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# Control of kidney, eye and limb expression of *Bmp7* by an enhancer element highly conserved between species

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**Abstract**

*Bmp7* is expressed in numerous tissues throughout development and is required for morphogenesis of the eye, hindlimb and kidney. In this study we show that the majority if not all of the *cis*-regulatory sequence governing expression at these anatomical sites during development is present in approximately 20 kb surrounding exon 1. In eye, limb and kidney, multiple distinct enhancer elements drive *Bmp7* expression within each organ. In the eye, the elements driving expression in the pigmented epithelium and iris are spatially separated. In the kidney, *Bmp7* expression in collecting ducts and nephron progenitors is driven by separate enhancer elements. Similarly, limb mesenchyme and apical ectodermal ridge expression are governed by separate elements. Although enhancers for pigmented epithelium, nephrogenic mesenchyme and apical ectodermal ridge are distributed across the approximately 20 kb region, an element of approximately 480 base pairs within intron 1 governs expression within the developing iris, collecting duct system of the kidney and limb mesenchyme. This element is remarkably conserved both in sequence and position in the *Bmp7* locus between different vertebrates, ranging from *Xenopus tropicalis* to *Homo sapiens*, demonstrating that there is strong selective pressure for *Bmp7* expression at these tissue sites. Furthermore, we show that the frog enhancer functions appropriately in transgenic mice. Interestingly, the intron 1 element cannot be found in the *Bmp7* genes of vertebrates such as *Danio rerio* and *Takifugu rubripes* indicating that this modification of the *Bmp7* gene might have arisen during the adaptation from aquatic to terrestrial life. Mutational analysis demonstrates that the enhancer activity of the intron 1 element is entirely dependent on the presence of a 10 base pair site within the intron 1 enhancer containing a predicted binding site for the FOXD3 transcription factor.


*Keywords:* Bone morphogenetic protein; BMP; BMP7; OP1; Kidney development; Eye development; Limb development; Ureteric bud

## Introduction

The bone morphogenetic protein (BMP) ligands comprise a large family of growth factors sharing structural homology with the transforming growth factor β proteins. Their expression is essential for key events in early embryonic patterning and development of multiple organ systems (Hogan, 1996). BMPs regulate processes as diverse as cell proliferation, apoptosis and differentiation in a spectrum of tissues. Despite the variety of cellular responses elicited by these growth factors, the signaling pathway downstream of BMPs is relatively simple, with receptor binding leading to phosphorylation and activation of the Smad1, 5 and 8 transcription factors that are translocated to the nucleus together with Smad4 (Massagué, 1998). Differential transcriptional responses to BMPs are largely regulated by association of the phosphorylated Smad complex with cell type specific auxiliary transcription factors that activate distinct genetic programs (Massagué and Wotton, 2000). Our previous work has shown that the developmental functions of BMP7 can be entirely replaced with the closely related BMP6 and largely replaced with the more distantly related BMP4 (Oxburgh et al., 2005). The spatial distribution and overall levels of BMP activities may thus explain their distinct roles in development. Consistent with this idea, exacerbated phenotypes were revealed by compound mutation of the *Bmp5*, *Bmp6* and *Bmp7* genes. These BMP family members are expressed in an overlapping

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fashion in the heart, but their individual inactivation fails to disrupt heart development. However, inactivation of *Bmp7* and *Bmp5* (Solloway and Robertson, 1999) or *Bmp7* and *Bmp6* (Kim et al., 2001) leads to retardation in heart development and defects in valve formation and septation. This strongly indicates that at least the 60A subgroup (*Bmp5*, *6* and *7*) is functionally redundant and that the collective expression of these ligands determines developmental function. In keeping with this conclusion, individual inactivation of these genes reveals phenotypes that closely correspond with their domains of unique expression (Dudley et al., 1995; Dudley and Robertson, 1997; Kingsley et al., 1992; Solloway et al., 1998). Collectively, these studies strongly suggest that developmental roles of BMPs are determined by the *cis*-regulatory sequences governing their expression rather than via distinct properties of individual ligands. Thus, a mechanistic understanding of BMP function in development will require characterization of the basis of tissue-specific expression of these growth factors. *Bmp7* is essential for development of the eye, kidney and hindlimb (Dudley et al., 1995, 1999; Oxburgh et al., 2004). Here we describe for the first time the *cis*-regulatory sequences controlling *Bmp7* expression in these tissues. We have identified an approximately 480 base pair evolutionarily conserved enhancer island within intron 1 of the *Bmp7* locus governing expression in all three of these tissues. Surprisingly, we find that this enhancer activity is entirely dependent on the presence of a stretch of 10 base pairs containing a predicted binding site for the transcription factor FOXD3.

