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Creation of a hybridization probe specific to chick bone morphogenetic protein-6

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NOTE TO USERS

CREATION OF A HYBRIDIZATION PROBE SPECIFIC TO CHICK BONE MORPHOGENETIC PROTEIN-6

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By

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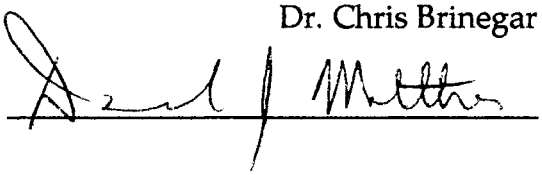

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ABSTRACT

CREATION OF A HYBRIDIZATION PROBE SPECIFIC TO

CHICK BONE MORPHOGENETIC PROTEIN-6

By Stephanya Freckelton

Developmental defects involving heart septation and valvulogenesis contribute to a high percentage of all deaths attributed to congenital defects. Understanding the mechanisms underlying cardiogenesis may help researchers develop cell- and gene-based therapies for these conditions. Studies conducted in the mouse and chick have shown bone morphogenetic proteins play key regulatory roles in the development of several organ systems. Recent studies indicate bone morphogenetic protein-6 (BMP-6) is expressed in a unique spatio-temporal pattern that coincides with heart septation and valve formation. Further studies are needed, however, to determine the regulation and role of BMP-6 in cardiogenesis. This study outlines the process of designing and generating a DNA probe, for use in future studies, that is specific to the chick BMP-6 gene, with minimal cross-reactivity to other BMPs.

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INTRODUCTION

Heart defects, specifically defects in septation and valve formation, have the highest mortality rate of all congenital defects, and are believed to occur in approximately 1% of all live births (Délot, 2003b). Many of the underlying mechanisms behind cardiac morphogenesis have yet to be clearly defined. Research to elucidate the genes and signaling pathways involved in the process of cardiogenesis are currently being conducted in several animal models.

One important class of molecules involved in many aspects of developmental regulation is the transforming growth factor-β (TGF-β) superfamily. Included in this family are the bone morphogenetic proteins (BMPs). Several of the BMPs have been shown to regulate key aspects of cardiac morphogenesis.

In this study the identification of a clone within a 48-hour chick plasmid cDNA library as putative chick bone morphogenetic protein-6 (BMP-6) is reported. The identity of the sequence was tested against other known BMP-6 sequences. BMP-6 expression in the developing heart, during the key phases of heart septation and valve formation, has previously been reported in the mouse

(Armstrong and Bischoff, 2004; Délot, 2003b; Nakajima et al., 2000) and chick (Somi et al., 2004).

A probe was designed for use in gene expression studies in the chick. Primers were carefully designed to amplify a region of the chick BMP-6 gene for use as a probe, which is highly specific and unique to BMP-6. Isotopic and non-isotopic northern blot analyses were performed to examine gene expression. Due to the very low expression of the molecule, and the inadequate sensitivity of the labeling procedures used, northern blot analysis was unsuccessful. The unique BMP-6 probe that was created was cloned into a pSTBlue-1 cloning vector for later synthesis of sense and antisense probes to be used in future *in situ* hybridization studies of chick cardiogenesis. Difficulties in designing a probe unique to one member of the highly conserved TGF-β superfamily, and the potential role for BMP-6 in organogenesis, specifically cardiogenesis, in the chick are discussed.

This thesis has been written in a format to allow for the inclusion of both a condensed write-up in journal manuscript format, and material that would have been excluded from a typical journal manuscript. An extended introduction to the information covered by the research is given, followed by the journal manuscript section, which consists of the body of the research

material, condensed into a format specified by the instruction to authors for journal submission. Extended materials and methods, results, and discussion follow. The reference section contains all references to literature cited throughout the entire thesis. Appendix A is included for additional figures and tables noted in the introduction and expanded document. Figures and tables cited in the introduction and expanded document that are also cited in the journal manuscript are shown only in the journal manuscript section to avoid repetition. Appendix B contains manufacturer provided figures of DNA ladders and vector maps used throughout this study, and a list of websites for research tools, chick genome resources, and suppliers of products and services used in this research. Appendix C consists of a glossary of acronyms used in this thesis.

Heart Development in the Chick

Morphological changes observed during all developmental stages of the chick have been well characterized, due to the importance of the chick as a developmental model (Hamburger and Hamilton, 1951; Huettner, 1950; Patten, 1952). Developmental stage of the chick is most commonly classified by the Hamburger-Hamilton (HH) system, by somite number, or by hours of

incubation (Table 1). The processes underlying cardiogenesis in the chick begin in the primitive streak stage. Most of the morphological changes required to form the heart are completed within the first five days of development.

By 16 hours of incubation (HH 3-4), the embryo is in the primitive streak stage (Huettner, 1950; Patten, 1952). The presumptive heart-forming region has been identified in the primitive streak stage embryo as bilateral segments of anterior mesoderm, adjacent to the anterior notochord. At 24 hours (HH 6) there is a thickening of the lateral splanchnic mesoderm, which later forms the paired heart primordia. Between 24 and 29 hours (HH 7-8) the visceral and somatic mesodermal layers separate to form the amnio-cardiac vesicles, and the mesoderm differentiates into outer epimyocardium, and inner endocardium layers (Fig. 1). These vesicles shift medially and fuse along the midline to form the linear heart tube (Figs. 2A,B; A). The heart tube expands and bends to the right side of the embryo by 33 hours (HH 9) (Fig. 2A,B; B-D). By 39 hours (HH 10-11) the heart has twisted dramatically, and is in the "looping" stage.

Cardiomyocytes begin uncoordinated contractions around 42 hours, and rhythmic contractions are generally seen by 44 hours (HH 11) (Huettner, 1950; Patten, 1952). Between 40 and 55 hours (HH 11-15), the heart tube dilates and elongates, and twists in a right-handed manner, realigning the prospective

chambers so that the forming atrium is to the left and cephalic to the ventricle (Fig. 2A,B; E-I). During this same period a constriction forms, initiating atrioventricular canal (AVC) formation, which will separate the two chambers. The atrium begins to divide into right and left chambers by 4 days (HH 22-23). Also by the fourth day of development, the myocardium shows distinct trabeculation, and endocardial cells begin to migrate, during a process called the endocardial-to-mesenchymal-cell transformation (EMT), to form the endocardial cushions. Cells from the endocardial cushions will septate the atrium and ventricle into right and left sides of the heart (HH 29), and are also involved in valve formation (HH 31-33).

The TGF-β Family

The TGF-β superfamily is a very large, diverse group of highly evolutionarily conserved proteins (Herpin et al., 2004). There are three main subdivisions of the TGF-β superfamily based on sequence homology and signaling mechanism: *i*) the TGF-β family, *ii*) activins, and *iii*) the BMP family. The wide chromosomal dispersal of related members of this superfamily, combined with the high degree of interspecies conservation, suggests an ancient origin for the TGF-β superfamily, and indicates that these proteins serve

a critical function in regulating key developmental events (Dickinson et al., 1990).

Members of this superfamily are synthesized as a large pre-pro-protein, consisting of an N-terminal pro-domain, a C-terminal biologically active peptide and hydrophobic signal (Herpin et al., 2004). The signaling peptide is cleaved to release a precursor protein, consisting of a pro-domain and a biologically active peptide. The precursor protein is N-glycosylated, and subsequently cleaved to release the mature active peptide. The active peptide contains the TGF-β superfamily consensus sequence. A high degree of sequence homology has been maintained in the mature peptide region, while the pro-domain displays moderate sequence homology (Burt and Law, 1994; Herpin, 2004).

Bone Morphogenetic Proteins

The BMPs are further subdivided based on sequence homology. There are two main groups: *i*) the Dpp subgroup, related to Drosophila *decapentaplegic* (*dpp*) consisting of BMP-2 and BMP-4; and *ii*) the 60A subgroup, related to Drosophila 60A protein which consists of BMP-5, BMP-6, BMP-7, BMP-8a and BMP-8b (Solloway, 1998; Zhao, 2003). Within the 60A subgroup, BMP-7 is

located on human chromosome 20, while BMP-5 and -6 are both located on human chromosome 6, indicating a more recent divergence between these two genes (Hahn et al, 1992). Four other BMP subgroups have been proposed: *i*) BMP-3a and BMP-3b; *ii*) BMP-9 and BMP-10; *iii*) BMP-12, BMP-13, and BMP-14; and *iv*) BMP-11 and growth/differentiation factor-8 (Chen, 2004).

Bone Morphogenetic Protein-6 Expression and Function

Bone morphogenetic protein-6 (BMP-6), also known as Vegetal-related-1 (Vgr-1), is expressed in a wide array of tissues during embryonic development. BMP-6 has been reported in stellate and Kupffer cells of the liver in rat (Knittel et al., 1997), in bovine and rat ovarian tissue and oocytes (Glister et al., 2004; Otsuka et al., 2001), in the smooth muscle cells of human fetal intestine (Perr et al., 1999) and in both healthy and sclerotic human blood vessels (Schluesener and Meyermann, 1995a). Early studies reported expression of BMP-6 in rat radial glial cells (Schluesener and Meyermann, 1994) and Schwann cells (Schluesener et al., 1995b). Other studies revealed expression of BMP-6 in the developing central nervous system, specifically the optic nerve, neocortex, and pyramidal cells of the hippocampus, but did not confirm expression in glial cells in rodents (Lyons et al., 1989; Tomizawa et al., 1995; Wall et al., 1993).

In the skeletal system, BMP-6 plays a role in ossification. BMP-6 is expressed in hypertrophic cartilage of the developing mouse skeletal system (Iwasaki et al., 1997; Lyons et al., 1989). The level of BMP-6 in bovine growth plate chondrocytes has been reported to increase with decreasing cell size in hypertrophic cartilage (Carey and Liu, 1995). Studies in the chick indicate that BMP-6 is expressed in osteoblasts, but not chondrocytes, and is required for ossification of the cartilage model (Mayer et al., 1996). BMP-6 appears to induce ossification by accelerating the rate of maturation of chondrocytes, and the rate of matrix mineralization in the chick (Boskey et al., 2002). In rodents, expression appears to be required to induce early differentiation, but is not required to commit cells to an osteoblastic fate (Ebisawa et al., 1999; Gitelman et al., 1995). BMP-6 null mice have been shown to be both viable and fertile, with a slight delay in ossification of the sternum being the only visible defect (Solloway et al., 1998). Other members of the BMP family may compensate for the loss of BMP-6 in knockout mice, minimizing any deleterious effects on survivability and fertility.

BMP-6 in the skin appears to play an important role in cellular differentiation and wound healing. BMP-6, for example, is strongly expressed in the suprabasal layers of the skin in mouse (Drozdoff et al., 1994; Lyons et al.,

1989; Wall et al., 1993), while keratinocyte differentiation appears to be promoted by BMP-6 expression (D'Souza et al., 2001; McDonnell et al., 2001). Increased expression of BMP-6 is seen in newly formed epithelium at the site of wound repair, and appears to play a role in regulating cell proliferation in the mouse (Drozdoff et al., 1994; Kaiser et al., 1998); over-expression leads to delayed healing, while under-expression results in cellular hyperproliferation (Kaiser et al., 1998).

BMP-6 is found in many normal and malignant samples of glandular tissues. A slight increase in BMP-6 has been reported in malignant prostate tissue over levels found in healthy prostate in rats and humans; however, the level does not correlate with tumor grade or potential for metastasis (Barnes et al., 1995). BMP-6 is also expressed in both normal and malignant human breast tissue (Clement et al., 1999). Unlike the prostate cancer study, the breast tumor samples in the study by Clement et al. showed a wide range of BMP-6 expression, which correlated with the presence or absence of epidermal growth factor receptor. BMP-6 was also shown to be expressed in serous acinar, but not ductal cells, of normal and cancerous tissue from human submandibular, parotid, and intraoral glands (Heikenheimo et al., 1999). While BMP-6 does not appear to be involved in tumor formation, there may be a link between BMP-6

expression in malignancies of glandular tissues such as breast and prostate, which metastasize to bone, due to its osteoinductive properties.

Bone Morphogenetic Protein-6 in Heart Development

The role of BMP-6 in cardiogenesis has come under question due to the lack of cardiac defects in BMP-6 null mice (Solloway, et al., 1998). Tissue explant studies in the chick have shown that BMP-6 does have a minor cardiogenic effect on non-precardiac mesoderm from the lateral plate, although BMP-6 is not required for cardiac induction (Barron et al., 2000). BMP-6 is expressed in a temporally and spatially defined pattern in the developing heart and appears to play a role in cushion formation, septation, and valve formation in the mouse (Armstrong and Bischoff, 2004; Délot, 2003b; Nakajima et al., 2000).

BMP-6 expression in the mouse heart has been observed from the heart tube stage through septation and valve formation (Kim et al., 2001). Expression is first observed in the myocardium of the linear heart tube at 8.5-9.5 days post coitum (d.p.c.), and by 9.5 d.p.c. BMP-6 is localized to outflow tract (OFT) myocardium and endocardium. Within 24 hours the signal strength decreases on the right side of the OFT, and is altogether absent on the right side in an

additional 24 hours. By 11.5 d.p.c., BMP-6 is also expressed in the developing atrioventricular canal (AVC). At the start of valvuloseptal morphogenesis (12.5 d.p.c.), BMP-6 is seen in the mesoderm of the valve leaflets, cushion mesenchyme, and endothelium of the forming aortic and pulmonary trunks.

While no cardiac defects are observed in BMP-6 null mice (Solloway et al., 1998), double null mutants for BMP-6 and BMP-7 die between 10.5 and 15.5 d.p.c. due to circulatory failure. These double null mice display several cardiac defects including delayed EMT, decreased OFT cushion size, abnormal ventricular trabeculation, and a dilated atrium. This indicates that BMP-7 may compensate for loss of BMP-6 in cardiogenesis and other developmental pathways.

Expression of BMP-6 during chick cardiac morphogenesis shares a very similar pattern to that described in the mouse (Somi et al., 2004). One of the primary differences noted between chick and mouse cardiogenesis in this study was the onset of BMP-6 expression. BMP-6 was not seen in the developing chick heart tube before HH 28, while expression is seen at the equivalent stage in the mouse. By the time fusion of the cushions is observed at HH 29, BMP-6 was observed in the mesenchyme of the OFT septum. By stage HH 30, BMP-6 was expressed in the endocardium of the AVC, ventricular septum

mesenchyme, and restricted to the atrial side of the tricuspid valve mesenchyme. During valvulogenesis (HH 31-33) BMP-6 was weakly expressed in the luminal walls of the aortic and pulmonary trunks, extending into the forming valve leaflets. At later stages, BMP-6 is expressed in the mesenchyme around the atrium and pulmonary vein.

JOURNAL MANUSCRIPT

Development
Articles
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SUMMARY

Developmental defects involving heart septation and valve formation are associated with the greatest percentage of deaths attributed to congenital defects. Recent advances in genetic research have created a hope that understanding the mechanisms underlying developmental processes will allow researchers to develop new cell- and gene-based therapies. Gene expression studies conducted in the mouse and in the chick have shown the regulatory importance of members of the transforming growth factor superfamily. The bone morphogenetic proteins are one group within this family, which are known to play key regulatory roles in the development of several organ systems. Bone morphogenetic protein-6 (BMP-6) has recently been shown to be expressed in a unique spatio-temporal pattern that coincides with heart septation and valve formation processes. Further expression studies to elucidate the regulation and role of BMP-6 in cardiogenesis should be conducted. This study outlines the process that was undertaken to design and clone a probe that is specific to chick BMP-6, and which should have minimal cross-reactivity with other BMPs, for use in future experiments.

INTRODUCTION

Heart defects, specifically defects involving septation and valve maturation, account for the majority of fatalities linked to congenital defects (Délot, 2003b). Many advances have been made in the last few decades, which have allowed researchers to investigate organogenesis at the cellular and molecular level. However, many aspects of the pathways regulating cardiogenesis remain unresolved. Continued research into the regulatory mechanisms behind cardiogenesis holds promise for cell- and gene-based preventative or therapeutic treatments.

Bone morphogenetic proteins (BMPs), a subset of the large and diverse transforming growth factor-β (TGF-β) superfamily, play an important role in development of several organ systems, including the heart (Dickinson et al., 1990; Herpin et al., 2004). Several studies have shown that BMP-6 is expressed in a distinct spatio-temporal pattern, but the role of BMP-6 in cardiogenesis remains unclear (Armstrong and Bischoff, 2004; Barron et al., 2000; Délot, 2003b; Kim et al., 2001; Nakajima et al., 2000; Solloway, et al., 1998). In tissue explant studies, BMP-6 has been shown to exert a minor cardiogenic effect on non-precardiac mesoderm from the lateral plate; however, cardiac induction is not dependent on BMP-6 (Barron et al., 2000). BMP-6 null mice show no

cardiac defects (Solloway et al., 1998). Expression patterns and delayed cardiac development in knockout mice suggest that BMP-6 does play a role in cushion formation, septation, and valve formation in the mouse (Armstrong and Bischoff, 2004; Délot, 2003b; Nakajima et al., 2000).

Less information is available regarding cardiogenesis in the chick, despite the importance of the chick as an experimental model. Developmental stages of the chick have been well classified on an anatomical level (Table 1; Hamburger and Hamilton, 1951; Hill, 2000; Huettner, 1950), and the chick has the potential to be a useful vertebrate model for genetic and developmental studies. Stages of heart development have been well described (Fig. 1; Huettner, 1950 and Figs. 2A, 2B; Patten, 1952), however the molecular mechanisms underlying various aspects of cardiogenesis have yet to be fully elucidated.

One study published in the last year examined the developmental expression pattern of BMP-2 through BMP-7 in the developing chick heart (Somi, et al., 2004). The results of the study reveal a regulatory role for BMPs in chick cardiogenesis that closely parallels that seen in mice. The only notable exception to the expression pattern of BMP-6 during cardiogenesis between

mice and chick embryos was in the onset of BMP-6 expression in the early heart tube stage.

This study reports the identification of a clone within a 48-hour chick plasmid cDNA library, which has significant sequence identity to a putative chick partial BMP-6 sequence. A unique region, specific to the BMP-6 gene was identified, and used to create a gene specific probe for use in later *in situ* hybridization studies of chick cardiogenesis. Difficulties in defining a region with minimal non-specific binding to closely related family members are discussed. The use of the chick as an animal model, and the role of BMP-6 in cardiogenesis and its possible medical implications are briefly discussed.

MATERIALS AND METHODS

Screening of chick embryo cDNA library

A 48-hour chick embryo plasmid cDNA library in pBluescript SK+ transfected DH5α *Eschericia coli* cells (previously constructed by Payam Shahi) was randomly screened (Ausubel et al., 1999). Colonies were grown overnight in 3.0 ml LB/ Amp (100 μg/ ml) at 37°C, 250 rpm. Cultures were then streaked on LA/ Amp (100 μg/ ml) plates, and incubated overnight at 37°C. Single, well-isolated clones were picked, and grown overnight in 5.0 ml LB/ Amp (100 μg/

ml) at 37°C, 250 rpm. Plasmids were purified from 4.5 mls of each sample using the QIAprep Spin Miniprep Kit (Qiagen).

Presence of cloned inserts was determined by EcoRI (Promega) digestion and electrophoresis on 1% TAE agarose gels. Samples were visualized by staining with 0.5 μg/ ml ethidium bromide and illuminated using a BioRad GelDoc. Insert size was determined by comparison to a simultaneously run Promega 1 kb DNA Ladder.

DNA sequencing

Isolated clones were sequenced using the Big Dye Terminator Cycle Sequencing Kit v. 1.0 (Applied Biosystems), using the half-reaction protocol. Samples were purified by ethanol/ sodium acetate precipitation prior to electrophoresis.

Identification of sequence

Prior to homology searches, sequences were run through VecScreen (NCBI; http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to identify and remove any potential vector sequence. Sequences were then run through species-specific BLAST searches against the databases held by The Institutes for

Genomic Research (TIGR; http://tigrblast.tigr.org/tgi/) for preliminary identification and additional BLAST searches of the NCBI databases (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were imported into the SDSC Biology Workbench (SDSC; http://workbench.sdsc.edu/) for further analysis. Sequence homology was determined by ClustalW alignment (Thompson et al., 1994) and Boxshade analysis (Hofmann and Baron, unpublished).

Probe design

The probe was designed to a unique region of a partial putative chick BMP-6 (TC122562; June 2004). The 10H12 clone from the cDNA library aligned in a non-coding region of the gene. Chick β-actin (NM_205518; June 2004) was selected as a positive control for hybridization studies. RT-PCR and the following primer pairs were used to generate RNA probes:

BMP-6 (TC122562): Right Primer 5′ GTTTCAGTGTAAATCCTAGAGAAG 3′,

BMP-6 (TC122562): Left Primer 5′ TCTGCACGCGGTTTTTAA 3′ (232 nt);

chick β-actin (NM_205518): Right Primer 5′ AGCCAACAGAGAGAAGATGA 3′,

chick β-actin (NM_205518): Left Primer 5′ TCTTCATGAGGTAGTCCGTC 3′ (236 nt).

Amplification and quantification of probe

RT-PCR was performed using Qiagen's OneStep RT-PCR Kit, according to the manufacturer's protocol. Sample concentration was determined by gel electrophoresis against Invitrogen DNA Mass Ladders. The sample was further amplified in a secondary PCR reaction using Qiagen's Taq Core kit, and purified with Amicon-30 Spin Filters.

Cloning of the probe into pSTBlue-1

The purified PCR product was cloned using Novagen's pSTBlue-1 PerfectlyBlunt Cloning Kit. An insert to vector molar ratio of 2:1 was used. The end conversion and ligation reactions were performed according to Novagen's protocol. Transformation of NovaBlue Singles competent cells was performed according to the manufacturer's protocol, followed by a 60-minute outgrowth at 37°C with shaking at 250 rpm. Cells were plated onto IPTG (0.5 mM)/ Xgal (80 μg/ ml)/ Amp (100 μg/ ml)/ LA plates and grown overnight at 37°C (Ausubel et al., 1999).

Confirmation of cloned probe

Plates were screened using blue-white screening of colonies. White colonies were picked and amplified by growth on secondary grid plates. White colonies were then screened by colony PCR and/ or plasmid isolation and restriction digestion for presence of the insert. PCR products were run on 2% TAE agarose gel with Invitrogen's Low Mass Ladder and Promega's 1kb DNA Ladder. Plasmid isolation was performed with Qiagen's QIAprep Spin Miniprep kit, and by the alkaline lysis method. BamHI, HindIII and EcoRI (Promega) were used for restriction analysis of isolated plasmid. Linearized plasmids were run out on 1% TAE agarose gel with Invitrogen's High Mass Ladder and Promega's 1 kb DNA Ladder.

Probe identity was confirmed by amplifying the probe from isolated pSTBlue-1 vector using SP6 and T7 or specific BMP-6 primers, and sending the PCR product out for sequencing by Gene Gateway (Hayward, CA) and Tocore (Menlo Park, CA). Sequence homology was determined by ClustalW alignment (SDSC) and Boxshade analysis (SDSC). The clone containing the probe was stored 1:1 in glycerol freezing media at -70°C (Ausubel et al., 1999).

