, an inhibitor of normal blood vessel formation (Troyanovsky et al., 2001). Angiomotin mediates the inhibitory effect of angiostatin on endothelial cell migration and tube formation in vitro (Bratt et al., 2002). XL106m02 is strongly expressed in the animal pole tissues of a gastrula stage embryo and at later neurula stages, is expressed in the lateral edge of the neural plate as well as the future brain and weakly in the epidermis. At tailbud stages, XL106m02 is strongly expressed in the brain, the dorsal eye, and possibly the developing heart as well as the branchial arches, neural tube, and somites. Thus, as is evident from the expression pattern, this protein may have other roles than angiogenesis in development (Fig. 2B).

XL096a24 was induced at a level of 11.9-fold. Although this clone shows no major homology to sequences available in the databases, it does display weak similarity to the *Xenopus* gene for aldolase C, a key glycolytic enzyme expressed mainly in regions of the brain (Yatsuki et al., 1998). We find that XL096a24 is strongly enriched in the animal pole region of the gastrula stage embryo. At later stages, the expression of XL096a24 becomes weak and expressed ubiquitously but stronger expression is seen in the brain and eye of the tailbud embryo (Fig. 2B).

XL087j15 (phosphoethanolamine methyltransferase) was induced at 10.8-fold. Expression of this enzyme is strong in the gastrula stage embryo, where it is detected in the animal pole tissues as well as the marginal zone mesoderm. At the neurula stage, XL087j15 is mostly located in the developing nervous system but is also expressed in the epidermis. At the tailbud stage, XL087j15 transcripts are ubiquitously expressed in the head region as well as the developing nervous system, including the brain, otic vesicle, and weakly in the somites (Fig. 2B) showing that, at the tailbud stage, the expression pattern of phosphoethanolamine methyltransferase resembles that of *BMP4*.

Lodestar protein (XL068a16) was induced at 8.1-fold. Lodestar has been shown to be vital for cell cycle progression and was originally identified as a maternal-effect gene essential for embryonic mitosis in *Drosophila*. Mutations in lodestar tangle chromosomes during anaphase (Girdham and Glover, 1991). Lodestar is confined to the animal pole tissues of the gastrula stage embryo. At the neurula stage, Lodestar is expressed in the epidermis. At the tailbud stage, Lodestar transcripts are detected in the eye, branchial arches, otic vesicle, and excluded from the cement gland (Fig. 2B).

4-Nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (NipSnap1; XL098n19) was induced at a level of 7.9-fold. To date, the biological function of the NIPSNAP protein family is unknown, however, according to expression of the *C. elegans* ortholog, it may encode protein motifs known to be involved in vesicular transport (Seroussi et al., 1998). *NipSnap1* transcripts are very strongly detected in the animal pole ectoderm. Expression analysis in neurula stage embryos shows ubiquitous expression and at tailbud stages, *NipSnap1* expression partially resembles that of BMP4 as it is expressed in the brain, eye, otic vesicle, and weakly in the branchial arches (Fig. 2B).

XL104e22 was induced at a level of 7.4-fold. This clone displays weak homology to *Xenopus* y-6/uPAR-Related Protein (LURP-1), which is a molecular marker for myeloid cells from the early tailbud stage in *Xenopus* embryos (Smith et al., 2002). XL104e22 expression is restricted to the animal pole region of the gastrula stage embryo. At later stages, XL104e22 is strongly expressed in the eye, branchial arches, brain, and somites (Fig. 2B).

Oracle 2 protein (XL063e22) was induced at a level of 7.0-fold. Oracle is a PDZ-LIM domain protein, and in the mouse, it is expressed in the adult heart and at low levels in skeletal muscle, as well as in the atrial and ventricular myocardial cells at embryonic day (E) 8.5. From E9.5, low expression of Oracle mRNA was detectable in myotomes (Passier et al., 2000). The expression of Oracle 2 in *Xenopus* is very strong in the animal pole of the gastrula stage embryo. At the neurula stage, transcripts can be detected in the developing somites. Concurrent with the previously described mouse data, *Xenopus* Oracle 2 is restricted to the heart anlage and somites at the tailbud stage. We find no expression of *Xenopus* Oracle 2 in other areas at this stage (Fig. 2B).