## Materials and methods

### *RNA purification, Northern analysis and RACE*

RNA was purified from embryonic day 13.5 (E13.5), E17.5 and adult kidneys using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. 50 μg total RNA was separated on a 1% formaldehyde denaturing gel alongside an RNA size ladder (ssRNA ladder, New England Biolabs). The gel was ethidium bromide stained and nucleic acid migration distances calculated. The gel was subsequently blotted to Hybond N nitrocellulose (GE Healthcare) (Sambrook et al., 1989) and hybridized with a $^{32}$P-labeled random primed probe (Rediprime, GE Healthcare) representing the entire *Bmp7* coding sequence using standard Northern blotting procedures (Sambrook et al., 1989). Autoradiographs were measured and compared to the size standards to determine molecular weights of detected bands. Three prime Rapid Amplification of cDNA ends (RACE) was performed on total RNA using the SMART RACE kit (Clontech) according to the manufacturer's instructions. 3′ termini of isolated clones were sequenced and aligned with the genomic sequence of *Bmp7* (chromosome 2, 172,510,951–172,583,260). *Bmp7* cDNA clones isolated from an embryonic kidney library (Stratagene) using the *Bmp7* coding sequence probe were sequenced, and their 5′ termini compared to the *Bmp7* genomic sequence.

### *Transgenic reporter constructs*

A phage clone spanning approximately 20 kilobases (kb) surrounding the first exon of *Bmp7* was isolated from a 129 SVJ genomic library (Stratagene) by screening using a $^{32}$P-labeled random primed probe representing the first exon of *Bmp7* using standard procedures (Sambrook et al., 1989). The genomic clone was restriction mapped and subcloned into the Hsp68lacZ reporter construct (Sasaki and Hogan, 1996) in five fragments (Fig. 1D). The following restriction enzymes were used to generate fragments: for 142:1, *Eco*RI–*Nsi*I, for 216:1 *Nsi*I–*Hin*dIII, for 216:2 *Hin*dIII–*Nsi*I, for 217:1, *Nsi*I–*Nde*I, for 217:2, *Nde*I–*Xba*I. Genomic DNA was digested, separated on 0.8% agarose gels and DNA was purified from bands of appropriate molecular weights using the Geneclean Spin kit (QBioGene) according to the manufacturer's instructions. Purified DNA was polished using T4 DNA polymerase (New England Biolabs) according to the manufacturer's instructions, and enzyme was heat inactivated for 20 min at 65 °C before ligation. The 480 base pair (bp) *Xenopus tropicalis Bmp7* intron 1 element was PCR amplified from genomic DNA using Platinum Hi-Fidelity PCR kit (Invitrogen) with 5′ phosphorylated oligonucleotides 5′-GGCTCGGACG TTCTTGGACG TCTCT-3′ and 5′-AGATCCTTAT AATCA-CAACC AGACA-3′. Hsp68lacZ plasmid was linearized with *Sma*I and dephosphorylated using Calf Intestinal Phosphatase (Both New England Biolabs) according to the manufacturer's instructions. Linearized plasmid was gel purified using the Geneclean spin kit. Plasmid and insert were ligated using the Takara ligation kit (Takara) according to manufacturer's instructions and transformed into chemically competent DH5α E. coli prepared according to standard procedures (Sambrook et al., 1989). Ampicillin-resistant colonies were screened by restriction mapping for the presence of genomic DNA and DNA was purified from positive clones using the Qiagen Maxiprep kit (Qiagen) according to manufacturer's instructions. Purified plasmid DNA was digested with *Not*I, separated on 0.8% agarose gel, and the anticipated molecular weight corresponding to the transgene was excised and purified using the Qiagen Gel Cleanup Kit (Qiagen). Transgene DNA was verified by agarose gel electrophoresis and suspended to a concentration of 3 ng/μl for pronuclear injection in a buffer containing 10 mM PIPES, 5 mM NaCl and 150 mM KCl.