RESULTS

Identification of clone as putative chick BMP-6

The clone 10H12, isolated from the 48-hour chick cDNA library was found to have significant sequence identity to a sequence identified as chick BMP-6 (Fig. 3). The sequence found in the TIGR database, TC122562 (June 2004) was described as a partial sequence, homologous to BMP-6. The 10H12 clone sequence aligned to the putative chick BMP-6 sequence with greater than 90% identity.

The identity of the putative chick BMP-6 from the TIGR database was determined by homology to known BMP-6 sequences from other organisms. The nucleic acid sequence for chick BMP-6 was translated in Sixframe (SDSC), and the longest open reading frame was selected. BMP-6 protein sequences from mouse, rat, and human were obtained from NCBI databases. Homology was compared using ClustalW (SDSC) and Boxshade (SDSC) (Fig. 4).

The TIGR sequence for chick BMP-6 is a partial cDNA sequence, therefore the sequence was translated in Sixframe for further analysis. The longest ORF of the translated sequence was used for all alignments. The longest ORF sequence is missing the N-terminal end of the known protein sequence found in the other species, which may be a region of the pre-pro-

protein, which is later cleaved to form the active protein. The dashes in the sequence alignment for BMP-6 (Fig. 4 and Fig. 5) indicate this missing N-terminal region. For the segment that is represented by the chick sequence, a very high degree of identity is seen across species.

Selection of a region for use as a specific BMP-6 probe

The bone morphogenetic proteins are a large highly conserved family. There is a high degree of sequence conservation both within species for a specific BMP family member, and between BMP family members within a given species. To find a region that was specific to chick BMP-6, the translated sequence was compared to other known chick BMP sequences. Protein sequences for chick BMP-2, -4, and -7 were obtained from NCBI databases. Homology was compared using ClustalW (SDSC) and Boxshade (SDSC) (Fig. 5). As expected, BMP-6 had a higher degree of sequence identity with BMP-7 than with BMP-2 and -4, which share a high degree of sequence identity with each other. The region with the highest degree of identity across all family members was at the C-terminal end of the protein sequence. This region contains the coding region for the biologically active peptide, and has the TGF-

β-1 family signature consensus sequence (Prosite_PDOC00223: [LIVM] - x (2) - P - x (2) - [FY] - x (4) - C - x - G - x – C).

A probe for chick BMP-6 needs to contain a sequence that is specific to BMP-6 and that has low sequence identity to other BMP family members. A composite of the previous protein alignments was constructed to show which regions are specific and unique to chick BMP-6 (Fig. 6). The region, which showed the highest conservation between species for BMP-6, with the lowest sequence identity between BMP family members, was selected for probe design. The amino acid sequence was then back translated to the original nucleic acid sequence, using the Sixframe output. Primer 3 (SDSC) was used to design the best primer pair, and the primer sequences were checked against the sequence alignments to ensure that they would amplify a region with high homology to BMP-6 and low homology to other BMP family members. The amplified region to be used as a BMP-6 probe was limited in length due to homology constraints. Oligo primers were synthesized to BMP-6 by Operon (Huntsville, AL): BMP-6 Right Primer 5′ GTTTCAGTGTAAATCCTAGAGAAG 3′, BMP-6 Left Primer 5′ TCTGCACGCGGTTTTTAA 3′ (product size = 232 bp).

Creation of a vector containing the BMP-6 probe

The BMP-6 primers were used to isolate the selected region of chick BMP-6 by RT-PCR. The product was further amplified by a secondary PCR reaction, and spin purified using Amicon-30 spin filters according to the manufacturer's protocol. NovaBlue competent cells were then transformed with pSTBlue-1 containing the amplified BMP-6 probe region. Presence of the insert was confirmed by colony PCR (Fig. 7). Plasmid isolation by alkaline lysis was also performed to confirm insert presence (Fig. 8).

The probe was amplified off of the isolated pSTBlue-1 plasmid using the BMP-6 specific primers, and sent for sequencing. The probe sequence provided by Tocore (Menlo Park, CA) confirms the identity of the probe (Fig. 9). While the sequence shows some mismatched bases, the majority of the amplified region aligns with high identity to the BMP-6 sequence.

DISCUSSION

Defects in cardiac septation and valve formation are common, potentially fatal, congenital defects. The fatalities that can result from these cardiac defects might be avoided with an increased understanding of the molecular mechanisms behind these processes. Recent advances in genome

sequencing, and genetic research are identifying the genes involved in cardiogenesis, and deciphering the complex molecular pathways underlying their regulation. Increased understanding of the molecular mechanisms of cardiogenesis may aid researchers in developing gene-based methods to prevent or treat heart defects.

Bone morphogenetic proteins are regulatory proteins that belong to a large superfamily of growth factors. Animal models, most notably the mouse, have been used for gene knockout and expression studies that display the importance of BMPs in the developing vertebrate embryo. BMPs have been found to play a crucial role in the development of several organ systems, including the heart. A unique spatio-temporal expression of BMP-6 has been shown during the critical stages of cardiac septation and valve formation in the mouse (Armstrong and Bischoff, 2004; Délot, 2003b; Nakajima et al., 2000).

The chick may also prove useful in studying gene expression during cardiogenesis. The mouse is often preferred by researchers because it is a mammal, and therefore more closely related to humans than an avian species. However, use of a mouse model has inherent drawbacks, including the expense of care of animals, a long gestation time, increased sensitivity to some genetic mutations resulting in early embryonic lethality, and increasing regulations

regarding the use of animals in research. In contrast, the chick is relatively easy and inexpensive to care for through embryogenesis, developmental stages have been well-defined, there is a shorter gestation time, harvesting of embryos at various developmental stages does not require sacrifice of the mother, and a high level of conservation is seen in regulatory genes shared by mammals and avians.

The majority of the experimental evidence for BMP regulation of aspects of cardiogenesis comes from studies in the mouse. One notable exception is the study by Somi, et al. (2004), which examined the spatio-temporal expression of BMP-2, -4, -5, -6, and -7 in the chick at several developmental stages. That study indicated that the role of BMP-6 in the chick parallels that seen in the mouse.

The purpose of this study was to create and clone a specific and unique hybridization probe to chick BMP-6 for future studies of the gene expression patterns during cardiogenesis by *in situ* hybridization against whole mount chick embryos. The clone 10H12 from the 48-hour chick plasmid cDNA library was found to be homologous to a partial sequence for a putative chick BMP-6. Sequence alignments against BMP-6 sequences from other species were conducted to confirm the identity of the chick BMP-6 sequence. The sequence

was analyzed to find a specific and unique region to be used as a chick BMP-6 probe. The segment of the gene captured in the cDNA library did not align with the coding region of the gene, and was therefore not chosen.

The size of the TGF-β superfamily, of which BMPs are only one sub-family, and the highly conserved nature of the members make it very difficult to find a suitable region to be used as a specific and unique probe. The putative chick BMP-6 sequence was compared to known BMP-6 sequences from other species to confirm its identity. There is an 80% sequence identity between the avian sequence and the sequences from human, mouse, and rat.

There is also a high degree of identity between members of the BMP family within each species. BMP-2 and BMP-4 comprise one subset of the BMP family, known as the Dpp subgroup (Solloway, 1998; Zhao, 2003). BMP-5, BMP-6, and BMP-7 belong to a different subset of the BMP family, known as the 60A subgroup (Solloway, 1998; Zhao, 2003). As expected, the putative chick BMP-6 sequence was more similar to BMP-5 and BMP-7 than to BMP-2 and BMP-4. Finding a sequence within the coding region of the gene that is specific to BMP-6, and that has low homology to the other BMP family members, especially BMP-5 and BMP-7, is essential to avoid non-specific binding of the

probe. Primer sequences were thus chosen to select a probe region with minimal homology to other BMP family members.

A bioinformatics-based approach was used to identify a 232 bp region of the BMP-6 sequence, which is expected to bind selectively (under high stringency hybridization conditions) solely to BMP-6. The fundamental requirement for high specificity meant that primer design was, of necessity, restricted to the sequence within (or tightly bracketing) this 232 bp region to avoid any unnecessary homology to other family members. While some minimal overlap of residues could not be avoided, multiple BLAST searches comparing the probe region against the NCBI database make it highly unlikely that the probe and other members of the BMP family would interact. Although a longer probe might be more desirable due to the ability to label to a higher specific activity, probe length was restricted by this need to avoid cross-reactivity with other BMP family members.

The amplified probe region was inserted into pSTBlue-1 cloning vector. NovaBlue cells transfected with the insert containing pSTBlue-1 vectors were then screened to confirm the presence and identity of the insert. Liquid cultures grown from these recombinant colonies were stored at -70°C in freezing media. Plasmid isolated from these cells can then be used to create

single-stranded DNA probes via end-labeling followed by denaturation, or single-stranded sense and antisense riboprobes via *in vitro* transcription using the SP6 and T7 primers (or the specific BMP-6 primers) for use in future gene expression studies.

Understanding the molecular mechanisms underlying cardiogenesis may yield significant advances in the prevention and/ or treatment of morbidity and mortality associated with congenital heart defects. Studies in the mouse and chick have provided a great deal of insight into some developmentally critical genetic pathways. The BMP family is part of a much larger superfamily of signaling molecules that play an important role in many aspects of embryogenesis, and BMP-6 has recently been shown to express a unique spatio-temporal pattern, which suggests a crucial role in septation of the heart and formation of the heart valves.

The most common congenital heart defects are those that involve improper septation and valvulogenesis; however, the mechanisms underlying these formative processes and the events that give rise to these developmental defects remain unclear. Further studies into the expression and regulation of BMP-6 thus seem necessary to further our understanding of the complex signaling pathways, and these interactions that are involved in specific aspects

of heart formation, if cell- and gene-based preventive measures and therapies are to be developed.

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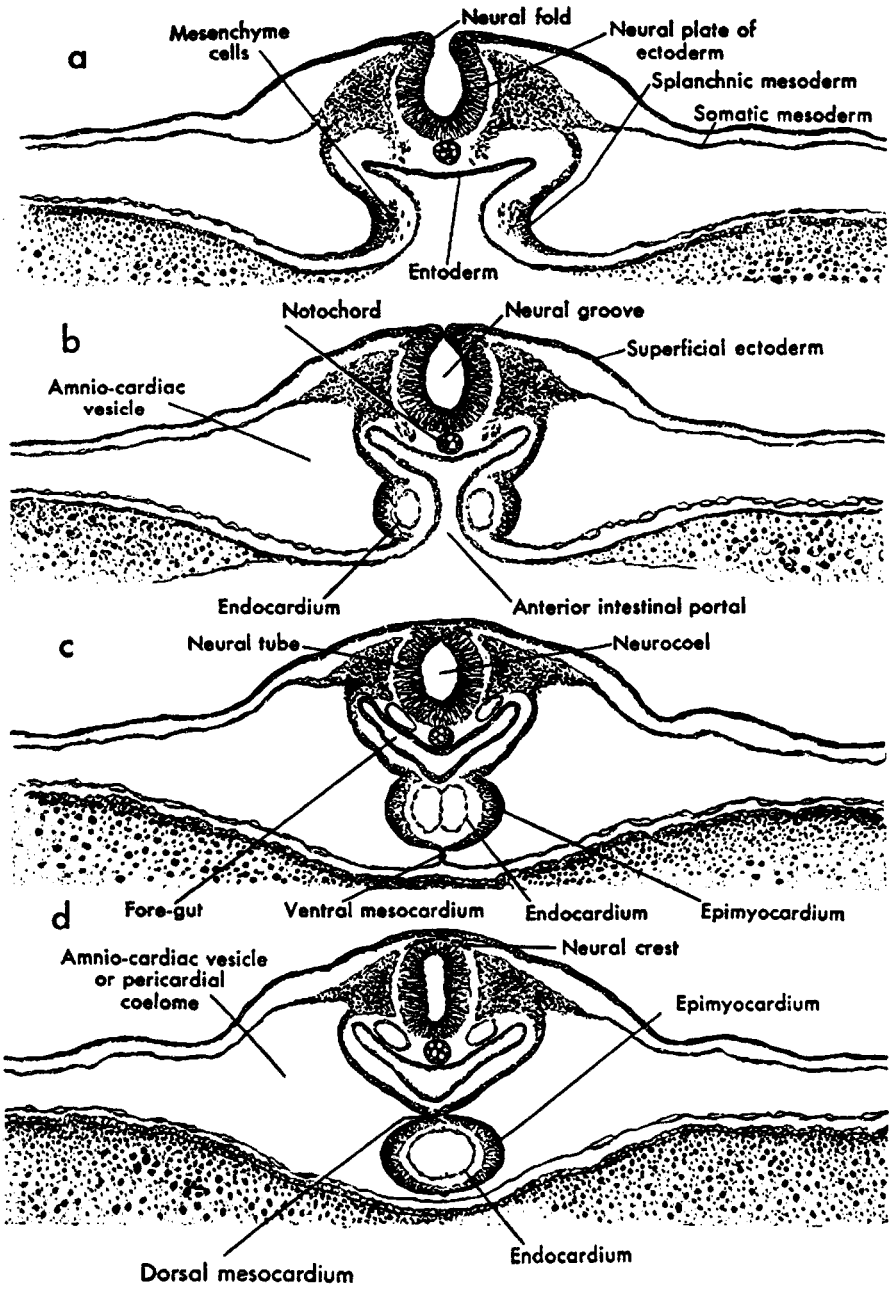

Fig. 1. Development of the chick heart from 24 to 29 hours (transverse sections). From Huettner, A.F. (1950). The Embryology of the Chick. In *Fundamentals of Comparative Embryology of the Vertebrate.*, pp.142- 233, Figure 109. New York: The MacMillan Company. a) 25-hour embryo, b) 26-27-hour embryo, c) 27-28-hour embryo, d) 29-hour embryo.

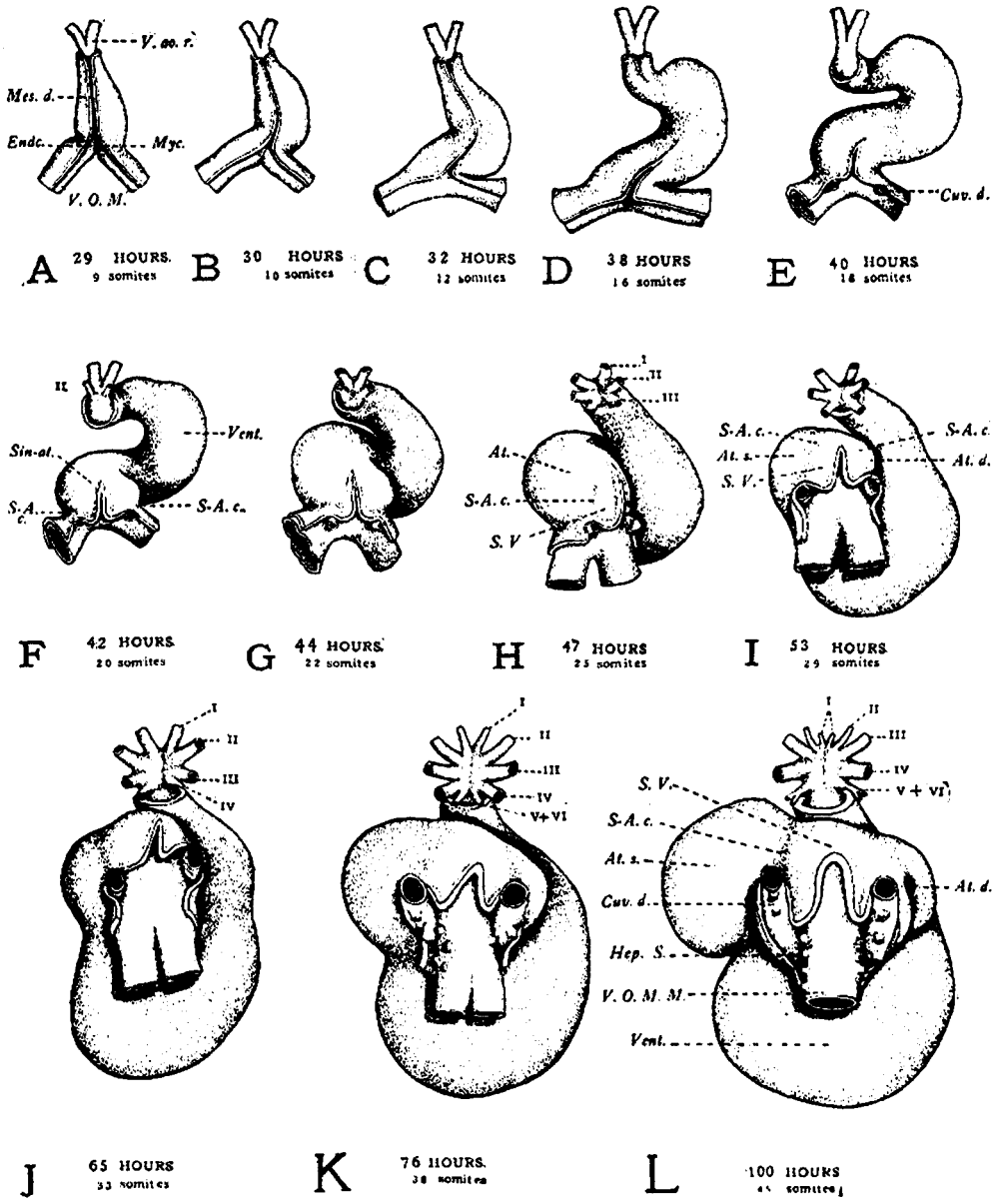

Fig. 2A. Development of the chick heart from 29 to 100 hours (dorsal aspect). From Patten, B.M. (1952). The Circulatory System. In *Early Embryology of the Chick,* pp.181-201, Figure 96. New York: The Blakiston Company. Abbreviations: (I-VI) aortic arches I to VI; (*At. d.*) atrium, right; (*At. d.*) atrium, left; (*Cuv. d.*) duct of Cuvier; (*Endc.*) endocardium; (*Hep. s.*) stubs of some of the larger hepatic sinusoids; (*Mes. d.*) dorsal mesocardium; (*Myc.*) cut edge of epi-myocardium; (*S-A. c.*) sino-atrial constriction; (*Sin-at.*) sino-atrial region (before its definite division); (*S.V.*) sinus venosus; (*V. ao. r.*) ventral aortic roots; (*Vent.*) ventricle; (*V.O.M.*) omphalomesenteric veins; (*V.O.M.M.*) fused omphalomesenteric veins.

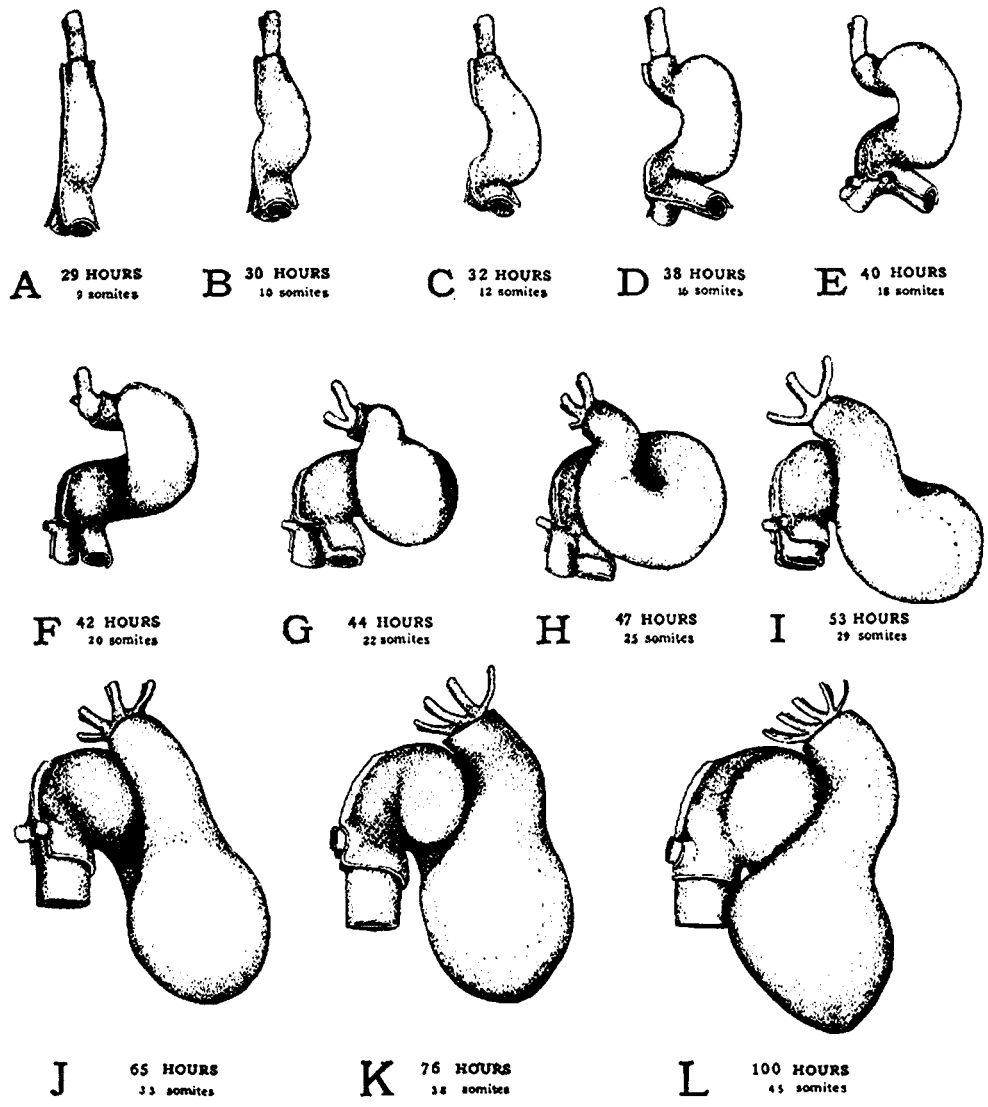

Fig. 2B. Development of the chick heart from 29 to 100 hours (dextral aspect). From Patten, B.M. (1952). The Circulatory System. In *Early Embryology of the Chick,* pp.181-201, Figure 94. New York: The Blakiston Company.

TIGR_BLAST.
Query = clone 10H12 (639 letters)
Subject = g_gallus|TC122562 homologue to UP|Q811S4 (Q811S4) Bone morphogenetic protein-6, partial (50%) Length = 4101
Minus Strand HSPs: Score = 2763 (420.6 bits), Expect = 2.2e-119, P = 2.2e-119
Identities = 593/640 (92%), Positives = 593/640 (92%), Strand = Minus / Plus

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Fig. 3. Alignment of clone 10H12 from the 48-hour chick plasmid cDNA library to a putative chick BMP-6 homologue in the TIGR database.

Fig. 4. Alignment of the translated putative partial chick BMP-6 sequence against BMP-6 protein sequences from mouse, rat, and human.

Sequences aligned with ClustalW and analyzed with Boxshade at the SDSC Biology Workbench site. Sequences obtained from NCBI, except chick (TIGR) longest open reading frame, obtained by translation with Sixframe in the SDSC Biology Workbench. (mouse = bone morphogenetic protein-6 [*Mus musculus*] NP_031582.1 [NM_007556], 510 AA; rat = bone morphogenetic protein-6 [*Rattus norvegicus*] NP_037239.1 [NM_013107], 506 AA; human = bone morphogenetic protein-6 precursor [*Homo sapiens*] NP_001709.1 [NM_001718], 513 AA; chick = putative BMP-6 [*Gallus gallus* BMP-6 Translated - Longest ORF, +1, 215 AA]). The TGF-β-1 family signature consensus sequence (Prosite_PDOC00223: [LIVM] –x(2)-P-x(2)-[FY]-x(4)-C-x-G-x-C) is located on the left-hand side of the seventh row of the alignment. The conserved consensus sequence for BMP-6 is IIAPKGYAANYCDGEC.