NADH dehydrogenase (XL085p07) was induced at a level of 7.0-fold. The NADH dehydrogenase complex, also called NADH coenzyme Q oxidoreductase, is the first complex in the electron transfer chain of mitochondria and catalyzes the transfer of electrons from NADH to coenzyme Q (CoQ). NADH dehydrogenase transcripts are very strongly detected in the animal pole ectoderm. Expression analysis of the neurula stage shows presence of transcripts in the developing nervous system with slightly stronger staining anteriorly. At the tailbud stage, NADH dehydrogenase transcripts can be found in the eye, branchial arches, pronephros, and somites (Fig. 2B).

DNA helicase homolog (XL072k08) was induced at a level of 6.1-fold. DNA helicases are required for DNA replication, recombination, and repair. DNA helicase homolog is weakly expressed in the animal pole tissues in the gastrula stage embryo. At the neurula stage, DNA helicase homolog is strongly expressed in the anterior neural region. At the tailbud stage, DNA helicase homolog transcripts are found in the otic vesicle, the eye, branchial arches, and weakly in the developing somites (Fig. 2B).

Xvent2 (XL106b07) was induced at a level of 4.4-fold. The homeobox gene, *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). In the gastrulating embryo, *Xvent2* is expressed in the ventral marginal zone and is not expressed in the organizer region (Schmidt et al., 1996). A description of the *Xvent2* expression pattern at later stages can be found in Table 1.

Xpo (XL077m12) was induced at a level of 3.8-fold. *Xpo* (*Xenopus*-posterior), a putative transcription factor, contains a single zinc-binding "CCHC" motif and is activated shortly after the midblastula transition (MBT) (Sato and Sargent, 1991); however, *Xpo* may function as a nontranscription factor, such as a RNA binding protein. *Xpo* transcripts accumulate at a relatively low level, which remains constant until gastrulation, then can mostly be found in the posterior ectoderm and mesoderm of tailbud stage embryos (Sato and Sargent, 1991). Interestingly, past work has suggested that *Xpo* may in fact be a BMP target gene as animal caps overexpressing *BMP4* transcribe high levels of ventral mesoderm markers, including *Xpo* (Re'em-Kalma et al., 1995). However, no human or mouse homologs exist for *Xpo*.

DISCUSSION

Here, we have demonstrated that *Xenopus* DNA microarrays in combination with animal cap ectoderm assays can be successfully used to identify genes that are directly induced by BMP signals. In the midst of the many genes that we have shown to be BMP inducible, we find several genes such as *Xvent2*, *XId3*, and *Xmsx-1* that have been known for some time to be a direct target of this growth factor. Moreover, a large majority (70%) of these genes are strongly expressed in the animal pole tissues of gastrulating embryos, a region enriched in BMP signals. We have also identified several new genes that have not been implicated in the BMP signaling pathway. It is true that additional analysis is required to define the roles of these genes during developmental processes; however, our analysis has assisted in uncovering potentially useful BMP target genes that should be studied in the future.

What we have shown here is that there is in fact a diverse set of potential direct BMP target genes that are involved in early development. Some of these BMP target genes appear to belong to a BMP synexpression group in that they are expressed in regions where *BMP2* or *BMP4* are expressed. However, some of these genes are expressed in areas where BMPs are not expressed, suggesting that these genes may be regulated by other factors. Perhaps these candidate genes may be BMP target genes in a specific tissue, or at a later stage in development, but fortuitously activated in our animal cap assay where dissociated cells are challenged with a high concentration of BMP2. Alternatively, this finding may be due to the sensitivity of these analysis techniques as whole-mount in situ analysis is likely to be less sensitive than RT-PCR.

On the other hand, it is often difficult to classify genes into a few defined categories, but this difficulty may reflect that BMPs have very dynamic and complicated expression patterns during development. Recently, accumulating work also suggests a substantial degree of crosstalk between the BMP pathway and TGF-β/activin, Wnt, Ca^{2+}/calmodulin, Erk–MAPK, and JAK–STAT pathways (von Bubnoff and Cho, 2001). In this regard, it may be expected for this pathway to have a very diverse set of target genes.

