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Expression domain of Alk2 BMP receptor in chick embryos by in situ hybridization

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EXPRESSION DOMAIN OF ALK2 BMP RECEPTOR IN CHICK EMBRYOS BY *IN SITU* HYBRIDIZATION

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ABSTRACT

EXPRESSION DOMAIN OF ALK2 BMP RECEPTOR IN CHICK EMBRYOS BY *IN SITU* HYBRIDIZATION

by Maryam Shenasa

Statistics show that congenital heart disorder occurs in approximately 1% of live infants, leading to early mortality. Studies have shown that Bone Morphogenetic Proteins (BMPs) are highly involved in cardiogenesis. BMPs are part of the TGFβ super-family and their cellular activities includes binding to heterodimeric receptors that through their intrinsic serine threonine kinase activity phosphorylate and activate downstream SMADs, which in turn carry out gene transcription. Alk2 is one of the essential type I receptors that has been reported to be involved in epithelial-to-mesenchymal transformation and heart looping.

We have cloned, sequenced, and analyzed chick Alk2 mRNA. Additionally, we examined its spatial expression pattern in the heart using *in situ* hybridization during H&H stage 18 and found it present in the outflow tract, atrium and ventricles.

ACKNOWLEDGEMENTS

I owe particular debt of gratitude to my advisor, Dr. Steven White, who always welcomed my questions in spite of his busy schedule. I have been very fortunate to have Dr. White as my mentor and guide during my graduate years at San Jose State University. His dedication to provide the necessary means and training for my research went as far as using his own personal funds and weekends to move this research forward. His enthusiasm and high energy were contagious and produced inspiration for progress. I am deeply grateful to him for kindly reading through the first versions of my thesis and providing me with the necessary feedback.

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THIS THESIS IS DEDICATED TO MOHSEN;

FOR HIS SUPPORT AND ENCOURAGEMENT

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1. INTRODUCTION

1.1. Heart Development and Morphology Changes

The heart undergoes an extremely complicated process of morphogenesis transiting through five stages of development in order to reach the final shape of a four-chambered heart. During these stages of development, there are numerous signaling errors that could occur, leading to congenital heart defects potentially causing serious blood flow or heart rhythm abnormalities. Statistical studies estimate that 0.5% to 1% of live newborns are born with this defect and place congenital heart disease as the leading cause of death from birth defects (Delot 27; Armstrong and Bischoff 459). Recently, an article in the Journal of the American Heart Association stated that:

> Cardiovascular malformations are the most common congenital anomaly, occurring in four to six infants for every 1000 live births...In adults, valvular heart disease remains a major cause of morbidity and mortality; approximately 82,000 valve replacements are performed each year in the USA...An increased molecular understanding of the processes controlling heart valve development and remodeling will continue to suggest new therapeutic modalities. (Armstrong and Bischoff 459)

Understanding pathways involved in heart differentiation and formation will help our understanding of this significant problem.

The heart is the first organ to differentiate and function. During the early gastrula stage, cardiogenic tissue development is directed by molecular signals acting on the precardiac mesoderm as it migrates into the heart-forming region (Nakajima et al. 119-27).

In chick embryos, this occurs around Hamburger & Hamilton (HH) stage 3.[1] Afterwards, left and right precardiac mesoderm form the pleura-pericardial cavity at stage 5 of development. During embryonic folding, the heart-forming regions migrate to the ventral midline of the body and fuse with each other to form the myocardial mantle, which is a straight symmetrical tube called the primitive heart tube. This folding and fusion occurs between HH stages 7 and 9 (Schlange et al. 259-70). At HH stage 10, the primitive heart tube spontaneously starts to beat (D. Wang et al. a1-15). Subsequently, the heart tube generates a right-sided bend (d-loop), and other cardiac segments such as the primitive right and left ventricles, outflow tract (OT), atrio-ventricular canal (AVC), atrium, and sinus venous begin to emerge. After completion of the d-loop, the extracellular matrix (cardiac jelly) between the myocardium and endocardium layers of the AVC and OT converts to mesenchymal cells, which migrate into adjacent cardiac jelly to form endocardial cushion tissue (Nakajima et al. 119-27). This is called the epithelial-to-mesenchymal transformation (EMT) which usually occurs in chick development at HH stage 16-17 (Delot 27-35). The heart valve and septa derived from cardiac cushions divide the heart into four functional chambers (Chang, Brown, and Matzuk 787-823).