Key: Conserved residues are magenta, identical residues are yellow, similar residues are green, and different residues are white. Gaps are indicated by a dash. In the consensus line, capital letters are conserved residues, lower case letters have high identity, and dashes indicate residues that are not conserved.

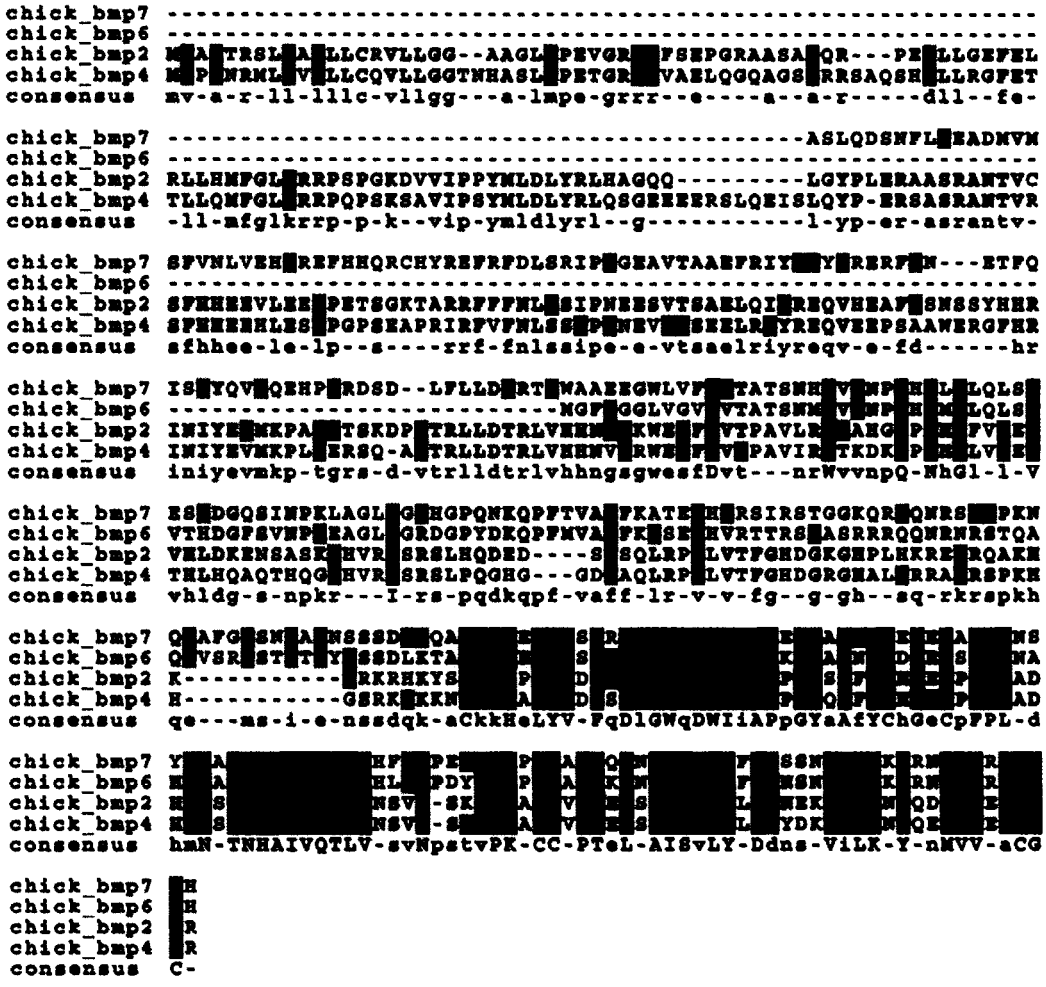

Fig. 5. Alignment of the translated putative partial chick BMP-6 sequence against other bone morphogenetic proteins from the chick.

Sequences aligned with ClustalW and analyzed with Boxshade at the SDSC Biology Workbench site. Chick bone morphogenetic protein-2, -4 and -7 obtained from NCBI. Chick BMP-6 sequence is the longest open reading frame, obtained by translation with Sixframe at the SDSC Biology Workbench. (bone morphogenetic protein-7 [*Gallus gallus*] AAF34179.1 [AF205877], 398 AA; putative BMP-6 [*Gallus gallus* BMP-6 Translated - Longest ORF, +1, 215 AA]; bone morphogenetic protein-4 [*Gallus gallus*] NP_990568.1 [NM_205237], 405 AA; bone morphogenetic protein-2 [*Gallus gallus*] NP_989689.1 [NM_204358], 392 AA). The TGF-β-1 family signature consensus sequence (Prosite_PDOC00223: [LIVM] –x(2)-P-x(2)-[FY]-x(4)-C-x-G-x-C) is located on the right-hand side of the sixth row of the alignment. The conserved consensus sequence for BMP-6 is IIAPKGYAANYCDGEC.

Key: Completely conserved residues are magenta, identical residues are yellow, similar residues are green, and different residues are white. Gaps in the sequence are indicated by a dash. In the consensus line, capital letters are conserved residues, lower case letters have high identity, and dashes indicate residues that are not conserved.

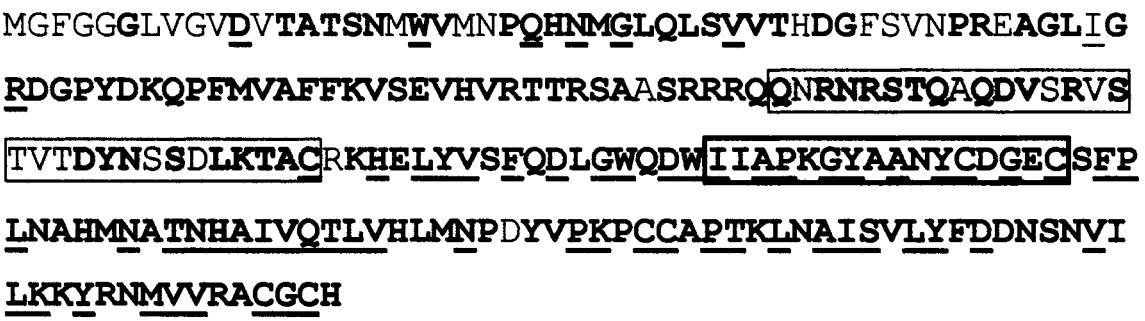

Fig. 6. Conserved and non-conserved BMP-6 amino acid residues based on alignments of chick BMP paralogs and BMP-6 orthologs used to identify optimal probe regions on which to base a specific and unique chick BMP-6 probe.
The sequence alignments of BMP-6 across species, and of various BMP family members within the chick were used to create a composite sequence to indicate which residues are conserved across species and across BMP family members. BMP-6 residues conserved across human, mouse, rat, and chick are shown in bold. Residues conserved across several BMPs within the chick are underlined. The chick homologue to a previously described unique sequence for BMP-6 (Schluesener and Meyermann, 1994): QSRNRSTQSQDVARGSSASDYNSSELKTAC (30 AA) is boxed and shaded. This sequence is within a region that appears to contain the highest number of residues conserved across species for BMP-6 with minimal conservation of residues within the bone morphogenetic protein family. This region was selected for probe design. The TGF-β-1 family signature consensus sequence (Prosite_PDOC00223: [LIVM] – x(2)-P-x(2)-[FY]-x(4)-C-x-G-x-C) is bordered in light grey. The conserved consensus sequence for BMP-6 is IIAPKGYAANYCDGEC.

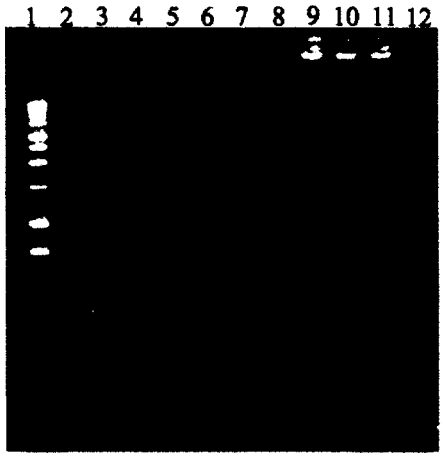

Fig. 7. Colony PCR of NovaBlue cells containing the pSTBlue-1 vector with the BMP-6 probe insert.

Colony PCR was performed on boil-preps of white colonies picked from the grid plates, using SP6 and T7 primers. Samples (lanes 2-11) were run out on 2% TAE agarose gel, and visualized by ethidium bromide staining. PCR product size was assessed with the Promega 1 kb DNA ladder (lane 1). Of the ten samples run, only one (circled in lane 2) amplified. The observed product size is close to the expected product size of ~310 bp. Colony PCR of this system provided poor results. Only one of ten white colonies was amplified, and the yield was low. A moderate percentage of the white colonies may be false positives.

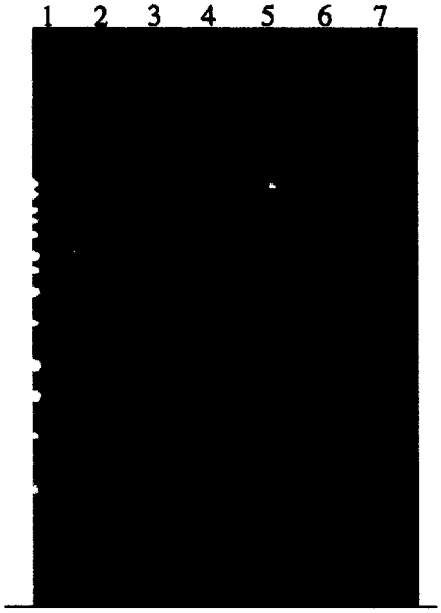

Fig. 8. Isolated and purified pSTBlue-1 vector containing the BMP-6 insert, visualized and quantified by agarose gel electrophoresis.
Plasmid isolated by the alkaline lysis method was purified by spin purification with Amicon-100 and Amicon-30 spin filters. Isolated plasmid (circled in lane 2) and filtrates (spin1, Amicon-100 in lane 3, and spin 2, Amicon-30 in lane 4) were run out on 1% TAE agarose gel with Promega's 1 kb DNA Ladder (lane 1) and Invitrogen's High Mass DNA Ladder (lane 5). The remaining lanes are empty. The isolated plasmid is shown at the expected size of ~ 4 kb, indicating the insert was present, and concentration was estimated at 5 ng/ 2 µl (90 ng total in 38 µl isolated plasmid). Plasmid yield was low with both Qiagen Miniprep plasmid isolation (results not shown) and alkaline lysis plasmid isolation. Significant loss of the plasmid during spin filtration is unlikely, because no bands are seen in the filtrate lanes (lane 3 and lane 4). Overnight growth of pSTBlue-1 transfected NovaBlue cells in LB/ Amp (100 µg/ml) at 37°C with shaking at 250 rpm resulted in relatively poor growth. For maximum growth, optimization of culture conditions for these cells should be determined.

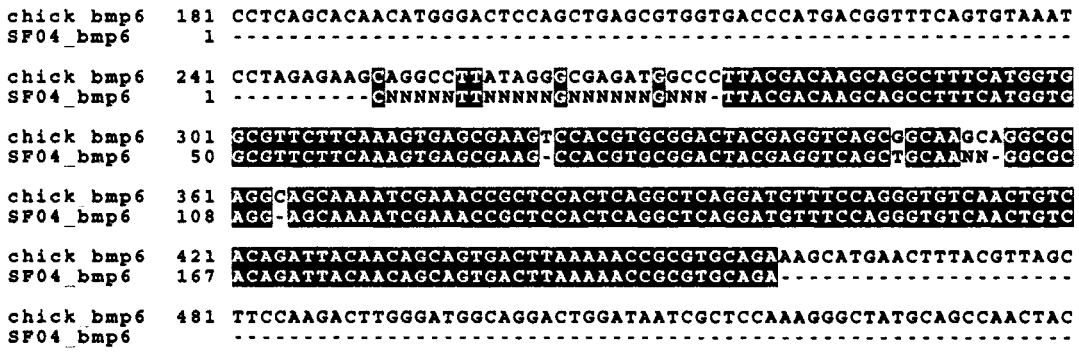

Fig. 9. Alignment of cloned BMP-6 insert in pSTBlue-1 vector against putative partial *G. gallus* BMP-6 sequence.

PCR amplified probe from isolated pSTBlue-1 plasmid was sequenced by Tocore (Menlo Park, CA) using the BMP-6 Right primer. The probe sequence (SF04_bmp6) was aligned with the putative partial chick BMP-6 sequence (chick_bmp6) in ClustalW and analyzed with BoxShade in the SDSC Biology Workbench. The sequence is shorter than the expected length. The probe sequence is degraded or unreadable at base 251- 275, after the 24 base region specified by the right primer (base 225- 249). The high degree of identity confirms that the insert in the pSTBlue-1 vector is the probe region specified by the BMP-6 primers. The insert may be used to create sense and antisense probes against chick BMP-6 for future *in situ* hybridization experiments.

Table 1. Staging of the chick embryo.
Hamburger-Hamilton (HH) stages are used to time chick development based primarily on the distinguishing morphological characteristics (Hamburger, V. and Hamilton, H., 1951). Earlier studies often used an hourly or somite based staging system. While the morphological characteristics of embryos of the same somite number are consistent, the timing of development of somite pairs can be highly variable. A reasonable estimate is that the first somite pair appears at 21 hours and one somite pair per hour is added up to 48 hours of incubation (Huettner, 1950). (Table from Hill, M. (2000). Embryology [electronic resource](version 2.2). Sydney: School of Anatomy, University of New South Wales. Accessed March 1, 2005; modified.)

HH Stage	Time of Incubation	Somite Number	Definitive Structures
1			Embryonic Shield
2	6-7 hours		Primitive Streak
3	12-13 hours		Intermediate Streak
4	18-19 hours		Definitive Streak
5	19-22 hours		Head Process
6	23-25 hours		Head Fold
7	23-26 hours	1-3	Neural Folds; Coelem
8	26-29 hours	4	Blood Islands
9	29-33 hours	7-9	Primary Optic Vesicles; Ant. Amniotic Fold
10	33-38 hours	10	3 Primary Brain Vesicles
11	40-45 hours	13	5 Hindbrain Neuromeres
12	45-49 hours	16	Telencephalon
13	48-52 hours	19-21	Atrioventricular Canal (AVC); Tail Bud
14	50-54 hours	22-23	Trunk Flexure; Visceral Arch I and II; Cleft 1 and 2; Premandibular Head Cavities
15	50-55 hours	24-27	Visceral Arch III; Cleft 3
16	51-56 hours	26-28	Wing Buds; Post. Amniotic Fold
17	52-64 hours	29-32	Leg Buds; Epiphysis
18	3 days	30-36	Somites Extend Past Leg Bud: Allantois
19	3-3.5 days	37-40	Somites Extend into Tail; Maxillary Process
20	3-3.5 days	40-43	Rotation Complete; Eye Pigmentation
21	3.5 days	43-44	Visceral Arch IV; Cleft 4
22	3.5-4 days		Somites Extend to Tail Tip
23	4 days		Dorsal Contour Curved Hindbrain to Tail
24	4.5 days		Toe Plate
25	4.5-5 days		Elbow and Knee Joints
26	5 days		First 3 Toes
27	5-5.5 days		Beak
28	5.5-6 days		3 Digits, 4 Toes
29	6-6.5 days		5^{th} Toe Rudiment
30	6.5-7 days		Feather Germs; Egg Tooth; Scleral Papillae
31	7-7.5 days		Webbing Between 1^{st} 2 Digits
32	7.5 days		Ant. Tip of Mandible Reaches Beak

33	7.5-8 days		Web of Radial Margin of Wing and 1st Digit
34	8 days		Nictitating Membrane
35	8.5-9 days		Toe Phalanges
36	10 days		Comb Primordium; Labial Groove; Uropygial Gland; Beak length (Ant. Angle of Nostril to Tip of Bill)= 2.5mm; 3rd Toe Tip to Middle of Metatarsal Joint) =5.4mm+/-0.3
37	11 days		Beak length=3.0mm; 3rd Toe=7.4mm+/-0.3
38	12 days		Beak length=3.1mm; 3rd Toe=8.4mm+/-0.3
39	13 days		Beak length=3.5mm; 3rd Toe=9.8mm+/-0.3
40	14 days		Beak length=4.0mm; 3rd Toe=12.7mm+/-0.5
41	15 days		Beak length=4.5mm; 3rd Toe=14.9mm+/-0.8
42	16 days		Beak length=4.8mm; 3rd Toe=16.7mm+/-0.8
43	17 days		Beak length=5.0mm; 3rd Toe=18.6mm+/-0.8
44	18 days		Beak length=5.7mm; 3rd Toe=20.4mm+/-0.8
45	19-20 days		Yolk Sac Half Enclosed in Body Cavity; Less Blood in Chorio-Allantoic Membrane
46	20-21 days		Hatched Chick.

EXPANDED MATERIALS AND METHODS

cDNA Library

The plasmid cDNA library utilized in this study was previously prepared by Payam Shahi (White lab, SJSU, unpublished). After 48 hours of incubation, the chick embryos were isolated and stored in RNAlater (Ambion) at -20°C. mRNA was isolated using Ambion's MicroPoly(A) Pure kit, and stored at -80°C.

cDNA was prepared using the Superscript Choice System for cDNA Synthesis (GibcoBRL/ Invitrogen), and subsequently ligated into pBluescript SK+ plasmid vector (Stratagene) at an EcoRI restriction site, using T4 DNA ligase (Promega). The resulting vector was used for transformation of Max Efficiency DH5α Competent E. Coli Cells (GibcoBRL/ Invitrogen). Colonies were grown and screened on IPTG (0.5 mM)/ Xgal (80 μg/ml)/ Amp (100μg/ ml)/ LA plates (Ausubel et al., 1999). Transformed colonies were picked and stored in 1.0 ml 96-well plates in LB/ Amp (80 μl/ ml) and grown for 6 hours at 37°C with shaking at 350 rpm (Ausubel et al., 1999). Prior to storage, glycerol freezing medium (Ausubel et al., 1999) was added 1:1 to the samples. Plates were labeled and stored at -80 °C.

Colony Screening

A total of 328 colonies were screened (Table 2). Plates were selected at random. The entire plate was screened whenever possible. Some plates were only partially screened due to time constraints and relocation of freezers and rearrangement of freezer contents beyond the author's control.

Colonies were picked from the stock freezer box (original 96-well plate) and grown in culture to produce a working stock. Each well was stabbed with a sterile P100 pipette tip and transferred to 3.0 ml sterile LB/ Amp (100 μg/ ml) in clean, sterile culture tubes. Tubes were incubated overnight at 37°C, with shaking at 250 rpm (Ausubel et al., 1999). A sample of each re-grown stock sample was taken at this point and stored 1:1 in 50% glycerol in Nunc vials and stored at -20°C to minimize any impacts of freeze-thaw effects on the original 96-well plates. Vials were labeled with box number, column and row (i.e. 2A1 = box 2, row A, column1).

Sterile inoculating loops were used to streak each culture onto LA/ Amp (100 μg/ ml) plates. The plates were incubated overnight at 37°C. A single well-isolated colony from each plate was picked and transferred to 5.0 ml LB/ Amp (100 μg/ ml) in clean, sterile culture tubes. The tubes were incubated overnight at 37°C, with shaking at 250 rpm (Ausubel et al., 1999). A second

sample was taken at this time and stored as above. The isolated colony was labeled as before with a number code suffix to signify the number of the colony.

The remaining 4.5 ml of culture was pelleted and stored at -20°C. Plasmids were purified from pelleted samples using the QIAprep Spin Miniprep kit (Qiagen). Presence of cloned inserts was determined by EcoRI (Promega) digestion (approximately 1 μg plasmid/ U enzyme activity) and electrophoresis on 1% TAE agarose gels. Samples were run with a 1 kb DNA Ladder (Promega) and visualized by staining with 0.5 μg/ ml ethidium bromide and illuminated using a BioRad GelDoc.

Clones that had an insert, which was fully cleaved by EcoRI restriction, were selected preferentially for sequencing, followed by clones that were linearized by EcoRI restriction. Isolated clones were sequenced using the Big Dye Terminator Cycle Sequencing Kit v. 1.0 (Applied Biosystems), using the half-reaction protocol. Samples were purified by ethanol/ sodium acetate precipitation prior to electrophoresis.

Due to the high number of plasmids that linearized with EcoRI digestion, a side study was conducted to determine the cause. One possibility was that residual salts from the plasmid prep might interfere with the restriction enzyme. Two samples were desalted and the EcoRI digestion was

repeated. Another possibility was that one of the restriction sites was damaged and could not be recognized by the enzyme. During the creation of the cDNA library, inserts were ligated to EcoRI adapters and placed in the vector at the EcoRI site. Digestion with EcoRI should remove the insert from the vector. HindIII and PstI were chosen for a double digestion study because they flank the EcoRI site. If the first site was damaged, then an EcoRI/ PstI digest will release the insert. If the second site was damaged, then the insert will be released with a HindIII/ EcoRI digestion.

Gene Selection

Sequences were stored at the SDSC Biology Workbench 3.2 site (http://workbench.sdsc.edu/) for analysis. Identification of isolated inserts was done by performing BLAST searches (NCBI) and by checking for sequence identity against species-specific databases at The Institutes for Genomic Research (TIGR). Sequences were run through VecScreen (NCBI) to remove any suspected plasmid DNA before the identity searches were conducted.

Identified housekeeping genes were recorded and omitted from further consideration. Genes with no homology matches, or with matches to unidentified clones were also omitted. A short list was compiled of genes with

potential for developmental significance that had no confirmed chick homologue. A single clone, which showed high sequence identity to a putative partial BMP-6 gene sequence was selected for further study.

Probe Design

The translated sequence alignments were used to identify a region unique to BMP-6 within the coding region, and a region in the non-coding segment of the gene where the 10H12 clone aligned. These regions were then identified in the nucleic acid sequence from the Sixframe output. Probes for RT-PCR were designed to these regions using Primer3 (SDSC), and the probe sequences were checked against the nucleic acid alignments, to ensure low sequence identity between the BMP-6 sequence and the corresponding region on other members of the BMP family. Oligo primers were synthesized to chick β-actin, BMP-6 (coding region), and the 10H12 clone by Operon (Huntsville, AL).

The RT-PCR probe synthesis was conducted using Qiagen's RT-PCR kit, on a thermal gradient due to the imprecision of T_m calculations provided by primer design software. RT-PCR used 48-hour total chick RNA provided by Xuezhi Li (White's lab, SJSU, CA). The RT-PCR products were run out on 1%

TAE agarose gel, and ethidium bromide stained for visualization. The optimal PCR temperature was chosen, and a secondary PCR reaction was performed using Qiagen's Taq Core kit. The PCR product was spin purified in Amicon-30 spin columns, and probe concentration was measured against mass standards. The 10H12 clone was not amplified in the first RT-PCR reaction and was redone following the same procedure.