What we have found here is alike to that found in recent microarray reports attempting to identify BMP target genes induced by ectopic expression of activated BMP type I receptors in C2C12 cells as well as BMP-induced expression in NIH3T3 fibroblasts. Extremely diverse sets of genes are induced by this factor in those cells. Although these studies focused mainly on both myoblast and osteoblast differentiation, it was shown that many extracellular matrix genes were up-regulated, muscle-related genes down-regulated, and transcription factors/signaling components modulated (Vaes et al., 2002; Korchynskyi et al., 2003). However, comparison of this data to our *Xenopus* data sets is difficult because we have implemented cycloheximide to identify "direct" BMP target genes and the duration of BMP treatment is only a few hours instead of several days. It is important to note that the C2C12 microarray experiments have not identified *Id3* or *msx1* as BMP targets. We believe that experiments attempting to identify direct BMP target genes without cycloheximide results in data sets, including both direct and indirect target genes. Although the use of cycloheximide may reduce the chance of identifying potential direct target genes, the likelihood of isolating bona fide direct BMP target genes increases (our unpublished observations). In fact, our RT-PCR analysis of 23 samples confirmed that 19 (83%) are direct BMP target genes.

In the accompanying study (Shin et al., 2005) based on neural induction by the inhibition of BMP signaling, we have noticed that some of the genes identified here are reduced in expression by BMP inhibition (such as *Xvent2*, *XId3*, *Xmsx-1*, *Xpo*); however, other direct BMP targets were not down-regulated in those experiments. This finding is because these two microarray experiments use distinctive approaches in that, here, to identify direct BMP target genes, we have used dissociated cells instead of intact animal caps, the protein synthesis inhibitor cycloheximide, as well as an exogenous BMP2 treatment as opposed to endogenous BMP by means of mRNA injection. Other variations could arise from additional differences in experimental conditions such as the source of reference RNA. For this reason, we believe that future experiments using a common reference RNA (such as a mixture of RNAs collected from several concurrent stages of development, i.e., blastula, gastrula, early neurula, late neurula, and tailbud stages) will allow for even more reliable comparisons between diverse microarray experiments such as the direct BMP target study with the study of genes involved in neural induction (Peiffer et al., 2003). Hybridization of a common reference sample simultaneously with each different experimental sample (Alizadeh et al., 1999; Eisen and Brown, 1999) will allow for the analysis of multiple data sets obtained using statistical methods such as the analysis of variance. In fact, in recent experiments, we have tried this approach and have found it to be advantageous in allowing for comparisons of various mi-

croarray experiments (unpublished observations). We have attempted to group a majority of these identified BMP target genes into several specific categories.

Transcription Factors

We verified the accuracy of our microarray approach by again identifying *XId3* (XL013b11), *Xmsx-1* (XL025i18), *Xvent2* (XL106b07), and *Xpo* (XL077m12) as being BMP target genes. However, we believe this report to be the first study to suggest *Xpo* as a direct BMP target gene. Thus, four of the 24 genes identified by microarrays are transcription factors.

Signal Transduction Components and Regulators

RACK1 (XL006p11) and XFGFR-2 (XL088k01) are components of signaling cascades. RACK1 acts as a signaling scaffold protein, important in recruiting other protein complexes for signaling (Yarwood et al., 1999). In fact, it is now known that there is an interaction between the integrin beta subunit cytoplasmic domain and RACK1. In regard to FGF signaling, crosstalk between FGF and BMP signals have been reported (Northrop et al., 1995; Ishimura et al., 2000). For example, the FGF and BMP4 signaling pathways appear to regulate the specification of the erythropoietic lineage, and BMPs have been shown to negatively regulate FGF signaling (Xu et al., 1999).

Cell Cycle

It also appears to hold true that BMPs play an important role in cell cycle regulation, as we have found several cell cycle regulators to be induced by BMPs. Lodestar (XL068a16), DNA helicase homolog (XL072k08), and Ubiquitin-carrier protein (XL051i05) have been implicated, at least in part, in cell cycle progression (Girdham and Glover, 1991; Ciechanover, 1994). *XId3* (XL013b11) potentially falls into this category as it is considered to be a negative regulator of cell differentiation and positive regulator of cell proliferation (Norton et al., 1998; Yokota and Mori, 2002).

Vascular System

Hematopoiesis is the generation, proliferation, and differentiation of hematopoietic stem cells (HSCs) into the erythroid, myeloid, and lymphoid lineages. In *Xenopus*, the HSC is derived from the ventral mesodermal that arises due to the inductive processes triggered by BMP4 (Huber and Zon, 1998). Several of the genes identified in our microarray screens are involved in the formation or maintenance of blood components. The genes that fall into this category are XL51b09 (Red cell anion exchanger), XL106m02 (Angiomoton-like 2), and XL063e22 (Oracle 2), which have all been implicated in either the development and/or maintenance of the vascular system and its components.