### *Subcloning and mutagenesis*

Six individual subclones were generated from the 217:1 genomic fragment by PCR with the Platinum Hi-Fidelity PCR kit (Invitrogen) using the following oligonucleotide combinations: 5′-GAACATTCTT GCCAAACCAT TCAG-3′ and 5′-CGCTATTCTA CGGTGGAAAC AGAC-3′, 5′-TTCCTGTGTT TGGGATGC-3′ and 5′-TTTCTGCTGG TGAGATGAC-3′, 5′-TGTTTCCACC GTAGAATAGC GTC-3′ and 5′-CCATTTTGGT CTCCCAGGTA GTG-3′, 5′-TGTGTGTGTG TGTATTACCC ACCG-3′ and 5′-CAACAGTGAC AATG-CTGAGA GACAG-3′, 5′-AGTATTTCTT CCCACCCCTT TCTG-3′ and 5′-AGACACCGCA GGCTGTATGT ATTAC-3′, 5′-CATCAGTAAA GCC-TGGTTGG ATTC-3′ and 5′-TTCCGCAATG TCCCGAAAC-3′. An 5′-TT-GGCGCGCC-3′ sequence containing an *Asc*I site was added at the 5′ end of each forward amplification oligonucleotide for directional cloning. Similarly, a PCR fragment representing the 480 bp intron 1 enhancer was generated using the oligonucleotide combination 5′-AACATTTGTG TCGGAAGGCA TCGCG-3′ and 5′-AGCCCCCAAC CCCCCACCCC ATAGA-3′. The 217:1 genomic clone was used as template and PCR products were phosphorylated using Polynucleotide Kinase and digested with *Asc*I (both New England Biolabs) according to the manufacturer's instructions before ligating into *Asc*I and *Sma*I linearized Hsp68lacZ plasmid. Ampicillin resistant clones were verified by restriction mapping and transgenes were purified.

For deletion mutagenesis, the intron 1 enhancer PCR fragment was polished using T4 polymerase and cloned into an *Eco*RV linearized pBSIIKS plasmid. Orientation of the insert was verified by restriction mapping, and a clone containing an insert that could be liberated by *Asc*I and *Sma*I digestion was selected. Six individual mutants of this enhancer element were generated by PCR using the following oligonucleotides: I, 5′-AACCCTGGTG TTCGCA-GAGG-3′ and 5′-TCTGGTTCCT GTACCAACAT-3′, II, 5′-CACGTTAAAC ATGTTGGTAC-3′ and 5′-ATTTCCAAAC CGGAGCCGCT-3′, III, 5′-TTG-GCCGGCC CTTTGAAATA-3′ and 5′-GGATGCCATT GTTAATTTGT-3′, IV, 5′-TGGCATCCCA AACACAGGAA-3′ and 5′-TTGTTAATTT GTTCCCAT-GC-3′, V, 5′-TTGGCAGCCC CGGCTCCTGC-3′ and 5′-CAGCCCTCAC TCGTGCTCGG-3′, VI, 5′-CCAGAATTAA CTGCAAAGTG-3′ and 5′-TTCCCTGCGA GGAACGGAAG-3′. This strategy results in deletion of: I, 40 nt, II, 9 nt, III, 40 nt, IV, 10 nt, V, 36 nt and VI, 28 nt. The 480 nt intron 1 enhancer pBSIIKS clone was used as template in each of these reactions. PCR products were phosphorylated, ligated, transformed into competent cells and verified by restriction mapping. The inserts of selected clones were sequenced for confirmation, and *Asc*I/*Sma*I cloned into the Hsp68lacZ plasmid from which transgenes were purified.

For site-directed mutagenesis, the 480 nt intron 1 enhancer pBSIIKS clone was used as template to replace three base pairs within the putative FOXD3 binding sites. Platinum Hi-Fidelity polymerase was used to perform PCR using