Northern Blot Hybridization: Non-Isotopic

The purified PCR products were labeled with Ambion's BrightStar Psoralen-Biotin Nonisotopic Labeling Kit according to the protocol. A 1 in 10 dilution series of each labeled probe was created, spotted onto a positively charged nylon membrane (Schleicher & Schuell BioScience/ Whatman), and fixed by UV cross-linking. The spot test was visualized with Ambion's BrightStar Biodetect protocol.

To determine expression patterns of BMP-6 in adult chicken, a pre-made tissue blot (Seegene) was used for hybridization. This blot was constructed with RNA isolated from the following organs: brain, heart, lung, liver, spleen, kidney, small intestine, testes, uterus, ovary, and gizzard. Probe hybridization and detection followed the lab protocol (White, 2004) using the Ambion

Brightstar detection kit. According to the Seegene manual, with proper probe removal and storage of the blot, the blot could be re-probed 3 to 5 times.

The β-actin probe was used for the first hybridization on the Seegene blot, to serve as a positive control. A low probe concentration (0.1 ng/ ml) was used in the first hybridization. Another hybridization at a high probe concentration (1.0 ng/ ml) was performed when results were not obtained at the first concentration. In between hybridizations, the blot was stored and cleaned for re-use according to Seegene's protocol.

Due to the limited number of hybridizations that can be done on a single membrane a second northern blot was made against a dilution of chick 48-hour total RNA (provided by Xuezhi Li, White's Lab, SJSU, CA), according to lab protocol (White, 2004). A 2-fold dilution of total RNA from 24 to 0.75 micrograms was made, and run out on a denaturing agarose gel. Northern transfer of the RNA was then carried out using a Turboblotter transfer pack (Schleicher & Schuell BioScience/ Whatman) for 2.5 hours. RNA was fixed to the membrane by baking for 10 minutes at 68°C, followed by UV cross-linking. The blot was stored at -20°C prior to use.

Hybridization against the chick 48-hour total RNA blot was carried out using Ambion's Brightstar kit. The β-actin probe was tested at a concentration

of 0.5 ng/ ml, with a 1.5 hour prehybridization, and an overnight hybridization at 42°C. The BMP-6 probe was then tested, using the same conditions. No observable signal was detected after a 75-minute exposure. A second hybridization was conducted with the probe concentration increased to 1.0 ng/ ml, extension of the hybridization time to ~44 hours, and a reduction of the washing temperature from 68°C to 55°C. The membrane was re-probed with β-actin under the same conditions as the first hybridization to determine if RNA is still detectable on the blot after multiple hybridizations.

Northern Blot Hybridization: Isotopic

To increase the sensitivity of the hybridization signal, a radiolabeled system was employed. PCR products, spin purified by Amicon-30 spin filtration, for each probe were end-labeled using Promega's 3'-End Labeling System and [α-S^{35}]-labeled dATP (Amersham). The labeling product was spin-purified with BioRad's Micro Bio-Spin 30 Chromatography Columns. Probe signal strength in counts per minute (cpm) per microliter was counted on a Beckman LS 6500 scintillation counter. A dot blot test of the radiolabeled probes was prepared from 1ng to 100 fg. One microliter of each dilution was spotted onto a positively charged nylon membrane (Schleicher & Schuell

BioScience/ Whatman) with a TE negative spot and fixed by UV cross-linking. Detection was performed according to lab protocol after a 48-hour exposure at -70°C.

A second northern blot of chick 48-hour total RNA was prepared as before, with a five-fold dilution from 25 to 1 μg per lane in duplicate. After fixation, the blot was split in half to perform hybridizations in parallel. A dot blot strip (10-fold dilution from 1 ng to 100 fg) was run simultaneously with each probe, to determine the signal threshold. Hybridization and detection were performed according to lab protocol (White, 2004) with a few modifications. The blot was prehybridized for 2 hours at 68°C. The probes were heat-denatured at 90°C for 10 minutes before being added to the prehybridization solution. Hybridization was carried out overnight at 42°C. Two low stringency washes were performed for 10 minutes at room temperature, followed by two high stringency washes at 50°C for 15 minutes each. The membranes were then blotted and sealed in plastic wrap. Film exposure took place at -70°C.

Construction of a Clone Containing the BMP-6 Probe

Novagen's pSTBlue-1 vector cloning system was used to create a clone containing the BMP-6 probe. The previously prepared spin-purified PCR product of the BMP-6 unique region was used as the insert. Insert concentration was determined by agarose gel electrophoresis against mass standards to obtain an insert to vector molar ratio of 2:1. The end conversion and ligation reactions were performed according to Novagen's protocol. Transformation of NovaBlue Singles competent cells was performed according to the manufacturer's protocol, followed by a 60-minute outgrowth at 37°C with shaking at 250 rpm. Cells were plated onto IPTG (0.5 mM)/ Xgal (80 μg/ ml)/ Amp (100 μg/ ml)/ LA plates and grown overnight at 37°C (Ausubel et al., 1999).

Blue-white screening of a diluted sample of transformed cells was used to pick colonies with inserts. White colonies were picked and used to create grid plates, which were grown overnight at 37°C, and used for further screening. Colony PCR and plasmid isolation with enzyme restriction were carried out to identify colonies with inserts. White colonies from the grid plates were grown up overnight in LB/ Amp (100 μg/ ml) at 37°C with shaking at 250 rpm for plasmid isolation (Ausubel et al., 1999).

The probe was amplified from the selected clone with BMP-6 specific primers. The PCR product of the BMP-6 probe (12 μl at 20ng/μl) was sent out for sequencing, with BMP-6 specific primers, to Gene Gateway (Hayward, CA) and Tocore (Menlo Park, CA). The probe sequence was then compared to the putative partial chick BMP-6 sequence using SDSC Biology Workbench, ClustalW, and Boxshade.

EXPANDED RESULTS

cDNA Library

The 48-hour chick embryo plasmid cDNA library used for screening in this study consists of twenty-eight 96-well plates, numbered 1 through 28. The final library is estimated to represent approximately 68% of the starting mRNA (Shahi, unpublished). Some wells may contain multiple colonies and/ or non-transformed colonies. Preliminary analysis of the library revealed that inserts range from 0.5 to 10 kb (Shahi unpublished). EcoRI restriction resulted in multiple insert bands in some clones, and some clones had an incomplete restriction resulting in linearized plasmid over 3kb in length (Shahi, unpublished).

Colony Screening

Isolated plasmids were cut with EcoRI to determine the presence and size of the insert. Two gels are shown as a representative sample of the results of the restriction digestion (Fig. 10). Some of the plasmids linearized with EcoRI digestion, and insert size could not be determined. A few plasmids showed multiple bands, indicating that the plasmid carried two inserts, or a

very large single insert with an EcoRI restriction site of its own. Insert size ranged from 300 bp to 1100 bp. Most inserts fell within the range of 500 to 900 bp.

Agarose gel electrophoresis shows the results of the tests to determine why EcoRI resulted in linearization of many of the isolated plasmids (Fig. 11). Desalting the plasmid did not change the results of EcoRI digestion. The tested samples were linearized following digestion. Double digestion revealed that one of the restriction sites is not being recognized by EcoRI. The samples were linearized following digestion with EcoRI alone, and with EcoRI/ PstI double digestion. Double digestion with HindIII/ EcoRI resulted in a 3 kb linear plasmid band, and an insert band. Only two samples were tested, but in both it was the first EcoRI site, which was not recognized by the enzyme. It is possible that these EcoRI adapters bound to the vector in a way that damaged this site in a percentage of the reactions.

Gene Selection

To find clones within the cDNA library that have a high sequence identity to previously unidentified chick homologues to developmentally important genes, the sequences were screened by performing homology

searches against the databases at TIGR and NCBI. Clones that contained segments of genes that are ubiquitous or well characterized were omitted from further consideration. Clones that had very low sequence identity to known sequences, or high identity to sequences that were described only as unidentified proteins or clones were also omitted. A sample of search results is shown (Table 3).

The sequencing output for clone 10H12 is shown (Fig. 12). The output was run through VecScreen, and bases matching vector sequence were removed prior to further sequence identity searches (Fig. 13). Clone 10H12, showed significant sequence identity to a putative partial chick BMP-6 sequence (TC122562, June 2004) in the TIGR database (Fig. 3). Links between sequences in the TIGR database allow a quick search to determine homology between BMP-6 protein sequences from human, mouse, rat and chick (Fig. 14). An 80% sequence identity is seen between chick and the other three species.

The partial BMP-6 mRNA sequence found in the TIGR database was conceptually reverse transcribed to obtain the DNA sequence, and translated using the Sixframe program (SDSC). The longest open reading frame (ORF) was identified as nucleotides 115 to 669 in the +1 frame. The translated ORF was used for alignments against BMP-6 from other species (Fig. 4) and against

other BMPs in the chick (Fig. 5) using ClustalW (SDSC) (Thompson et al., 1994) and Boxshade (SDSC).

Probe Design

Little information is available in the literature about the primers and probes used in BMP-6 studies. Some studies use antibodies or protein probes. A handful of articles listed the primers used to amplify the BMP-6 probe used in their study. Most of these studies used either the human or mouse sequence to design their primers. Known primer pairs for BMP-6 were compiled (Table 4) with known information on literature citation, species, amplified region, and product length.

Information from the alignment of BMP-6 across species, and alignment of chick BMPs, was used to form a composite sequence (Fig. 6). The composite was designed to show the region unique to BMP-6 that was previously described (Schluesener and Meyermann, 1994), as well as the residues conserved across species for BMP-6, and the residues that are conserved amongst BMP family members. This information was used to designate a region that could be used as a probe specific to BMP-6.

The Sixframe output showing the longest ORF in frame +1 was used to identify the nucleic acid sequence corresponding to the probe region selected from the protein sequence. Primers were designed to the selected region using Primer 3 (SDSC). The primer pairs were compared back to the annotated Sixframe output to ensure that they amplified the proper region, with minimal sequence identity to regions that are conserved across BMP family members (Fig. 15). As an extra precaution, the primer pairs were added to an alignment of chick BMP family members, to ensure that the primers would not bind to and amplify other BMP family members (Fig. 16). Some identity between BMP family members could not be avoided, but all precautions were taken to minimize the potential for cross-hybridization. The probe region amplified by the selected primers was used in a BLAST search of the NCBI databases to ensure that the probe did select BMP-6, and did not select other BMP family members.

Three primer pairs were designed in Primer 3 (SDSC) and synthesized by Operon (Table 5). One primer pair for BMP-6 was chosen to amplify a segment within the coding region that is unique to BMP-6. A second primer pair was designed against the putative chick BMP-6 in the non-coding region where the 10H12 clone aligned. A pair of primers to chick β-actin was also

created to amplify a positive control probe for use in future hybridization studies.

Northern Blot Hybridization: Non-Isotopic

The amplified probes were labeled with Ambion's BrightStar Psoralen-Biotin Nonisotopic Labeling Kit. A 1 in 10 dilution series of each labeled probe was created, spotted onto a positively charged nylon membrane (Schleicher & Schuell BioScience/ Whatman), and fixed by UV cross-linking. The spot test was visualized with Ambion's BrightStar Biodetect protocol (Fig. 19). The spot test confirms that the probe was labeled efficiently and can be observed clearly down to a concentration of 1 pg. Application of TE as a negative control did not create any appreciable signal above background staining.

To determine expression patterns of BMP-6 in adult chicken, a pre-made tissue blot (Seegene) was used for hybridization. This blot was constructed with RNA isolated from the following organs: brain, heart, lung, liver, spleen, kidney, small intestine, testes, uterus, ovary, and gizzard (Fig. 20). Probe hybridization and detection followed the lab protocol (White, 2004) using the Ambion Brightstar detection kit.

The positive control β-actin probe was tested first. In the first hybridization of β-actin (0.1 ng/ ml), the probe was, accidentally, not heat denatured prior to addition to the hybridization chamber. The hybridization was repeated. There was no observable signal, and very little background hybridization was seen (Fig. 21,A). Due to the limited number of times the blot could be used, the β-actin probe concentration was increased to 1 ng/ ml for the next hybridization. The blot showed no observable signal, and a significant amount of background (Fig. 21,B). In between hybridizations, the blot was stored and cleaned for re-use according to Seegene's protocol. Hybridization was allowed to proceed overnight at 68°C, this temperature may have been too high to allow for adequate binding of the probe.

Due to the limited number of hybridizations that can be performed on a single membrane, a second northern blot was made using 48-hour total chick RNA. A 2-fold dilution of total RNA from 24 to 0.75 micrograms was made, and run out on a denaturing agarose gel (Fig. 22). Hybridization against the chick 48-hour total RNA blot was carried out using Ambion's Brightstar kit.

The β-actin probe was tested at a concentration of 0.5 ng/ ml, with a 1.5-hour prehybridization, and an overnight hybridization at 42°C. The positive control probe bound to the membrane as expected, with very little background

staining (Fig. 23). The BMP-6 probe was then tested, using the same conditions. No observable signal was detected after a 75-minute exposure (Fig. 24, A). A second hybridization was conducted with the BMP-6 probe concentration increased to 1.0 ng/ ml, extension of the hybridization time to 44 hours, and a reduction of the high stringency washing temperature from 68°C to 55°C. There was no observable signal after an exposure of 90 minutes (Fig. 24, B). The membrane was re-probed with β-actin under the same conditions as the first hybridization to determine if RNA is still detectable on the blot after multiple hybridizations. The signal was weaker than on the first hybridization, but it was detectable (Fig. 25).

Northern Blot Hybridization: Isotopic

Spin-purified PCR products for each probe were end-labeled using Promega's 3'-End Labeling System and [α-S^{35}]-labeled dATP (Amersham). The labeling product was spin-purified with BioRad's Micro Bio-Spin 30 chromatography columns. Probe signal strength in counts per minute (cpm) per microliter was counted on a Beckman LS 6500 scintillation counter (Fig. 26).

A significant drop in the cpms per microliter between the labeling reaction and the spin-purified product indicates that unincorporated labeled

nucleotides were removed from the probe solution efficiently. The β-actin probe was labeled with a total of $1.7x10^6$ cpm, and the probe for BMP-6 was labeled with a total of $8.0x10^6$ cpm. For radiolabeled probes, a cpm of 10^6/ ml final concentration is optimal. Due to time constraints, the total probe concentration used in the hybridizations was 10-fold lower than optimal. Although the manufacturer's protocol states that double-stranded DNA may be labeled in the presence of cobalt ion (provided in the labeling buffer), blunt-ended PCR products may be labeled at a lower efficiency. Heat-denaturation of the PCR product prior to labeling (which was not done) might have increased the labeling efficiency. A dot blot test of the radiolabeled probes reveals that the signal strength of the BMP-6 probe is half that of the β-actin probe, which is consistent with the cpm counts (Fig. 27). The signal was relatively weak after a 48-hour exposure at -70°C, indicating that longer exposure times were necessary.

A second chick 48-hour total RNA blot was prepared as before, with a five-fold dilution from 25 to 1 μg per lane in duplicate (Fig. 28). After fixation, the blot was split in half to perform parallel hybridizations. A dot blot strip of the unlabeled probe in a ten-fold dilution from 1 ng to 100 fg with a TE negative spot was run simultaneously with each northern blot membrane.

The 48-hour chick total RNA northern blot and dot blot were exposed on a single film for both probes for 2 weeks at -70°C (Figs. 29, 30). The dot blot strips show that both the β-actin and BMP-6 probes can detect concentrations as low as 100 fg under the conditions used. The 48-hour chick total RNA northern blot had a weak positive signal at the highest concentration (25 μg per lane) with the radiolabeled β-actin probe. The signal strength could be estimated from the simultaneously run dot blot strip to fall between 10 and 1 pg. No positive signal was seen with the BMP-6 probe. No signal was seen on Seegene's tissue blot with the radiolabeled β-actin probe (results not shown).

Construction of a Clone Containing the BMP-6 Probe

Novagen's pSTBlue-1 vector cloning system was used to create a clone containing the BMP-6 probe sequence. The previously prepared Amicon-30 spin-purified PCR product of the BMP-6 unique region was used as the insert. Transformation of NovaBlue Singles competent cells was performed according to the manufacturer's protocol. Cells were plated onto IPTG (0.5 mM)/ Xgal (80 μg/ ml)/ Amp (100 μg/ ml)/ LA plates and grown overnight at 37°C (Ausubel et al., 1999).

All controls were run during the cell transformation (results not shown). The negative insert control showed limited growth. Approximately 70% of colonies that did grow were blue. This indicates that there is a relatively high potential for false positive (white) colonies. The positive insert control had a large number of colonies. Over 90% of the colonies were white. There is a small potential for false negatives. The vector control had a large number of colonies, all of which were blue. Colonies containing no plasmid could easily be avoided, as no false positives were found with plasmids carrying no insert. Some clones containing an insert may have been missed in the screening process because they showed as a false negative. A relatively large percentage of false positive colonies that appeared to have the plasmid with the insert complicated the screening process. The occurrence of a significant percentage of both false positive and false negative colonies has been regularly observed in our lab. The Novagen protocol notes that some insert-carrying vectors will turn blue, possibly due to the presence of a small amount of functional *lacZ* α-peptide expressed due to use of a secondary translation initiation site or generation of a fusion peptide. False positive white colonies may arise due to self-ligation of vectors with damaged ends or vector-vector ligation.

Screening of the clones for the insert was done by several methods. Colony PCR using the boil-prep method had limited success. In most instances, no amplified product was obtained. A representative gel, where a PCR product is obtained in only one of ten colonies screened, is shown (Fig. 7). Plasmid isolation with Qiagen's QIAprep Miniprep had several limitations. The colony had to be regrown overnight in culture to perform the plasmid isolation. Robust growth was not seen with overnight incubation under the conditions used. An initial screening of isolated plasmids with EcoRI digestion shows the failure of the restriction enzyme to cleave the insert from the plasmid (Fig. 31).

Due to the extremely low yield obtained using the Qiagen plasmid prep kit, the positive clone obtained by colony PCR (Fig. 7) was grown-up again for isolation by the alkaline lysis method. Alkaline lysis produced a higher yield than the Qiagen plasmid prep kit, however the yield was still low in comparison to a pGEM control vector (results not shown). The clone was grown up overnight in 8 culture tubes containing 5.0 mls each. Plasmid was isolated by alkaline lysis, and pooled together. The resulting sample was concentrated using an Amicon-100 spin filter down to 200 µl, and stored at -20°C. The sample was further concentrated with an Amicon-30 spin filter and resuspended in sterile millipore water.

The sample and filtrates were run out on a 1% TAE agarose gel (Fig. 8). The linearized plasmid ran out at approximately 4 kb. The expected vector (3851 bp) plus insert (232 bp) size is 4083 bp which indicates that the insert is present.

The isolated plasmid was also linearized by enzymatic digestion with HindIII and with BamHI, and run out on 1% TAE agarose gel (results not shown). The uncut plasmid ran at 6 kb, which indicates that the plasmid may be damaged or forming multimers. Similar results were obtained by others in the lab using the same cloning vector system. Contamination with genomic DNA seems unlikely, because a smear of bands is not seen on the gel.

PCR amplification was done to increase the yield of product for sequencing (Fig. 32). No amplification was obtained with the SP6 and T7 primers. The insert was amplified with BMP-6 specific primers. The PCR product was spin purified and sent out for sequencing to two companies (Fig. 33). The sequences returned from GeneGateway were longer than the insert size of 232 bp. This indicates that there may be contaminating sequences in the sequencing machine that created a background signal, which was erroneously reported as part of the sequence. The sequences returned from Tocore (Menlo Park, CA) were shorter than the expected insert size, which may be due to dye-

blob contamination obscuring part of the sequence or early truncation during the sequencing reaction.

The insert sequences were aligned with the putative partial chick BMP-6 sequence confirming that the insert is the amplified BMP-6 probe region (Figs. 9, 33). The insert aligns to the gene sequence with high sequence identity. Some mismatching occurs in the sequence returned from both companies. The additional residues on the sequence returned from GeneGateway (Hayward, CA) do not align with the gene sequence in an appreciable way, indicating that the extra signal is background noise. The sequencing was performed on a PCR product, therefore the extra signal is unlikely to be vector sequence. However, VecScreen (NCBI) analysis reveals that bases 303-363 of the insert sequence returned from GeneGateway have a moderate sequence identity with the E. coli lactose operon (gnl|uv|J01636.1:1-7477). No identity to a vector sequence was found for the bases between the expected end of the insert at base 232 and the region identified as sharing identity with the *lacZ* operon starting at base 303.

EXPANDED DISCUSSION

Despite the large number of articles that are available regarding the expression patterns of particular genes, it can be difficult to compile information on any one product in a given experimental system, due to a wide range of experimental procedures in various experimental models. Few direct comparisons of materials and protocols exist in the literature. Therefore the researcher must be careful in drawing conclusions about gene expression taken from an array of sources.

For a given gene product, experiments may be conducted *in vivo* in several different experimental models at various developmental stages. Different animal models often do not express the same genes at the same developmental stage. Experiments may also be carried out *in vitro*, with cell or tissue cultures. The results may or may not reflect the mechanisms that actually occur within the body. Interpreting gene knockout studies, for example, may be hampered by early lethality or by compensation by another gene product.

Probes used in one species may have been designed against the gene or protein sequence from a different species. Variation in the sequence between species may be significant, and could skew the results of the experiment.

Researchers may also use antibodies, protein probes, or nucleic acid or oligo probes. Purified proteins used in antibody production may contain protein mixtures, especially when the protein of interest belongs to a family of proteins with similar biochemical properties, which may interfere with isolation of a single component. Distribution of mRNA may not accurately reflect protein distribution due to factors such as targeting, translational control, and regulated mRNA degradation.

The complexity of the TGF-β signaling pathway increases the difficulty of deciphering the mechanisms involved in regulation and expression of BMP family members. Signal regulation occurs at all stages in the pathway. The presence and concentration of various receptors, signaling molecules, enhancers, and inhibitors can all vary with developmental stage, and by tissue and cell type to fine tune gene expression. In addition, the BMP signaling pathway interacts with several other complex signaling pathways.

In the BMP signaling pathway, the BMP molecule, which may exist as a hetero- or homo-dimer, binds to a Type II BMP receptor (Zhao, 2003). Type II receptors may also exist as hetero- or homo-dimers. Three Type II receptors for BMP are known: BmpRII, ActRIIa, and ActRIIb. The BmpRII receptor is known to form alternate splicing variants, which increases the receptor complex

variability (Nohe et al., 2004). The Type II receptor is a constitutively active serine/ threonine protein kinase. The resulting Type II receptor-BMP complex then binds to a Type I receptor dimer.

The Type I receptor may also exist as a hetero- or homo-dimer. There are three known Type IA receptors: Alk1, ActRI (Alk2), and BmpRIa (Alk 3). Only one TypeIB receptor, BmpRIb (Alk 6), is known. The Type I receptor requires binding of the Type II receptor-BMP complex to be activated. The activated Type I receptor then activates a Smad signaling molecule by phosphorylation.

Different pairings of molecules in the BMP and receptor dimers will bind with varying affinity and modulate specificity of binding. Type II receptors have been shown to bind strongly with high affinity (Herpin et al., 2004). Type I receptors bind weakly with low specificity (Herpin et al., 2004). The binding strength of the Type I receptors is enhanced when binding a Type II receptor-BMP complex.