Enzymes

Several enzymes were induced by BMP2 in the presence of CHX, including propionyl coenzyme A carboxylase (XL022f20), phosphoethanolamine methyltransferase (XL087j15), and NADH dehydrogenase (XL085p07). Unfortunately, the expression patterns of the enzymes have proven not be as informative as many are expressed ubiquitously in developing embryos at some stages. However, it is interesting to note that all of the enzymes in our list appear to be expressed very strongly in the animal pole tissues, a region of high BMP activity and at the tailbud stage, we find expression of many of these enzymes in regions where *BMP2* or *BMP4* is expressed.

Unknown Genes

XL049i08 (CG6618 gene product), XL019c19 (unknown protein), XL096a24 (no homology), and XL080e12 (no homology) did not have any significant homology to proteins of known function in available databases.

Among several BMPs, BMP2, BMP4, and BMP7 are the best characterized in early development. These BMPs have different expression patterns as well as different loss of function phenotypes. However, it is not clear whether actions of BMP2, BMP4, and BMP7 trigger the same cellular machinery to activate identical sets of target genes, differing quantitative levels of gene expression, or whether they serve to activate different sets of target genes, providing quantitative different responses. In the near future, it will be interesting to see whether the cellular responses to BMPs are qualitatively different. Different sets of target genes may be induced, depending upon how much BMP is available for the cell, at least partially. This appears to be true, as we have repeated the aforementioned microarray screens with a higher amount of BMP2, and we have obtained a somewhat different group of genes (our unpublished observations).

EXPERIMENTAL PROCEDURES

Generation of *Xenopus* cDNA Microarrays

Microarrays were generated by a homemade robotic spotter using normalized neurula and tailbud (stage 15 and stage 25) cDNA libraries spotted on Corning CMT-GAP glass slides. (For detailed specifics of this process, refer Tran et al. [2002] and Shin et al. [2005].) We were able to make improvements to our spotting capabilities through the application of pin microtapping and microvibration technology as well as spotting methods (Tran et al., 2002). Incorporation of these techniques results in an increased amount of uniformity of spot sizes.

RNA Preparation, Probe Generation, Array Hybridization, and Slide Scanning

Xenopus embryos were obtained by in vitro fertilization of eggs with testes homogenates. Embryos were dejellied in 2% cysteine and cultured in 0.1× Barth's saline solution, and animal cap ectodermal fragments were removed at the blastula stage (stage 8). Total RNA was isolated from animal cap ectodermal explants and dissociated cells using TRIzol Reagent (Gibco), followed by lithium chloride precipitation. This RNA was used in both the microarray experiments as well as the RT-PCR reactions. Microarray probe generation, slide hybridization, scanning, and data analy-

sis were preformed exactly as described in Shin et al. (2005).