There are three classes of smad molecules: R-smads, Co-smad, and I-smads (Goumans, 2000 and Zhao, 2003). R-smads bind to the Co-smad, translocate into the nucleus of the cell and interact with DNA binding proteins to activate or repress transcription. Five vertebrate R-smads are known. BMPs

signal though smad 1, smad 5, and smad 8. TGF-βs and activins signal through smad 2 and smad 3.

I-smads inhibit gene transcription. The inhibitory smads are smad 6 and smad 7. Smad 6 functions by competitive inhibition at the Type I receptor binding site and by competitive binding of the Co-smad, while smad 7 interacts with smurf 1 and smurf 2 to modify R-smads for degradation. (Herpin et al., 2004 and Nohe et al., 2004).

Cellular regulation of the BMP pathway occurs at all levels (Nohe et al., 2004). Extracellular factors such as cerberus, dan, gremlin, and noggin can act as BMP antagonists. On the intracellular level, I-smads can antagonize BMP signaling. Within the nucleus various cofactors and transcription factors can enhance or antagonize the BMP signal. The BMP signaling pathway has also been found to interact with the following signaling pathways: TGF-β/ activin, MAPK, Ras, Erk, p38, Tab1/ Tak1, Jak/ Stat, and calcium/ calmodulin.

BMP-6 has been found to bind to all three Type II receptors, and to the ActRI, BmpRIa, and BmpRIb receptors with varying affinity (Ebisawa et al., 1999). BMP-6 signaling appears to occur primarily through smad 1 and smad 5, with a preference for smad 5. Smad 6 acts as an inhibitory smad in the BMP-6 signaling pathway. The presence and concentration of the various receptors in

each cell or tissue type will modify the BMP-6 signal. Another study indicates that BMP-6 uses only the ActRI Type I receptor to signal through smad 1 and smad 5 (Zwijsen et al., 2003). It is reported elsewhere that BMP-6 may bind ActRI, BmpRIa, or BmpRIb, however it preferentially binds the ActRI receptor (Kim et al., 2001).

BMPs are one subgroup of a large class of transcription factors known as the TGF-β superfamily. The size of the TGF-β superfamily and the highly conserved nature of its members, create a problem for researchers trying to create a probe specific to only one molecule. Homology between species is a useful guide for creating primer pairs and probes to a specific gene region, when the gene sequence for the animal model under study is unknown or in question. However, sequence variations between species, and the possibility of alternate splicing variants can present a challenge in creating a species-specific probe based on homology to other species.

A greater challenge in creating a probe specific to a single member of the BMP family is the homology within species between members of the BMP family. BMP-2 and BMP-4 comprise one subset of the BMP family, known as the Dpp subgroup. BMP-5, BMP-6, and BMP-7 belong to a different subset of the BMP family, known as the 60A subgroup. Chromosomal location in the

human indicates that the divergence between BMP-5 and BMP-6 is more recent than that between BMP-6 and BMP-7 (Hahn et al, 1992). Finding a sequence within the coding region of the gene that is specific to BMP-6, and that has low homology to the other BMP family members, especially BMP-5 and BMP-7, is essential to avoid non-specific binding of the probe. Primer sequences must be carefully chosen to select a probe region with minimal sequence identity to other BMP family members if a unique and specific probe is to be produced.

There are many methods available to determine gene expression. Reverse transcriptase PCR can be used on a sample of isolated RNA to detect the presence of an mRNA in the tissue or organism under study. However, when total RNA is used, this data does not provide any information on the concentration at which it is expressed, or the tissue location where it is expressed. RNA from isolated tissue samples can be used to determine tissue expression patterns. However, harvesting sufficient quantities of tissue to determine tissue expression patterns during early developmental stages is both time-consuming and laborious.

To get a quantitative measure of gene transcription, northern blot hybridization can be a useful tool. However, northern blot analysis has several limitations. Careful attention must be paid through all stages of blot

preparation to avoid RNase contamination, which could degrade the sample RNA. Hybridization conditions such as temperature and length of prehybridization and hybridization may need to be optimized for each probe being used. Detection of a gene product by northern blot hybridization is highly dependent on the probe. Mismatched bases or self-annealing of the probe may inhibit binding to the target sequence. Labeling efficiency of the probe can also greatly affect the sensitivity of the system.

In addition the expression of the gene in the organism and/ or tissue at the developmental stage under question may affect results. UniGene at NCBI has expression profiles for some genes. There is no entry for BMP-6 in the chick at this time, but the expression profile for BMP-6 in the mouse, rat, and human indicate that the expression level is very low compared to a ubiquitously expressed gene such as β-actin (Tables 7 and 8). Due to its use as an experimental model in many gene expression studies, the mouse BMP-6 UniGene entry is more thorough in regards to the tissue types and developmental stages that were tested. Comparisons of the three profiles for BMP-6 expression indicate the degree to which expression may vary between organisms, and within a single organism by tissue and developmental stage.

An expression profile for chick β-actin is also available through UniGene (Table 8). Assuming expression levels of BMP-6 in the chick parallel those found in the mouse, rat, or human, there is likely to be up to a hundred-fold more β-actin expressed than BMP-6. The very low expression of BMP-6 may have been responsible for the inability to find BMP-6 expression in the northern blot hybridizations performed in this study. It is unlikely that enough RNA could be loaded onto a gel to provide sufficient target to produce a detectable signal.

In situ hybridization offers another alternative to examine gene expression. In addition to increased sensitivity, *in situ* hybridization allows a visualization of exactly where the gene of interest is being expressed in an organ or tissue. *In situ* hybridization has its disadvantages as well, in terms of reagent trapping, low signal to noise ratios, and the need to optimize all steps of the procedure for each specific probe.

The BMP-6 probe that was created in this study can be used for future *in situ* hybridization studies. The presence of SP6 and T7 binding sites allow for the synthesis of sense and antisense probes. Once amplified the probe may be labeled isotopically or non-isotopically for signal detection. The length of the

probe is sufficient to provide unique binding to BMP-6, yet small enough to get through to bind to its target.

Understanding the role that BMP-6 plays in heart formation may aid researchers in developing preventive and therapeutic procedures to treat some common forms of developmental heart defects. The most common congenital heart defects are those that involve improper septation and valvulogenesis, processes in which BMP-6 has been shown to play a role. Further studies into the expression and regulation of BMP-6 are necessary to advance our understanding of the complex signaling pathways and their interactions, which are involved in specific aspects of heart formation.

Lessons learned from the obstacles that arose during the course of this project may be of benefit to future students following a similar line of research. Some of the difficulties encountered involve the use of online resources, and bioinformatics tools. Other problems arose in the lab during the course of the experimental procedures.

Online databases can be searched to identify clones of interest. However, many database entries consist of clones that have no attributed gene product at the time the BLAST search is performed. While a query sequence may align with a high degree of sequence identity, little information can be

obtained about the nature of the query sequence when it aligns to an unidentified clone in the databases.

A few organism specific databases are now available. However, these databases may not exist for the organism under study, or may not recognize a gene that is homologous to a gene from another organism. It is therefore useful to perform BLAST searches against multiple databases.

Variations in naming conventions can also complicate online searches. For some genes, there is no clear naming convention. For example bone morphogenetic protein-6 may be found written in long form or as an abbreviation, the abbreviation may be written in upper or lower case letters, and the family member number may be separated by a space, a dash, or it may not be separated. Some reference materials may only be found under the older name vegetal related-1 (vgr-1). It is therefore necessary to use all possible naming conventions in both literature and database searches, to ensure the retrieval of as much information as possible.

Bioinformatics is an essential tool for many areas of biological research. To study the expression of a gene during development, a specific probe needs to be created. Designing a hybridization probe against one member of a large superfamily creates additional challenges. Care needs to be taken to find a

region that will bind with a high specificity to the gene of interest, but that will have a low identity to other members of the gene family. Many of the bioinformatics tools necessary to perform the database searches, sequence translations, alignments, probe design, and other analyses can be found at the SDSC Biology Workbench site.

In expression studies, the number of target molecules is an important limiting factor in the sensitivity of the system being used. The EST profiles for BMP-6 in mouse, rat, and human were only discovered at the end of the experimental procedures in this study. An expression profile for chick BMP-6 could not be found. However, using the other profiles as a general guideline for vertebrate expression, it is reasonable to assume that BMP-6 expression in the chick is very low. While RT-PCR is sensitive enough to amplify chick BMP-6 from a sample of total RNA, a sufficient number of target molecules may not exist for detection by northern blot hybridization under the conditions used in this study. Prior knowledge of the EST expression profile for the gene of interest in the organism under study, or a related species could provide helpful guidelines for the detection methods to be pursued.

Cloning of the probe sequence into an expression vector revealed additional problems. The pSTBlue-1 vector exhibited poor growth during

overnight incubations. Plasmid isolations of overnight cultures revealed a very low yield, and isolated plasmids did not cut properly during enzymatic digestion. Several individuals in the lab obtained these results. Modification of the growth media, temperature, antibiotics used, and length of incubation may need to be performed to find optimal growth conditions.

Blue-white screening of the transformed NovaBlue cells with the pSTBlue-1 vector was hindered by the presence of false positive and false negative colonies. A significant number of false positive (white) colonies were obtained. As a result, many "positive" colonies had to be screened by colony PCR before an insert-carrying clone was identified, while insert-carrying clones that show as a false negative may be missed. Similar results were obtained by others in the lab using the same vector system. Therefore, colonies were screened by both colony PCR, and plasmid isolation followed by enzymatic digestion, in an attempt to identify insert-carrying colonies. Plasmid or PCR products from the identified positive colonies were then sent for sequencing to confirm the presence and identity of the insert.

Sequencing of the insert by more than one company revealed another potential source of error. One sequence (Tocore) was slightly truncated, but showed a high sequence identity for the insert to the region of the gene that was

specified as the probe region. The other sequence (GeneGateway) provided the full insert sequence, but had a long string of additional residues. The additional sequence did not align to the gene sequence in an appreciable way. After a gap of approximately 70 bases the additional residues show homology to the *lacZ* operon. While an increase in sensitivity may provide the full insert sequence, the additional bases could potentially be erroneously identified as insert sequence. The additional sequence may have resulted from running into the vector sequence during the initial PCR amplification, or it may represent contamination during the sequencing reaction.

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APPENDIX A: FIGURES AND TABLES IN EXTENDED DOCUMENT

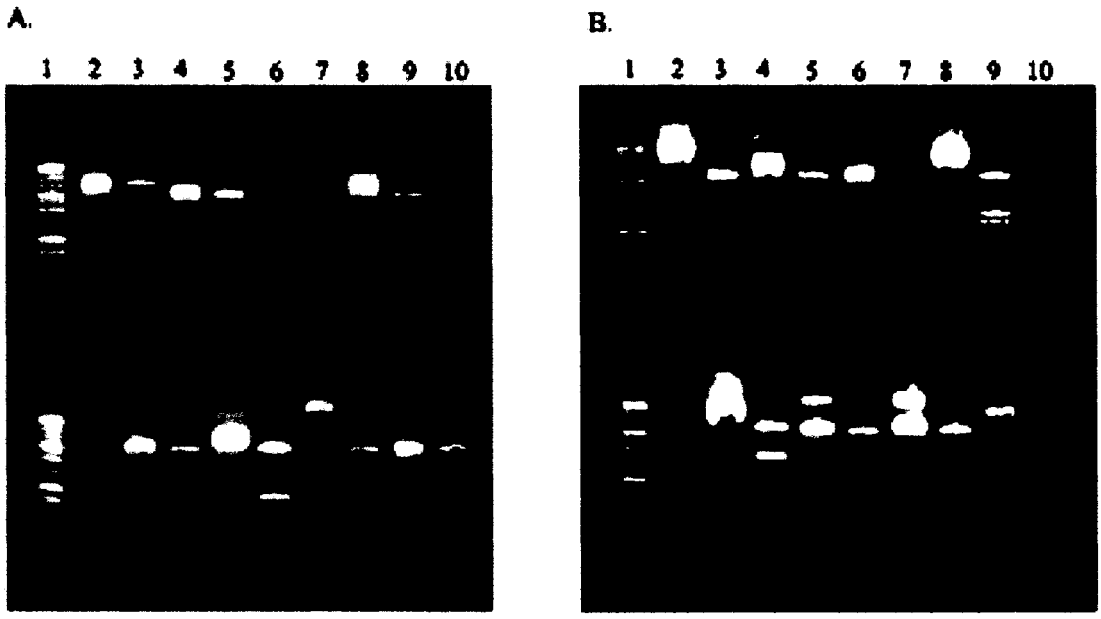


Fig. 10. Restriction digest of isolated plasmids from the 48-hour chick cDNA library.

Plasmids were isolated by Qiagen's Qiaprep Spin Miniprep kit. EcoRI was used for the restriction digestion. Promega's 1 kb DNA Ladder was used to estimate size. A) samples 10A9 through 10H9 were loaded in pairs of uncut and cut plasmid. B) samples 10A12 through 10H12 were loaded in pairs of uncut and cut plasmid. In both gels the top lanes are as follows: lane 1 = ladder, lane 2 = uncut A, lane 3 = cut A, lane 4 = uncut B, lane 5 = cut B, lane 6 = uncut C, lane 7 = cut C, lane 8 = uncut D, lane 9 = cut D, lane 10 is empty. In both gels the bottom lanes are as follows: lane 1 = ladder, lane 2 is empty, lane 3 = uncut E, lane 4 = cut E, lane 5 = uncut F, lane 6 = cut F, lane 7 = uncut G, lane 8 = cut G, lane 9 = uncut H, lane 10 = cut H.

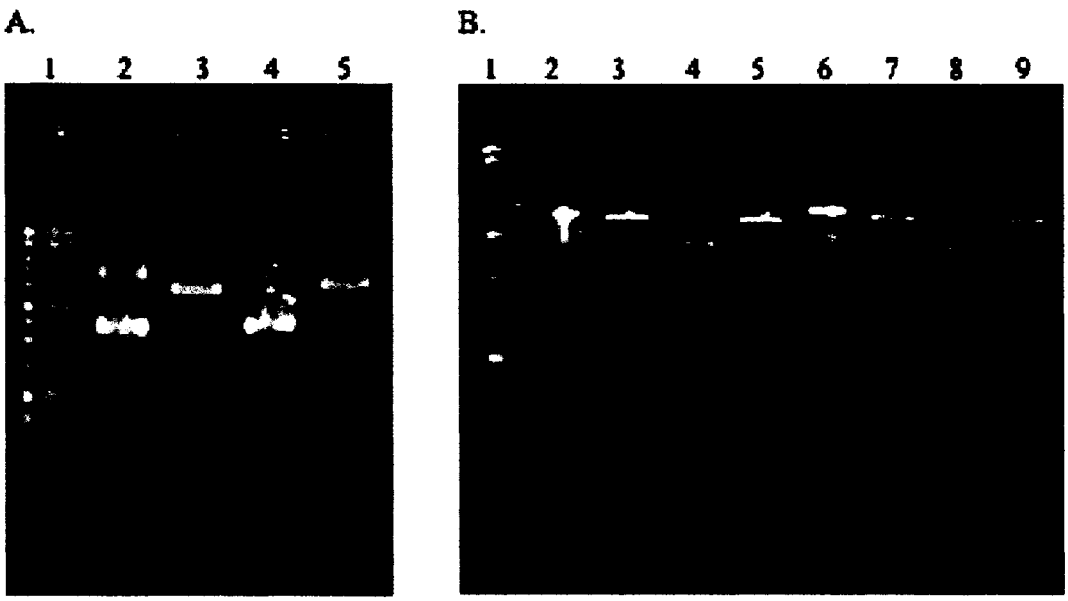


Fig. 11. Desalting and double digestion of plasmids that linearized upon EcoRI digestion.

Two samples that linearized on EcoRI digestion were chosen for further testing. The samples were purified to remove any possible salt contamination and the EcoRI digestion was repeated. A) Secondary EcoRI digestion of desalted plasmids. Lane 1 = Promega 1 kb ladder, lane 2 = uncut 2A1, lane 3 = cut 2A1, lane 4 = uncut 2F1, lane 5 = cut 2F1. B) Double digestion of plasmids using HindIII and PstI. Lane 1 = Promega 1 kb ladder, lane 2 = uncut 2A1, lane 3 = EcoRI cut 2A1, lane 4 = HindIII/ EcoRI cut 2A1, lane 5 = EcoRI/ PstI cut 2A1, lane 6 = uncut 2F1, lane 7 = EcoRI cut 2F1, lane 8 = HindIII/ EcoRI cut 2F1, lane 9 = EcoRI/ PstI cut 2F1.

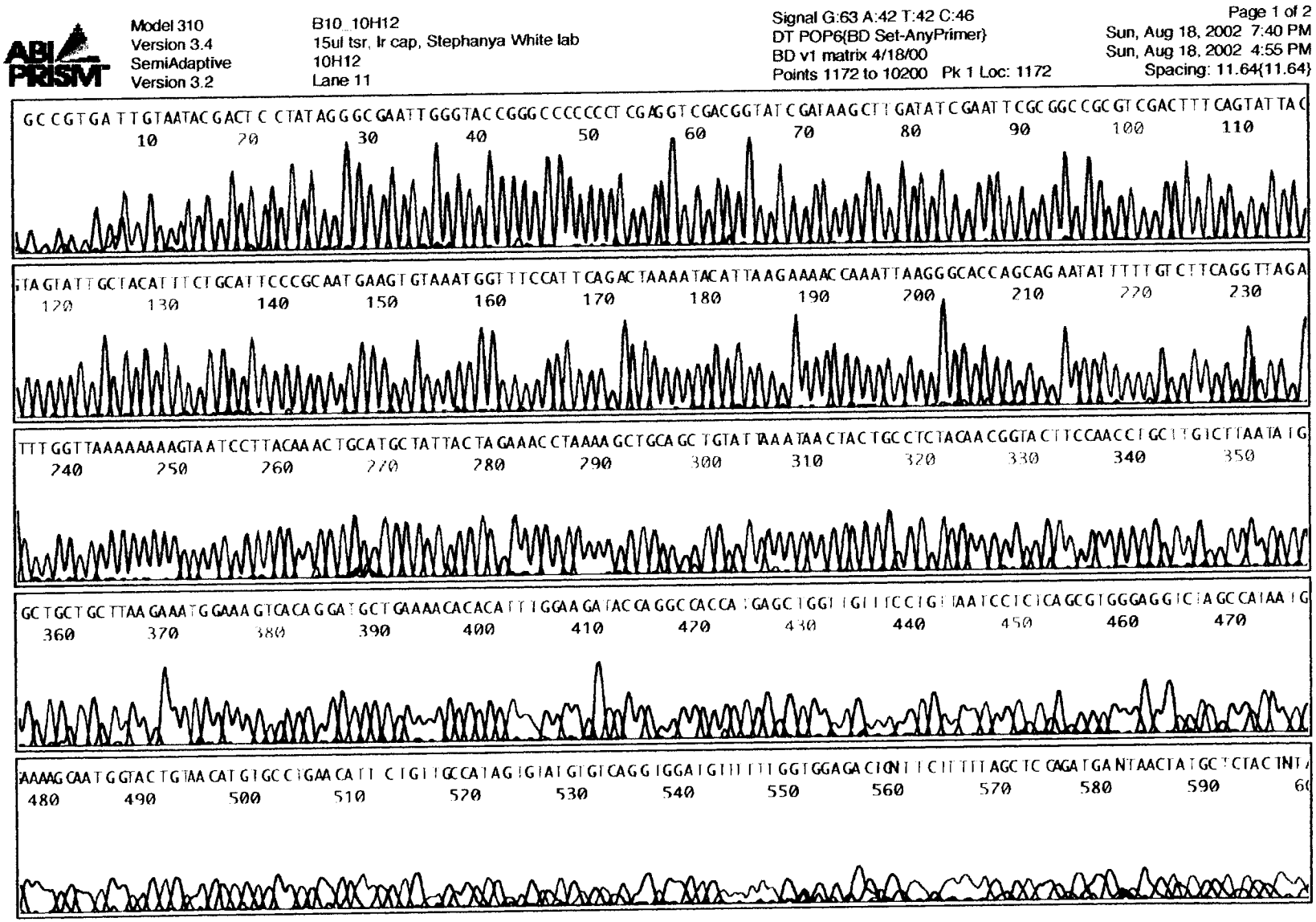
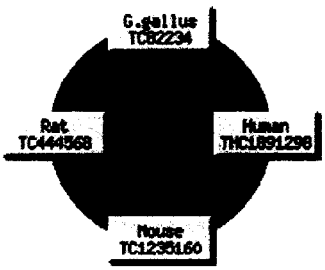

Model 310
Version 3.4
SemiAdaptive
Version 3.2
B10_10H12
15ul tsr, Ir cap, Stephanya White lab
10H12
Lane 11
Signal G:63 A:42 T:42 C:46
DT POP6{BD Set-AnyPrimer}
BD v1 matrix 4/18/00
Points 1172 to 10200 Pk 1 Loc: 1172
Page 1 of 2
Sun, Aug 18, 2002 7:40 PM
Sun, Aug 18, 2002 4:55 PM
Spacing: 11.64{11.64}

Fig. 12. ABI sequencing output for clone 10H12.

>10H12
TTTCAGTATTAGTAGTATTGCTACATTTCTGCATTCCCGCAATGAAGTGTAAATGGTTTCCATTCAGACT
AAAATACATTAAGAAAACCAAATTAAGGGCACCAGCAGAATATTTTTGTCTTCAGGTTAGATTTGGTTAA
AAAAAAGTAATCCTTACAAACTGCATGCTATTACTAGAAACCTAAAAGCTGCAGCTGTATTAAATAACTA
CTGCCTCTACAACGGTACTTCCAACCTGCTTGTCTTAATATGGCTGCTGCTTAAGAAATGGAAAGTCACA
GGATGCTGAAAACACACATTTGGAAGATACCAGGCCACCATGAGCTGGTTGTTTCCTGTTAATCCTCTCA
GCGTGGGAGGTCTAGCCATAATGAAAAGCAATGGTACTGTAACATGTGCCTGAACATTCTGTTGCCATAG
TGTATGTGTCAGGTGGATGTTTTTTGGTGGAGACTGNTTCTTTTTAGCTCCAGATGANTAACTATGCTCT
ACTNTANATNGANGTCAAAGTGAACTGGCTCTNTTATANCTACCTTCGTTANTATTAAACCGATCCAGTT
AAGACCTCAGAANCTGATTANNTGCCTTTCCTACCTTGAACNGGAATTCANAATTTGTNTNTACCTTGTT
CCTTAATCA

Fig. 13. Clone 10H12 sequence.
The sequence for the clone 10H12 was run through VecScreen (NCBI) and the first twenty-two residues were removed due to identity to vector sequence prior to further analysis.



#	TC	Putative function
1		bone morphogenetic protein 6
2		transforming growth factor-beta bone morphogenetic protein 6 precursor
3		Vgr-1 protein bone morphogenetic protein-6 bone morphogenetic protein 6 [Mus musculus]
4		bone morphogenetic protein-6 bone morphogenetic protein 6 [Rattus norvegicus]

Sequence1	Sequence2	% Identity	Match length	p-value	Recip. best hits
Human|THC1891298	G.gallus|TC82234	80.00	763	1.1e-104	*
Human|THC1891298	Rat|TC444568	75.00	1903	9.6e-219	*
Mouse|TC1235160	G.gallus|TC82234	80.00	762	1.7e-104	*
Mouse|TC1235160	Human|THC1891298	80.00	1916	1.9e-260	*
Mouse|TC1235160	Rat|TC444568	80.00	1857	5.8e-252	*
Rat|TC444568	G.gallus|TC82234	80.00	748	1.2e-103	*

Fig. 14. Sequence identity of BMP-6 protein sequences of chick, human, mouse, and rat in the TIGR database.

An 80% sequence identity is seen between the chick and human, mouse, and rat. The lowest level of identity is between rat and human, at 75%.

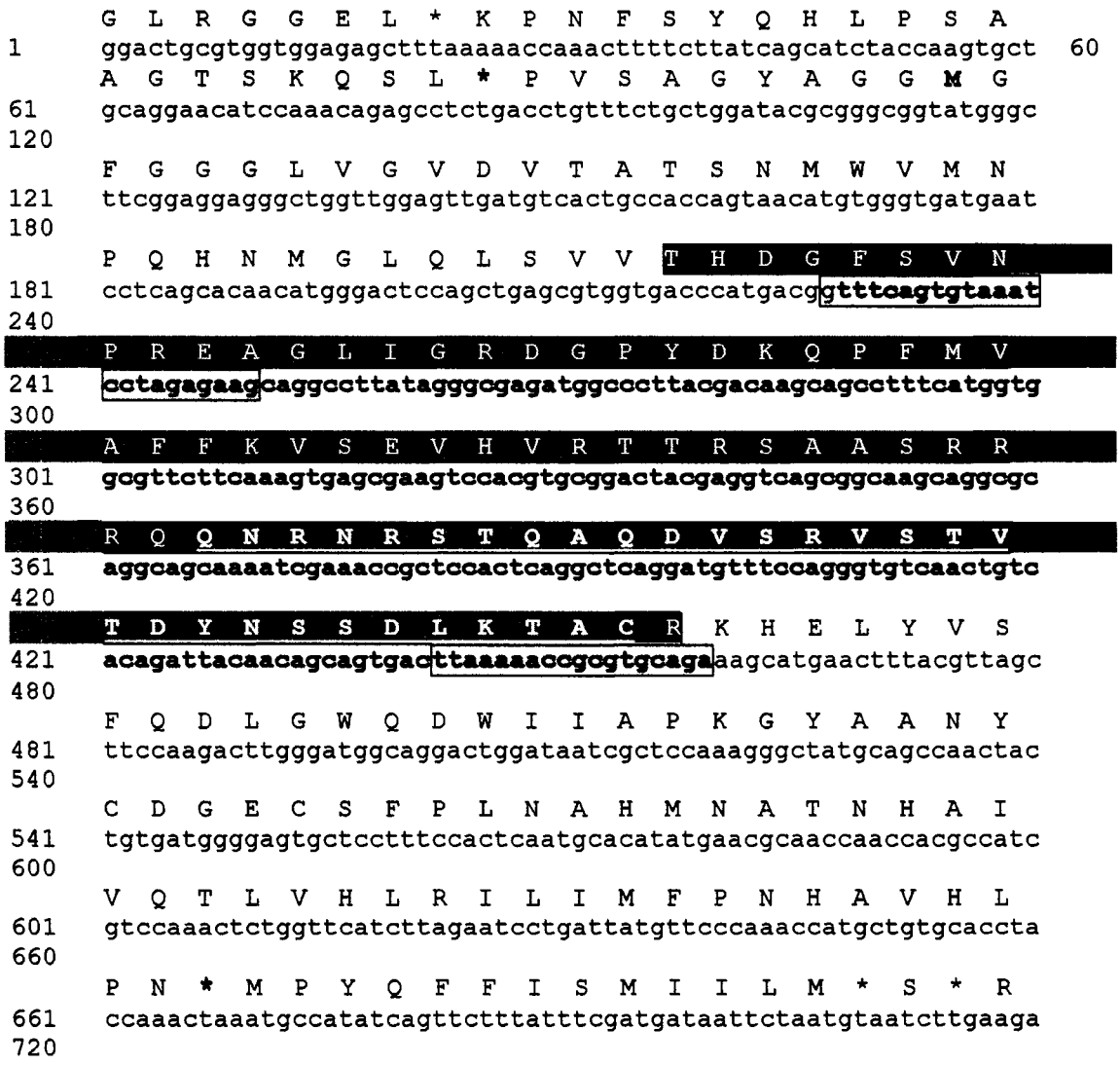
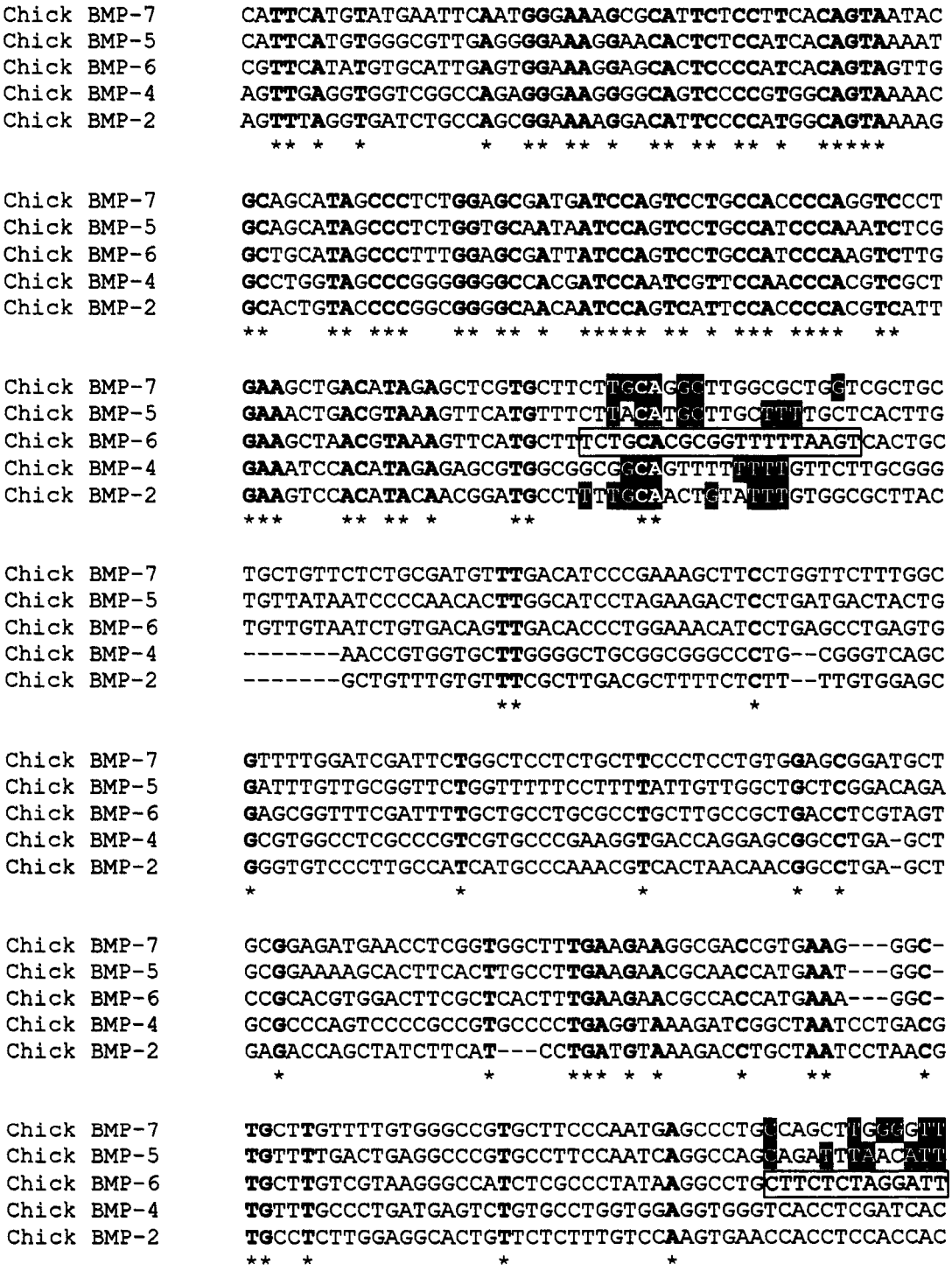


Fig. 15. Selection of a unique chick BMP-6 probe region and primer sequences using the translated sequence of the putative chick BMP-6.

A Sixframe translation performed in the SDSC Biology Workbench of the putative partial BMP-6 sequence provided the longest ORF (frame +1), which was used for probe selection and primer design. The protein sequence region unique to BMP-6 is shown boxed in dark grey with white lettering. The corresponding nucleic acid region selected for probe design is shown in bold. Selected primer sequences are shown in the nucleic acid sequence, boxed in light grey with black lettering. The protein region previously identified as unique to human BMP-6 is underlined and shown in bold (Schluesener and Meyermann, 1994).


Chick BMP-7
Chick BMP-5
Chick BMP-6
Chick BMP-4
Chick BMP-2

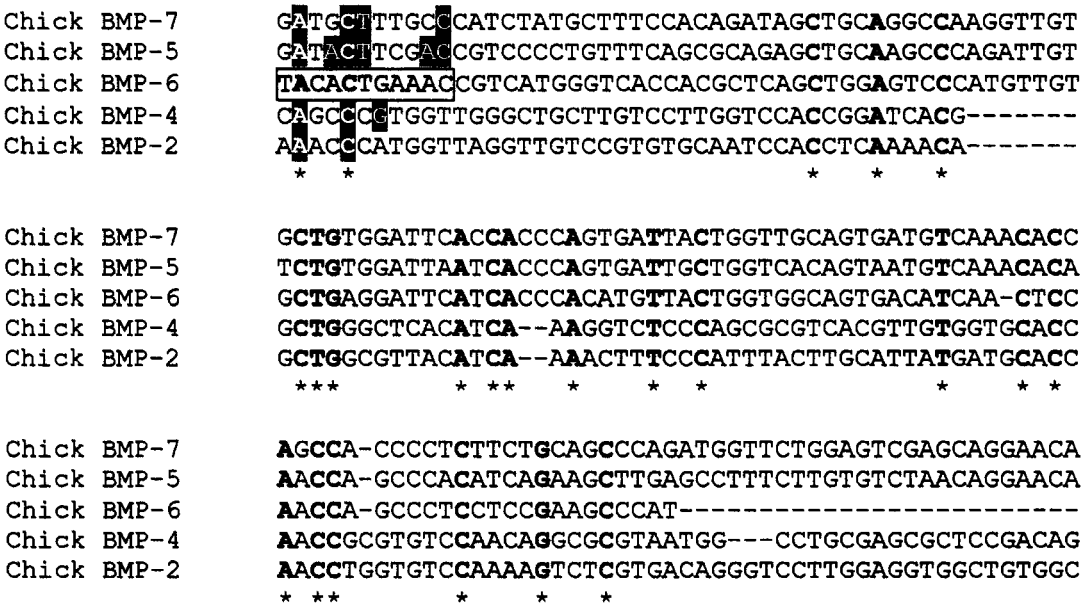


Fig. 16. Alignment of chick BMP family members, showing the BMP-6 primer sequences, and the selected probe region.

Chick bone morphogenetic protein gene sequences were aligned with ClustalW in the SDSC Biology Workbench. Sequences for BMP-2, -4, -5 and -7 were obtained from Genbank at NCBI. The primer sequences are shown boxed and shaded in light grey. The region selected for the unique chick BMP-6 probe is bounded by the two primer sequences. Conserved residues are shown in bold and marked below the alignment with an asterisk (*). Identical residues within the primer-binding region are shown boxed in dark grey with white lettering. (bone morphogenetic protein-7 [*Gallus gallus*] GBVRT:957233; bone morphogenetic protein-5 [*Gallus gallus*] GBVRT:1881822; putative partial BMP-6 [*Gallus gallus*] TC122562 (TIGR database); bone morphogenetic protein-4 [*Gallus gallus*] GBVRT:472929; bone morphogenetic protein-2 [*Gallus gallus*] GBVRT:472927).

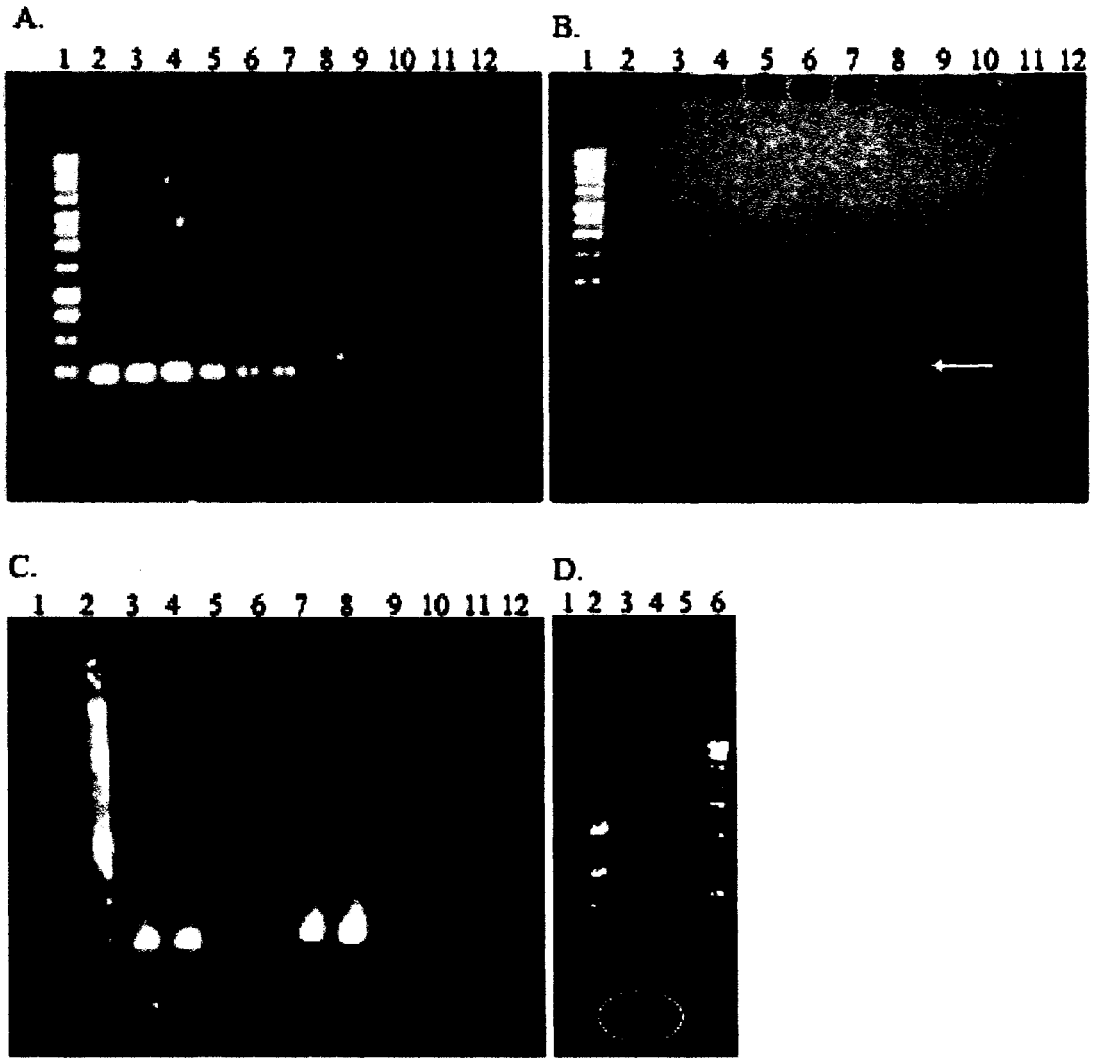


Fig. 17. Probe synthesis by RT-PCR, secondary amplification, and spin purification.

Using total chick RNA, and specific primers, the probes were synthesized by RT-PCR with the Qiagen RT-PCR kit. Presence of product was determined by 1% TAE agarose gel electrophoresis, stained with ethidium bromide. A) and B) RT-PCR amplification on a temperature gradient of T = 55.0 +/- 6.0 (Table 6). BMP-6 amplified well, while β-actin was only weakly amplified (indicated by an arrow). Clone 10H12 failed to amplify. Lane 1(both gels) = Promega 1kb DNA Ladder, lanes 2-7 = BMP-6, lane 8-11 and lane2-3 of gel B= 10H12, lane 4-10 of gel B = β-actin. C) Secondary PCR of probes using optimal T_m determined by RT-PCR on temperature gradient. Lane 2 = Promega 1 kb DNA Ladder, lane3-4 = BMP-6, lane 5-6 = 10H12, lane 7-8 = β-actin. D) Purified probe products after spin purification (indicated by a circle). Lane 2 = Invitrogen Low Mass ladder, lane 3 = BMP-6, lane 4= β-actin, lane 5 = Promega 1kb DNA Ladder.

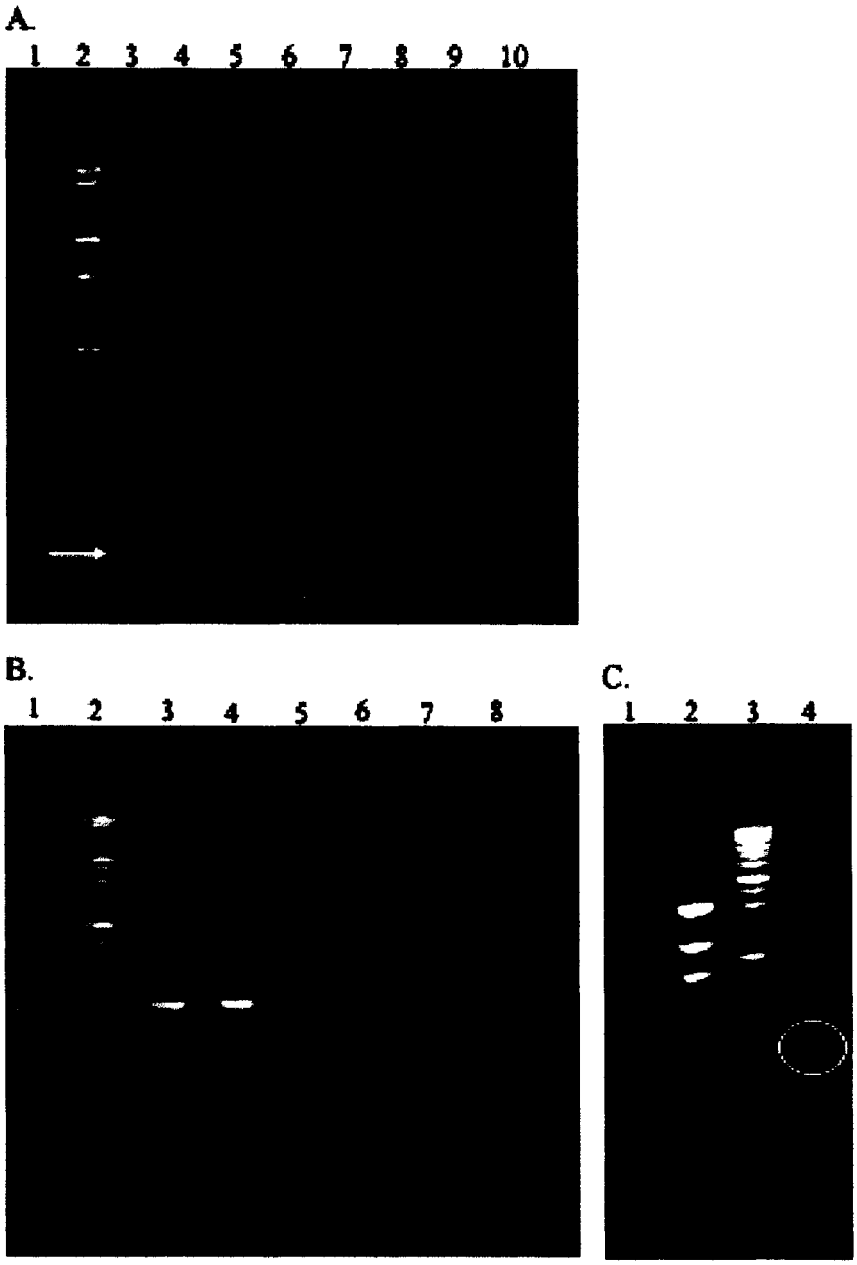


Fig. 18. Probe synthesis of 10H12 by RT-PCR, secondary amplification, and spin purification.

The process outlined in Fig. 16 was repeated for 10H12, which did not amplify in the first RT-PCR reaction. A) RT-PCR of 10H12 on a temperature gradient (indicated by an arrow). Lane 2 = Promega 1 kb DNA Ladder, lane 3-8 = 10H12; B) Secondary amplification at the optimal T_m. lane 2 = Promega 1 kb DNA Ladder, lane 3-4 = 10H12; C) Probe product after spin purification (indicated by a circle). Lane 2 = Invitrogen Low Mass ladder, lane 3 = Promega 1 kb DNA Ladder, lane 4 = 10H12.

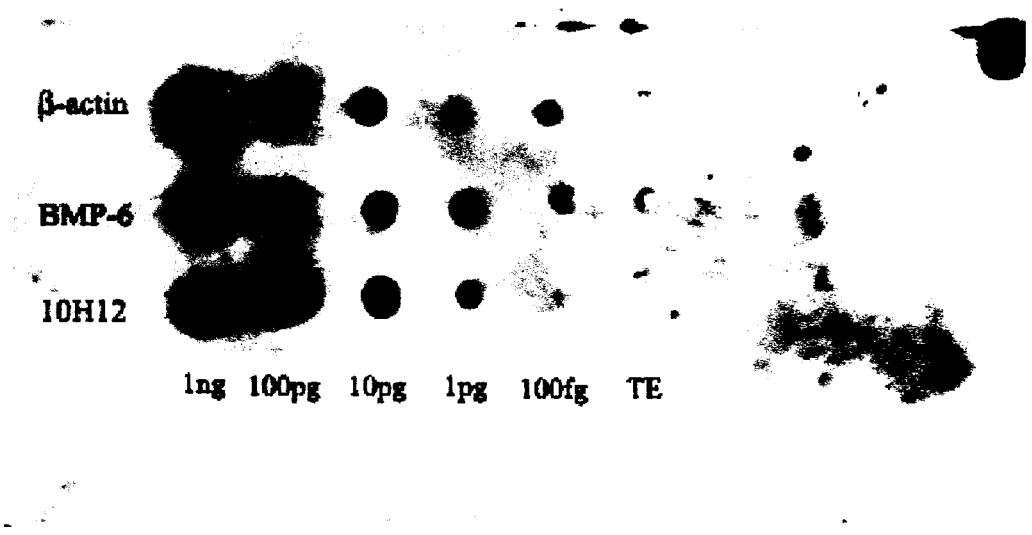


Fig. 19. Dot blot test of biotinylated probes.
A ten-fold dilution series of the labeled probe was prepared from 1 ng to 100 fg. One microliter of each dilution was spotted onto a positively-charged nylon membrane (Schleicher & Schuell Bioscience/ Whatman) with a TE negative control spot. Detection was performed according to Ambion's protocol.