RT-PCR Analysis

The protocol for RT-PCR is essentially as previously described (Blitz and Cho, 1995), except that in this case, the reaction products were visualized on ethidium bromide stained agarose gels (Fig. 1B). Unless otherwise indicated, the basic PCR protocol was 94°C, 5 min, 28 cycles (94°C, 30 sec, 55°C, 30 sec, 72°C, 30 sec), 72°C, 5 min. All of the target genes identified by microarray analysis shown in Table 1 (excluding XL087j15) were subjected to RT-PCR analysis. Specific PCR cycles and primer pair sequences for the induced genes from Set1 slide are as following: XL013b11 (*XId3*); 27 cycles, 5′-TGCGGTCCATGTCAAGC-3′ and 5′-CAGAGAGGTTAGAACGGCTCAG-3′, XL022f20 (Propionyl Coenzyme A Carboxylase); 28 cycles, 5′-CGCGATGGTGCTACTTGTC-3′ and 5′-GGTGTGCTTAATGGCATCC-3′, XL008i07 (no hits); 28 cycles, 5′-TCGGCTGGTAGTCACTAGTC-3′ and 5′-CTACCTGAGAGTGAGGAGCG-3′, XL051i05 (Ubiquitin carrier protein); 28 cycles, 5′- GCAGGAGGTATGTTTCGAATG-3′ and 5′-CATTCAGTGCTGACTCTGGG-3′, XL029c21 (Nucleoporin-like Rab); 30 cycles (annealing temp; 50°C), 5′-GATGATAGATCTTCTGCAATCCC-3′ and 5′-CCAAGGTCACTCAGCAGGTC-3′, XL015o18 (Murin endogenous retrovirus); 30 cycles (annealing temp; 50°C), 5′-TAGCGTAACCCCTGTTTACTG-3′ and 5′-AACATGAGTACAATGACTAGGGC-3′, XL051b09 (Red cell anion exchanger); 29 cycles, 5′-GAGCTGCATGAGTTGAC-3′ and 5′-CACTTCCTGGCATATCC-3′, XL049i08 (CG6618 gene product); 28 cycles, 5′-CCAAGCAGCACATGC-3′ and 5′-GCTGCTCATACTCTCCAC-3′, XL053b17 (ribosomal protein L15); 28 cycles, 5′-GGATGTCATGCGTTTCCTTC-3′ and 5′-TGTGGAGTCTTCACCAACCC-3′, XL006p11 (RACK1); 28 cycles, 5′-CACAGCCATGACTGAGC-3′ and 5′-CCACAGCCTCAGTGTTC-3′, *Xmsx-1* (27 cycles) primers were as previously described (Su et al., 1991), XL019c19 (Unknown protein); 30 cycles, 5′-AGGTGCTCGGGCATCTTC-3′ and 5′-GAGATCATGCCGGGCTG-3′. Specific PCR cycles and primer pair sequences for the induced genes from Set2 slide are as following: XL088k01 (FGFR-2); 28 cycles, 5′-GCAGGTAATTCGGCACGAG-3′ and 5′-GTGGGCAGTGGAGATCAAG-3′, XL106m02 (Angiomoton-like 2); 30 cycles, 5′-CACACAGGCTATGCTCGG-3′ and 5′-CCTAGGACTTCCACTGCC-3′, XL096a24 (no hits); 28 cycles, 5′-GTAAATCAGTCTTGATTTGGGC-3′ and 5′-GCATCCATAGATACCAGGACAC-3′, XL068a16 (Lodestar protein); 28 cycles, 5′-TGTCAGAGCTCAAGACCATAAGG-3′ and 5′-GATTCCCACCAACCAGATTG-3′, XL098n19 (NipSnap1); 28 cycles, 5′-CCCGTCACTGCTGTTATGTAG-3′ and 5′-AGATCACAGTGGTAATCTGGG-3′, XL104e22 (no hits); 31 cycles, 5′-AGTAGAGCTTTAATGATCGTTCC-3′ and 5′-CAACTTAGTCTGGATGGACAG-3′, XL063e22 (Oracle 2); 31 cycles, 5′-GTATTCCACCGCGGTG-3′ and 5′-CCGTCTGTGTTGACTCC-3′, XL085p07 (NADH dehydrogenase); 30 cycles, 5′- CATCCTCTTGACTGCTCTCTG-3′ and 5′-AGCCCACTGCTGATGGTC-3′, XL072k08 (DNA helicase homolog); 31 cycles, 5′-CGTTTGGTGGCATTCAAC-3′ and 5′-GCACAGCCTCGTAGCCAG-3, *Xpo*; 28 cycles, 5′-CGAGGCCATTGTATCCTTCC-3′ and 5′-CGCATCAGTCTCAGGCTTC-3′. *Xvent2* (28 cycles) and *Histone H4* (24 cycles) primers were as described previously (Blitz et al., 2003).

Whole-Mount In Situ Hybridization Analysis

In situ hybridization was performed essentially as described previously (Harland, 1991), except that BM purple (Boehringer-Mannheim) was used as the chromogenic substrate. As cDNAs were directionally cloned into pBSIISK-, library inserts were PCR amplified using T3 and T7 primers. Antisense RNA probes were generated by using a T7 RNA polymerase (New England Biolabs). Whole-mount in situ hybridization analysis was performed for three different stages of early embryonic development (stage ~10.5; gastrula, stage ~15–18; neurula, stage ~30–32; tailbud). Each expression pattern was analyzed in at least two different batches of embryos. There was some variation in the tailbud staining patterns from gene to gene and this variability is likely to come from differences in staining time, probe penetration, as well as the relatively weak expression of several genes analyzed.

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