Fig. 20. Seegene's Zooblot of adult chicken tissues.
Manufacturer-provided image of the gel used to make the tissue blot. RNA from adult chicken tissues were harvested and run out on a gel in the following order (left to right): brain, heart, lung, liver, spleen, kidney, small intestine, testis, uterus, ovary, and gizzard. The separated RNAs were then transferred onto a nylon membrane to create the tissue blot.

A.

B.

Fig. 21. Hybridization and visualization of the biotinylated β-actin probe on Seegene's Zooblot.

The β-actin probe was tested first as a positive control. Expression of β-actin is expected across all tissues. A) The blot was hybridized with β-actin (0.1 ng/ ml) once without prior heat denaturation of the probe. The blot was re-hybridized without an intermediate stripping of the blot. No signal was obtained, but little background is seen. B) The probe concentration was increased to 1.0 ng/ ml. The blot had been stored and cleaned for re-use according to Seegene's protocol. No signal is seen, and very high background staining is present.

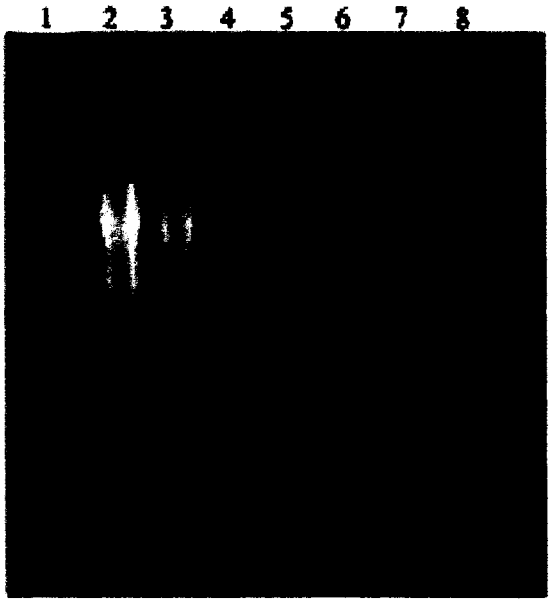


Fig. 22. Northern blot of 48-hour chick total RNA.
A northern blot of total chick RNA was created according to the lab protocol (White, 2004). A 2-fold dilution series of total RNA from 24 to 0.75 micrograms per lane was run out with RNA millennium markers on a denaturing gel. Standard precautions to avoid RNase contamination were taken. The gel was post-stained and visualized on the BioRad GelDoc. RNA was then transferred onto a positively charged nylon membrane (Schleicher & Schuell Bioscience/ Whatman), heated at 68°C for 10 minutes and UV cross-linked to fix. Lane 1 = Ambion's RNA Millennium Markers, lane 2-8 = 2-fold dilution of total chick RNA from 24 to 0.75 μg.

Fig. 23. Hybridization and visualization of the biotinylated β-actin probe on the 48-hour chick total RNA northern blot.

The β-actin probe was tested first as a positive control. The blot was hybridized with a β-actin probe (0.5 ng/ ml) with a 1.5 hour prehybridization step and overnight probe hybridization at 42°C. Ambion's detection protocol was followed. Film exposure = 60 minutes. A strong positive signal is seen at the highest concentration, which diminishes with sample dilution as expected. Background staining is minimal.

A.

B.

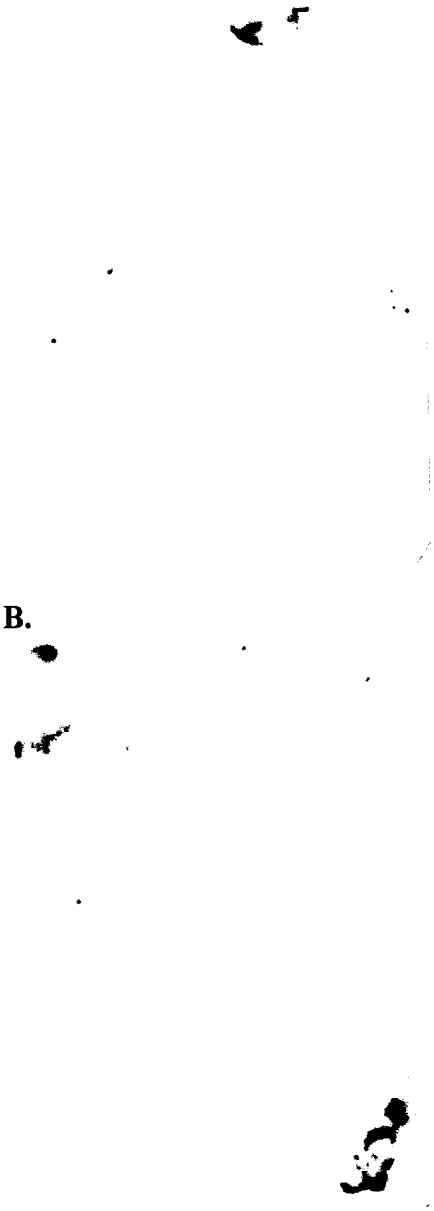


Fig. 24. Hybridization and visualization of the biotinylated BMP–6 probe on the 48-hour chick total RNA northern blot.

The BMP-6 probe was tested next. A) The blot was hybridized with BMP-6 probe (0.5 ng/ ml) with a 1.5 hour prehybridization step and overnight probe hybridization at 42°C. Ambion's detection protocol was followed. Film exposure = 75 minutes. B) The blot was hybridized with BMP-6 probe (1.0 ng/ ml) with a 1.5 hour prehybridization step and 44 hour probe hybridization at 42°C. Ambion's detection protocol was followed, with the following modification: high stringency washes were reduced from 68°C to 55°C. Film exposure = 90 minutes. No signal is seen and background staining is low.

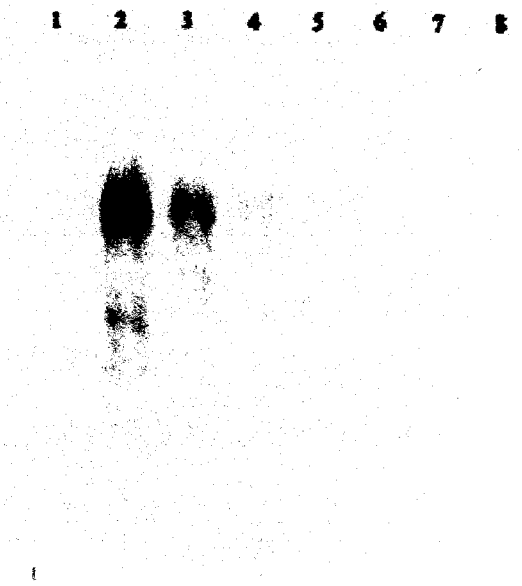


Fig. 25. Hybridization and visualization of the biotinylated β-actin probe on the 48-hour chick total RNA northern blot after multiple uses.
The 48-hour chick total RNA blot with a 2-fold dilution series from 24 to 0.75 micrograms per lane was re-hybridized with the β-actin probe after three previous hybridizations. The blot was stripped and stored between each use. The blot was hybridized with β-actin (0.5 ng/ ml) with a 1.5 hour prehybridization step and overnight probe hybridization at 42°C. Ambion's detection protocol was followed. Film exposure = 60 minutes. A weak positive signal is seen at the highest concentration, which diminishes with sample dilution as expected. The signal strength may have diminished due to loss of sample material through the stringent washing procedures. However, enough sample remains to detect molecules expressed at high levels. Background staining is minimal.

USER: 1 COMMENT:

PRESET TIME : 1.00

DATA CALC : CPM H# :YES SAMPLE REPEATS: 1 PRINTER : STD

COUNT BLANK : NO CPK : NO REPLICATES : 1 RS232 : OFF

TWO PHASE : NO AQC : NO CYCLE REPEATS : 1 DISK : OFF

SCINTILLATOR: LIQUID LUMEX: NO LOW SAMPLE REJ: 0 RWM LIST : OFF

DW LIMIT : NO HALF LIFE CORRECTION DATE: none

ISOTOPE 1: 3H %ERROR: 0.00 FACTOR: 1.000000 BKG. SUB: 0

ISOTOPE 2: 14C %ERROR: 0.00 FACTOR: 1.000000 BKG. SUB: 0

WIDE OPEN WINDOW %ERROR: 0.00 FACTOR: 1.000000 BKG. SUB: 0

SAM NO	POS	TIME MIN	H#	3H CPM	3H %ERROR	14C CPM	14C %ERROR	WIDE CPM	WIDE %ERROR	LUMEX %	ELAPSED TIME
1	[illegible]	1.00	103.2	2592272	0.12	2662502	0.12	5254794	0.09	[illegible]	[illegible]
2	[illegible]	1.00	102.9	22417.00	1.34	22025.00	1.35	44460.00	0.95	[illegible]	[illegible]
3	[illegible]	1.00	104.1	2631647	0.12	2455828	0.13	5087502	0.09	[illegible]	[illegible]
4	[illegible]	.00	104.6	10174.00	1.98	10670.00	1.94	20870.00	1.56	[illegible]	10.47

Fig. 26. Labeling efficiency of probes with [α-S^{35}]-dATP end labeling.
Labeling efficiency was determined by Cpm counts. The whole labeling reaction, and the spin-purified labeled probes were tested. 1) β-actin, 2) β-actin spin-purified, 3) BMP-6, 4) BMP-6 spin-purified. The β-actin probe was labeled with a total of 1.7×10^6 cpm. The BMP-6 probe was labeled with a total of 8.0×10^6 cpm.

A.

B.

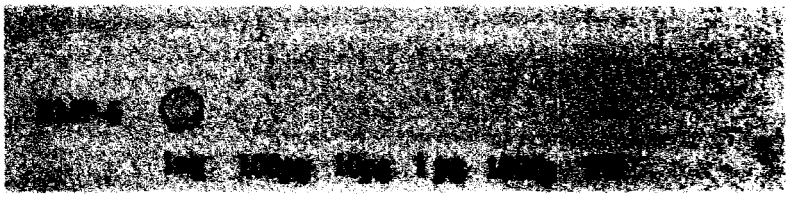


Fig. 27. Dot blot test of radiolabeled probes after 48-hour exposure at -70°C. A ten-fold dilution series of the [α-S^{35}]-labeled probe was prepared from 1 ng to 100 fg. One microliter of each dilution was spotted on the nylon membrane with a TE negative spot. Detection was performed according to the lab protocol (White, 2004).

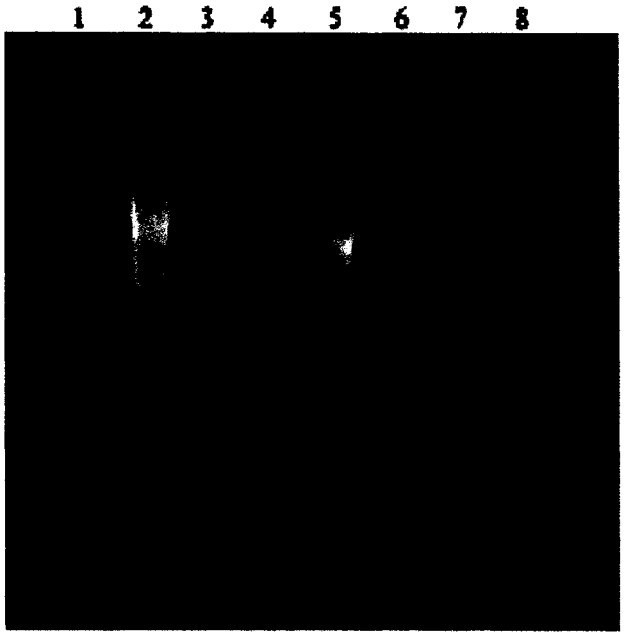


Fig. 28. Northern blot of 48-hour chick total RNA for parallel hybridizations. A northern blot of total chick RNA was created according to the lab protocol (White, 2004). A 5-fold dilution series in duplicate of total RNA from 25 to 1 micrograms per lane was run out with RNA Millennium Markers on a denaturing gel. Standard precautions to avoid RNase contamination were taken. The gel was post-stained and visualized on a BioRad GelDoc. RNA was then transferred onto a positively charged nylon membrane (Schleicher & Schuell Bioscience/ Whatman), heated at 68°C for 10 minutes and UV cross-linked to fix. Lane 1 = RNA Millennium Markers, lane 2-4 = 5-fold dilution of total chick RNA from 25 to 1 μg, lane 5-7 = 5-fold dilution of total chick RNA from 25 to 1 μg, lane 8 = Ambion's RNA Millennium Markers. After fixation the membrane was split in half for parallel hybridizations.

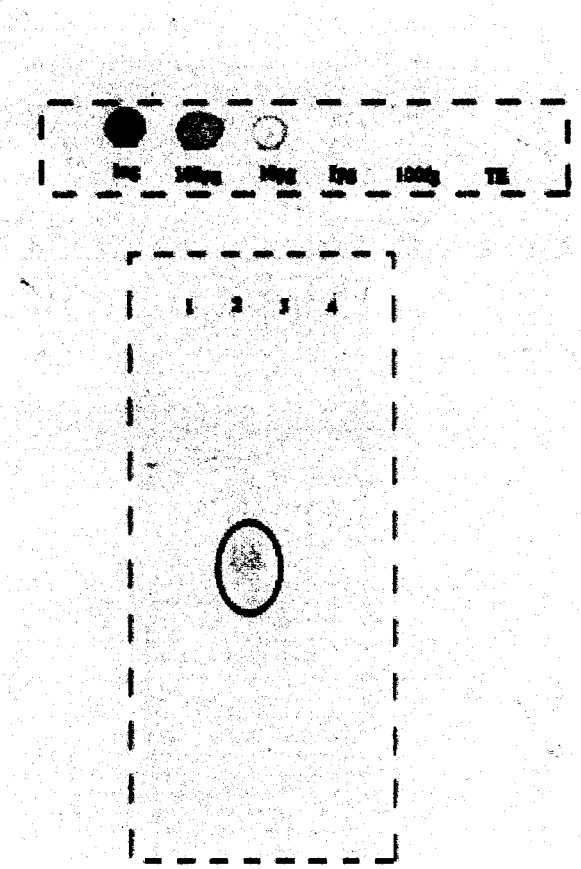


Fig. 29. Hybridization and detection of 48-hour chick total RNA with [α-S^{35}]-labeled β-actin probe after a 2-week exposure at -70°C.
Northern blot of 48-hour chick total RNA was hybridized with [α-S^{35}]-labeled β-actin probe. Lane one = Ambion's RNA Millennium Markers, lane 2 = 25 μg total RNA, lane 3 = 5 μg, lane 4 = 1 μg. A simultaneously run dot blot of the probe from 1 ng to 100 fg with a TE negative control is shown at the top of the film for signal strength comparison. A weak positive signal (circled) is seen at the highest concentration of total RNA in lane 2.

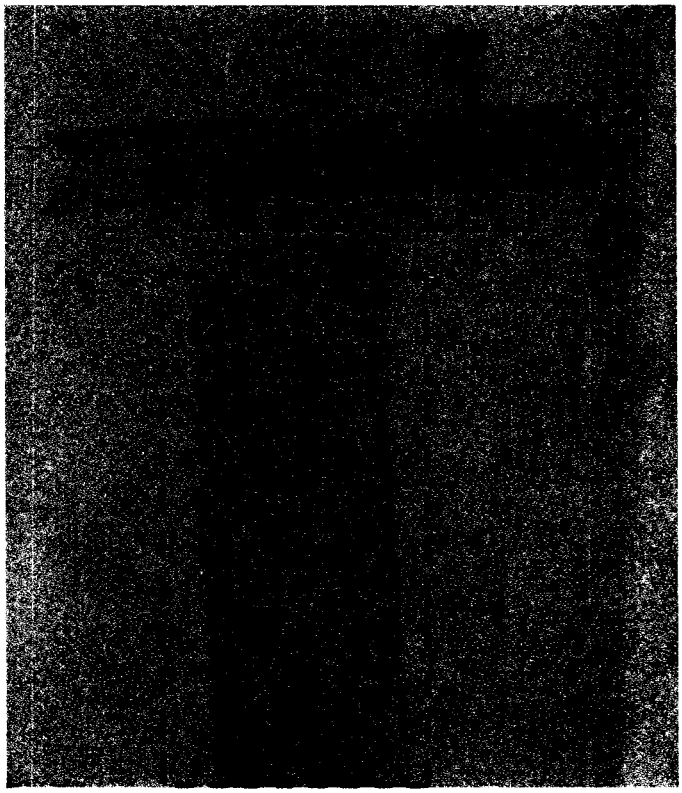


Fig. 30. Hybridization and detection of 48-hour chick total RNA with [α-S^{35}]-labeled BMP-6 probe after a 2-week exposure at -70°C.
Northern blot of 48-hour chick total RNA was hybridized with [α-S^{35}]-labeled BMP-6 probe. Lane 1 = 25 μg total RNA, lane 2 = 5 μg, lane 3 = 1 μg, lane 4 = RNA Millennium Markers. A simultaneously run dot blot of the probe from 1 ng to 100 fg with a TE negative control is shown at the top of the film for signal strength comparison. No positive signal was obtained.

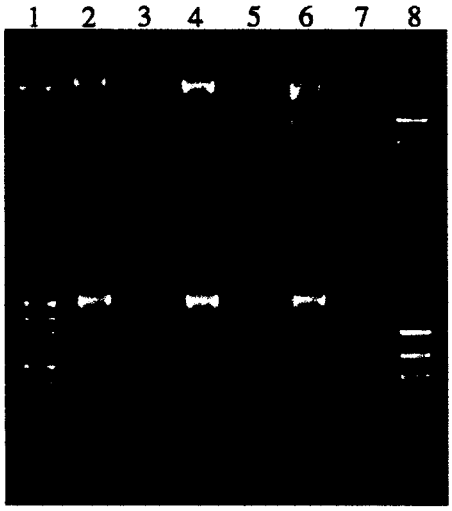


Fig. 31. EcoRI restriction of pSTBlue-1 isolated from white colonies by QiaPrep miniprep.
EcoRI restriction of isolated plasmids was performed to confirm the presence of the insert. (top and bottom). Lane 1= Promega 1kb DNA Ladder, lane 2-7 = paired uncut and cut plasmid samples, lane 8 = Invitrogen Low Mass Ladder. Expect 230 bp insert and 3850 bp vector. The plasmids are running very high in the gel, indicating possible contamination, cross-linking, or formation of multimers. The failure of the restriction enzyme to cleave the insert from the plasmid is probably due to contaminants or the altered conformation of the plasmid.

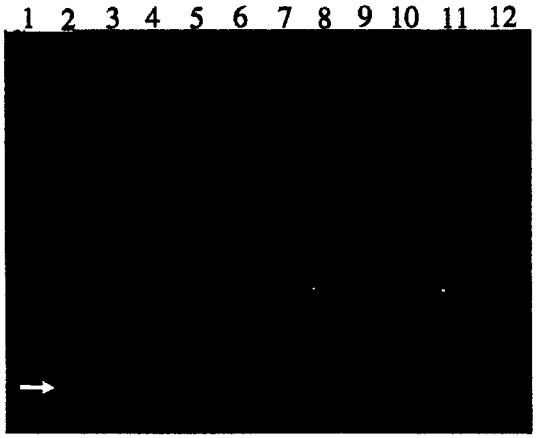


Fig. 32. PCR amplification of BMP-6 insert from isolated pSTBlue-1 plasmid. PCR amplification of the insert from the plasmid was performed to confirm the presence of the insert (indicated by an arrow). Lane 1= Promega's 1kb DNA Ladder, lanes 2-5 = BMP-6 insert amplified with BMP-6 specific primers, lane 6 = Invitrogen's Low Mass standard, lanes 7-10 = BMP-6 probe amplified with T7 and SP6 primers, lane 11 = Invitrogen's High Mass standard, lane 12 = isolated insert-containing pSTBlue-1 plasmid (circled).

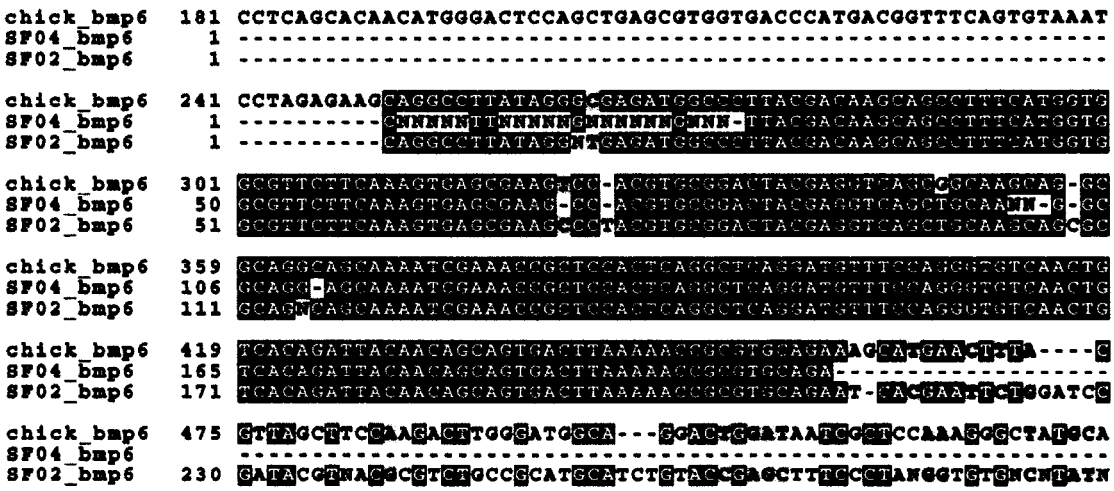


Fig. 33. Alignment of the BMP-6 insert in pSTBlue-1 vector against the putative chick BMP-6 sequence.

PCR amplified insert from isolated pSTBlue-1 plasmid was sequenced using the BMP-6 Right primer. Sample SF02_bmp6 sequenced by Gene Gateway (Hayward, CA), and sample SF04_bmp6 sequenced by Tocore (Menlo Park, CA), were aligned with the putative chick BMP-6 sequence (chick_bmp6) in ClustalW and analyzed with BoxShade in the SDSC Biology Workbench.

Table 2. Colonies screened.

The 48-hr chick plasmid cDNA library consists of twenty-eight 96-well plates. Plates were selected at random, and screened in full whenever possible. A total of 328 individual colonies were screened by plasmid isolation and insert sequencing, to identify clones with high sequence identity to developmentally important genes, particularly those involved in cardiogenesis, with no confirmed chick homolog. One clone was identified that shared a high sequence identity with a putative partial chick BMP-6 gene located in the TIGR database.

Plate #	Column Screened	Row Screened	Complete	Number Screened
1	1	A-H	N	8
2	1	A-H	N	8
10	1-12	A-H	Y	96
16	1-12	A-H	Y	96
22	1-12	A-H	Y	96
24	1-3	A-H	N	24

Table 3. Sequences omitted from further consideration.
A partial list of screened clones is shown to give a representative sample of the results obtained during database homology searches. The blast search function in the TIGR database was used to identify gene homologies. When no matches were made in the TIGR database, the sequences were screened against BLASTX and Low Homology BLAST at NCBI. Sequences that had significant sequence identity to known ubiquitous genes and genes that had sequence identity to other sequenced clones with no identified product were omitted. (x = no homology found.)

OMIT SEQUENCES: ubiquitous genes or low homology (unknowns)

Clone	TIGR_blast hit
16C8	heat shock protein
16A11	heat stable protein phosphatase 2A inhibitor
10D12	heat shock protein 90
10B12	heat shock protein (TRAP-1)
22H10	ribosomal protein L11
16G1	40S ribosomal protein S6
10D11	ribosomal protein L5
22B12	ribosomal protein L11
22F11	40S ribosomal protein S23
22B9	ribosomal protein L22
22C7	40S ribosomal protein S23
16C1	hnRNP protein
10E12	protein phosphatase 1Beta
16F10	M-phase phosphoprotein 6
16C11	cleavage and polyadenylation specificity factor
16H10	importin Beta-1 subunit
22H7	NADH-ubiquinone oxidoreductase chain 1
22A7	eukaryotic translation initiation factor
2G1	fbox protein fb17
22F6	cohesin complex subunit
10F11	guanine nucleotide binding protein

Clone	BLASTX Hit	Discontiguous MegaBLAST Hit
16A8	hypothetical protein (human)	G. gallus clone
22C10	x	G. gallus clone
22D10	ORF2 (P.spixii)	G. gallus clone
22D11	x	L. corniculatus chrom.2
16B1	x	BAC clone (human)
16D10	hypothetical protein (mouse)	G. gallus clone
10A12	GPI-anchored protein, caprin	GPI-anchored protein (human chrom.11)
16H1	x	M. gallopavo sequence
16E11	x	G. gallus clone
16F11	beta-gal.	cosmid (C. elegans)

22G10	unnamed protein (human)	x
16E8	x	x
16Ff8	x	x
16D8	x	x
16B8	x	x
10D1	permease, helix-loop-helix	pheromone receptor (mouse)
22C12	x	x
22D11	x	chrom.13 (mouse)
22F10	U1 snRNP, RNPC7	RNPC7 (RNA-bind. region containing 7, human)
2E1	unnamed protein (A. gambiae)	chrom.9 (human)
16B11	x	x
16B10	x	x
16C10	x	x
22C3	recG (mycobacterium)	G. gallus clone
16F5	unnamed protein (mouse)	G. gallus clone

Table 4. Published BMP-6 primer sequences.

Primer pairs found in a literature search of BMP-6 research have been collected. Species is indicated by a lower case later (h= human, m= mouse). The orientation of the primers and region being amplified are noted when known. The size of the amplified product is noted when known. [* Upper case letters indicate residues from the human gene sequence; ** Note: reverse primers are given in the 3' to 5' direction]. Literature citations: 1. (Barnes et al., 1995), 2. (Clement et al., 1999), 3. (Heikenheimo et al., 1999), 4. (Kaiser et al., 1998), 5. (Knittel et al., 1997), 6. (Liu et al., 2004), 7. (Ong et al., 2004), 8. (Rickard et al., 1998), 9. (Solloway et al., 1998), 10. (Tamada et al., 1998).

Lit. Cit.	Primer Name	Primer Sequence	Prod. Size
1	hBMP6 downstream	5' AGTTTCAAAGTCTCATCGTC 3'	459 bp
	hBMP6 upstream	5' TCCAGTGCTTCAGATTACAA 3'	
2	hBMP6 sense	5' GTCGTAATCGCTCTACCCAGTCC 3'	529 bp
	hBMP6 antisense	5' CTGGGTAATAAGGCACTGGCATG 3'	
3	hBMP6	5' AC(A/G)GC(A/G)ACTAGCAATCTGTGG 3'	500 bp
	hBMP6	5' (G/A)CAGCA(C/T)GGTTTGGGGAC(G/A)TA 3'	
4*	BMP6	5' agggatccCAACAGAGTCGTAATCGC 3'	N/A
	BMP6	5' agataagcttGTTGCATTCATGTGTGCG 3'	
5	hBMP6 forward	5' GCAGAAGGAGATCTTGTCGG 3'	614 bp
	hBMP6 reverse	5' GTCTCTGTGCTGATG 3'	
6	hBMP6 forward	5' CGCCTCTGTTCAGTTCATTCCC 3'	N/A
	hBMP6 reverse	5' ACCCTGTGGCGTGGTATGCTG 3'	
7	hBMP6 promoter forward	5' TCTTACTAGGTTGCCCAGCCTGATCTCGAG 3'	4.3 kb
	hBMP6 promoter reverse	5' AGAGGTCATCGCTAGTAAGAATGATCTGAC 3'	
8**	BMP6 noncoding 3' UTR	5' TGGTCTGTAGCAAGCTGAGTT 3'	458 bp
	BMP6 noncoding 3' UTR	3' GAGTACAGCAAATGGAGGATT 5'	
8**	BMP6 coding propeptide	5' ACATGGTCATGAGCTTTGTGA 3'	528 bp
	BMP6 coding propeptide	3' GTAGAGCGATTACGACTCTGT 5'	
9	mBMP6 exon2 forward	5' TCCCCACATCAACGACACCA 3'	112 bp
	mBMP6 exon2 reverse	5' TCCCCACCACACAGTCCTG 3'	
10	hBMP6 exon1 forward	5' CTGGGGCGGAGGGCGCAGTG 3'	230 bp
	hBMP6 exon1 rev.	5' TGCATCTCCCGCTTCTCCTGCGTC 3'	

Table 5. Primers used for probe amplification.

Primer Name	Sequence (5' to 3')	Len.	T_m (°C)	Exp. Size
10H12 right	AAAACATCCACCTGACACAT	20	56.3	218 bp
10H12 left	TACTTCCAACCTGCTTGTCT	20	58.35	
BMP-6 right	GTTTCAGTGTAAATCCTAGAGAAG	24	59.44	232 bp
BMP-6 left	TCTGACGCGGTTTTTAA	18	55.34	
ckActin right	AGCCAACAGAGAGAAGATGA	20	58.35	236 bp
ckActin left	TCTTCATGCGGTAGTCCGTC	20	60.4	

Table 6. Temperature gradient and optimal temperature for RT-PCR.
RT-PCR was performed on a thermal gradient to determine the optimal melting temperature (T_m) for each primer pair. The 10H12 and BMP-6 primer pairs had an expected T_m of 57°C. The β-actin primer pair had an expected optimal T_m of 59°C.

Block Position	Temperature (°C)	Expected Optimal T_m	Experimental Optimal T_m
1	49.0		
2	49.2		
3	49.8		BMP-6
4	50.9		β-actin
5	52.3		
6	53.8		
7	55.5		10H12
8	57.1	BMP-6, 10H12	
9	58.7	β-actin	
10	60.0		
11	60.9		
12	61.5		

Table 7. UniGene expression profiles for mouse, rat, and human BMP-6. Number of transcripts in each tissue pool is given in transcripts per million (TPM). The gene column gives the number of ESTs for the gene of interest in each tissue pool, and the total column lists the number of all ESTs found in the pool. BMP-6 EST expression by developmental stage is also shown. A) mouse, B) rat, and C) human. The heart pools are boxed.

A. Mm.374781- Bmp-6: Bone morphogenetic protein-6 (*M. musculus*)

bone	0	0 / 34152
bone marrow	9	1 / 110460
whole brain	16	3 / 184598
amygdala	0	0 / 1816
ganglia	0	0 / 25923
brain stem	0	0 / 2373
cortex	5	1 / 182305
mesencephalon	0	0 / 106
pineal gland	0	0 / 3878
diencephalon	0	0 / 19043
circumventricular organs	0	0 / 347
gallbladder	0	0 / 1555
intestine	0	0 / 82548
stomach	223	7 / 31256
liver	0	0 / 104680
pancreas	0	0 / 103377
eye	11	2 / 176039
heart	0	0 / 52305
respiratory system	22	1 / 43548
lymph node	0	0 / 15768
spleen	21	2 / 92555
thymus	0	0 / 125582
mammary gland	19	7 / 356179
muscle	0	0 / 26069
female genital	26	1 / 38205
male genital	19	2 / 104678
bladder	59	1 / 16717
kidney	85	10 / 117350
blood	194	3 / 15385
connective tissue	0	0 / 32452
skin	23	2 / 84177
endocrine system	0	0 / 54707

ear	53	2 / 37105
limb	0	0 / 27698
extraembryonic tissue	18	1 / 55307
embryonic tissue	0	0 / 71381
whole head	0	0 / 78582
mouth	0	0 / 32819
olfactory system	0	0 / 3902
vascular	83	1 / 11969
adult (7wk+ post natal)	21	21 / 974791
juvenile (7-49d post natal)	30	8 / 265589
neonate (0-7d post natal)	31	3 / 95368
female germ line	42	1 / 23574
fetus (12-21d.p.c.)	31	17 / 543693
early gestation (<7d.p.c.)	0	0 / 8137
pre-implantation (0-6.5 d.p.c.)	35	5 / 142328
mid-gestation (8-12d.p.c.)	0	0 / 170627
gastrula (6.5-7.9d.p.c.)	142	6 / 42114
late gestation (12-21d.p.c.)	0	0 / 85676
embryo (no stage given)	0	0 / 37462
male	15	9 / 596380
female	31	8 / 250290
mixed pool by gender	25	7 / 271415
virgin	57	4 / 69719
lactating	0	0 / 49550
pregnant	29	2 / 68507
fibroblast	0	0 / 607
mixed (normal and tumor)	0	0 / 6999
neoplasia	22	9 / 396760
non-neoplasia	0	0 / 18386
normal	21	66 / 3133435

B. Rn.40476- Bmp-6: Bone morphogenetic protein-6 (*R. norvegicus*)

Tissue Pool	TPM	Gene Total
brain	15	1 / 63380
colon	0	0 / 8087
eye	40	1 / 24482
heart	0	0 / 13394
kidney	0	0 / 10605
liver	0	0 / 9793
lung	192	3 / 15572
muscle	0	0 / 3097
ovary	0	0 / 7722
pancreas	0	0 / 958
placenta	163	4 / 24453
pituitary gland	61	1 / 16290
spleen	124	1 / 8045
testis	0	0 / 11389
thymus	0	0 / 8065
embryo	0	0 / 26237
adult	6	1 / 145669

C. Hs.285671- BMP-6: Bone morphogenetic protein-6 (*H. sapiens*)

Tissue pool	TPM	Gene Total
bladder	0	0 / 21754
blood	40	2 / 49988
bone	0	0 / 55055
bone marrow	0	0 / 36594
brain	6	3 / 469643
cervix	24	1 / 41294
colon	0	0 / 180153
eye	29	5 / 169020
heart	0	0 / 59062
kidney	14	2 / 139072
larynx	0	0 / 27598
liver	7	1 / 131343
lung	10	3 / 289012
lymph node	0	0 / 128237
mammary gland	0	0 / 140234
muscle	10	1 / 99982
ovary	94	9 / 95693
pancreas	0	0 / 197911
peripheral nervous system	0	0 / 25034
placenta	46	11 / 238006
prostate	22	3 / 133748
skin	6	1 / 165707
small intestine	0	0 / 14098
soft tissue	0	0 / 23774
spleen	0	0 / 19302
stomach	0	0 / 108315
tongue	0	0 / 28966
testis	0	0 / 136587
thymus	0	0 / 6844
uterus	0	0 / 181685
vascular	77	2 / 25893
embryo	23	13 / 557204
juvenile	0	0 / 59630
adult	13	13 / 975147

Table 8. UniGene expression profile for chick β-actin.

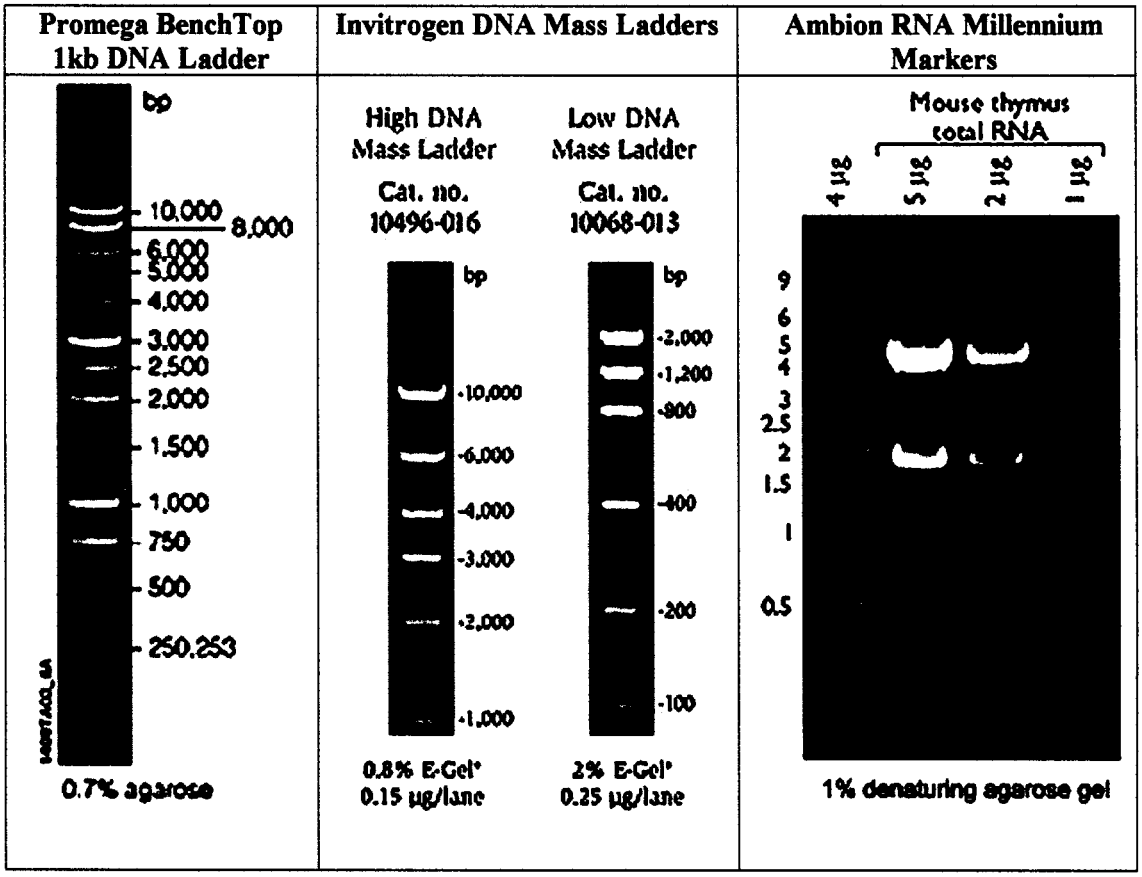
Number of transcripts in each tissue pool is given in transcripts per million (TPM). The gene column gives the number of ESTs for the gene of interest in each tissue pool, and the total column lists the number of all ESTs found in the pool. A breakdown of β-actin expression in the chick by developmental stage is not available. The heart pool is boxed.

Gga.8939- LOC396526: Beta-actin. (*G. gallus*)

Tissue Pool	TPM	Gene	Total
brain	762	48	62968
bursa of fabricius	4575	199	43494
cartilage	142	2	13996
female genital	796	18	22600
gastrointestinal tract	579	10	17257
head	1074	30	27913
heart	492	8	16260
liver	63	1	15774
male genital	244	2	8192
muscle	494	8	16187
pancreas	680	5	7348
soft tissue	1734	6	3459
spleen	2723	9	3305

APPENDIX B: ADDITIONAL RESOURCES

Commonly Used Ladders



In 2 µl of Invitrogen's High Mass Ladder there is 100 ng at 10,000 bp, 60 ng at 6,000 bp, 40 ng at 4,000 bp, 20 ng at 2,000 bp, and 10 ng at 1,000 bp.

In 2 µl of Invitrogen's Low Mass Ladder there is 100 ng at 2,000 bp, 60 ng at 1,200 bp, 40 ng at 800 bp, 20 ng at 400 bp, 10 ng at 200 bp, and 5 ng at 100 bp.

Sources:

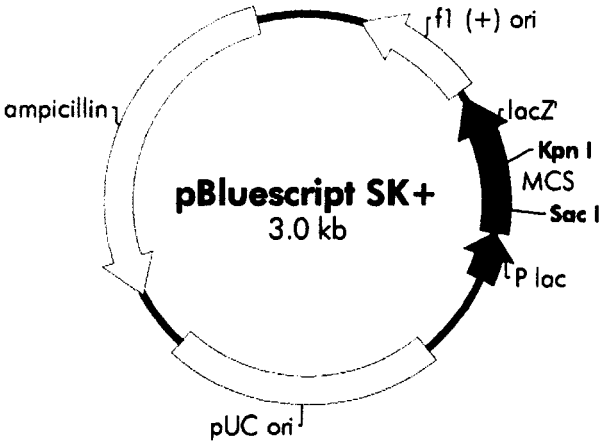
http://www.promega.com/figures/popup.asp?fn=1409ta&partno=G7541&product=Bench Top+1kb+DNA+Ladder#

http://www.invitrogen.com/content.cfm?pageid=3973

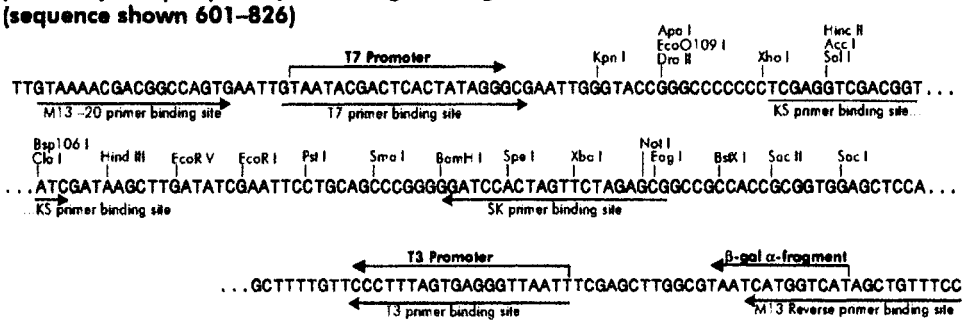
http://www.ambion.com/catalog/CatNum.php?7150

Stratagene pBluescript SK+ Vector

f1 (+) origin 138–444
β-galactosidase α-fragment 463–816
multiple cloning site 653–760
***lac* promoter 817–938**
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



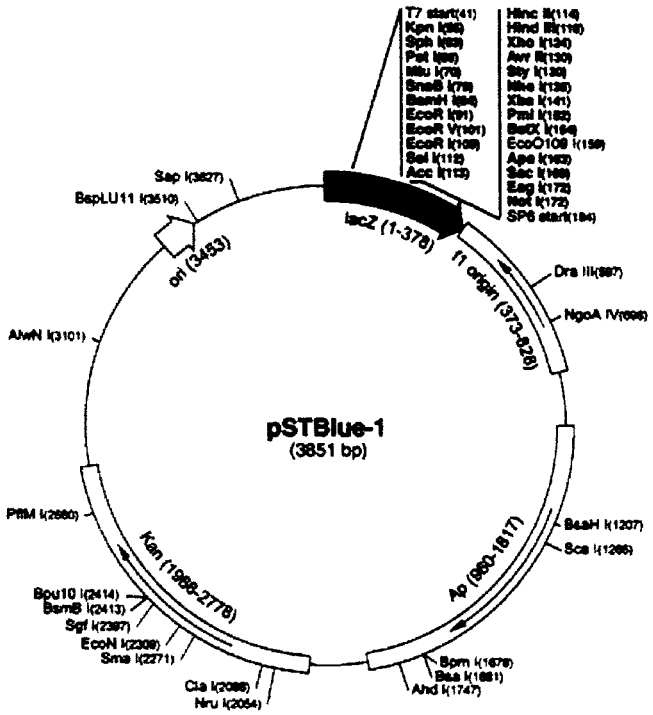
pBluescript SK (+/–) Multiple Cloning Site Region (sequence shown 601–826)



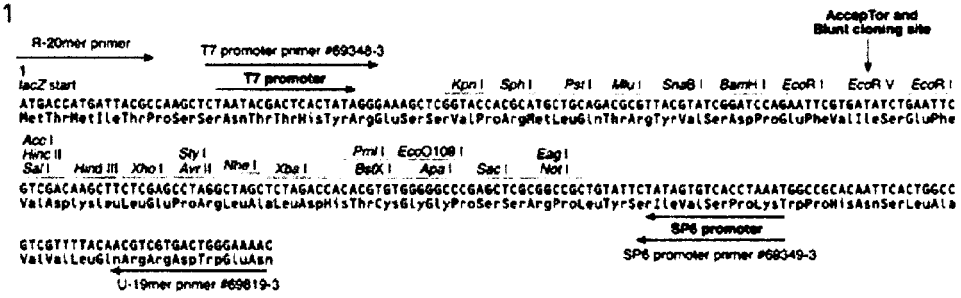
Source: http://www.stratagene.com/vectors/maps/pdf/pBluescript_SK_plus.pdf

Novagen pSTBlue-1 Vector

pSTBlue-1 sequence landmarks	
lacZ start codon	1
lacZ α-peptide ORF	1-378
T7 promoter	24-40
T7 transcription start	41
multiple cloning region (*Kpn* I - *Not* I)	51-178
SP6 promoter	185-202
SP6 transcription start	184
f1 origin	373-828
bla coding sequence	960-1817
Kan coding sequence	1966-2778
pUC origin	3453



pSTBlue-1



Source: http://www.emdbiosciences.com/docs/docs/PROT/TB214.pdf

Online Research Tools

National Center for Biotechnology Information:
- o http://www.ncbi.nlm.nih.gov/BLAST/
- o http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html
- o http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene

San Diego Supercomputer Center, Biology Workbench v3.2:
- o http://workbench.sdsc.edu/

Swiss Institute of Bioinformatics, Expert Protein Analysis System (ExPASy):
- o http://www.expasy.org/prosite/

The Institutes for Genomic Research:
- o http://tigrblast.tigr.org/tgi/

Online Chick Genome Resources

ARKdb:
- o http://www.thearkdb.org/

AvianNET:
- o http://www.chicken-genome.org/index.html

BBSRC ChickEST Database:
- o http://chick.umist.ac.uk/

Ensembl Chicken:
- o http://www.ensembl.org/Gallus_gallus/index.html

Gallus gallus EST and In Situ Hybridization Analysis database (GEISHA):
- o http://geisha.biosci.arizona.edu/

Company Websites

Ambion:
- o http://www.ambion.com/

Applied Biosystems:
- o http://www.appliedbiosystems.com/

Gene Gateway:
- o http://www.genegateway.com/

Invitrogen:
- o http://www.invitrogen.com/content.cfm?pageid=1

Operon:
- o http://www.operon.com/

Novagen:
- o http://www.emdbiosciences.com/html/NVG/home.html

Promega:
- o http://www.promega.com/

Seegene:
- o http://www.seegene.com/new_seegene/site_renewal/

Schleicher & Schuell:
- o http://www.schleicher-schuell.com/icm11be.nsf/(html)/FramesetBioScience

Tocore:
- o http://tocore.com/

APPENDIX C: GLOSSARY

AVC – atrioventricular canal

BLAST – basic local alignment search tool

BMP – bone morphogenetic protein

EMT – endocardial to mesenchymal transformation

HH – Hamburger-Hamilton system of staging chick embryos (see Table 1)

NCBI – National Center for Biotechnology Information (see Appx. B)

ORF – open reading frame

SDSC – San Diego Supercomputer Center (see Appx. B)

TGF – transforming growth factor

TIGR – The Institutes for Genomic Research (see Appx. B)