[1] All embryos in this thesis are staged according to Hamburger and Hamilton (1951).

The figure below (Nakajima et al. 120) illustrates pictorially the changes that occur initially in the precardiac mesoderm, and later to the heart tube to form the four-chambered heart.

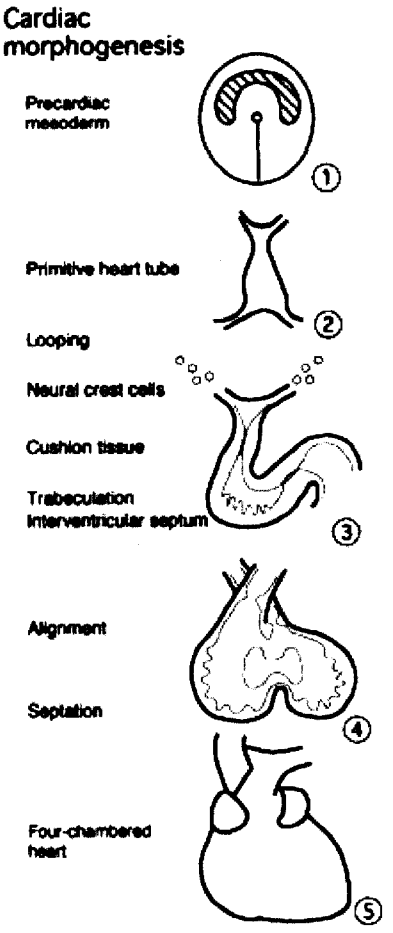

Figure 1. Illustration of Heart Differentiation: This figure was reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. 1. Precardiac mesoderm specification. 2. During embryonic folding, the heart-forming regions migrate to the ventral midline of the body and fuse with each other to form the myocardial mantle, which is a straight symmetrical tube called the primitive heart tube. 3. The heart tube generates a right-sided bend (d-loop), and other cardiac segments begin to emerge. 4, 5. Epithelial-to-mesenchymal transformation of the cardiac jelly occurs and the heart valve and septa derived from cardiac cushions divide the heart into four functional chambers.

Numerous studies have shown that Bone Morphogenetic Proteins play an important role in heart formation and development (Delot 27-35; Inai et al. 383-96; Miyazono et al. 251-63; Nakajima et al. 119-27; Schlange et al. 259-70).

Mice with mutations in BMP pathways exhibit severe heart developmental defects; many so profound, that the fetus succumbs to early gestational lethality (Dudley and Robertson 349-62; Roelen et al. 541-9; Gaussin et al. 2878-83).

Experiments culturing heart tissue from regions of the developing heart in a collagen gel containing media, provide strong supporting evidence that elimination of specific BMPs at a given stage could potentially inhibit the tissue from further differentiation. Finally, *in situ* hybridization experiments have started to clarify the location and timing of BMP expression in the developing heart. The importance of BMPs and their receptors in heart formation and differentiation is well documented.

1.2. Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor beta (TGFβ) gene family and play multiple roles in the developing embryo including morphogenesis and pattern formation. TGFβ super-family ligands initiate signaling through heterodimeric receptors which are transmembrane serine/threonine kinases. Based on their structure and functional properties, these receptors are divided into two subgroups: type I and type II receptors (Chang, Brown, and Matzuk 787-823). When a ligand binds to a type II receptor, it recruits and transphosphorylates the type I receptor. This activates the type I receptor and allows it to associate with receptor-regulated SMAD[2] proteins, and phosphorylate them. Once these R-SMADs[3] are phosphorylated, they associate with a common partner, SMAD4, and translocate to the nucleus (Macías-Silva et al. 25628-36). In the nucleus the R-SMAD/co-SMAD complex can initiate transcription of target genes. TGFβ molecules regulate processes as diverse as tumor progression, wound healing, and embryonic development in vertebrates (Grazul-Bilska et al. 787-800; Desgrosellier et al. 201-10; Waite and Eng 763-73). The super-family consists of more than 35 members, including TGFβs, BMPs, GDFs,[4] activins, inhibins, MIS,[5] Nodal, and leftys. Figure 2 shows a family tree of the TGFβ super-family by using a PILEUP program

[2] Small Mothers Against Decapentaplegic are a class of proteins that modulate the activity of TGF beta ligands.

[3] Receptor associated SMADs are also referred to as R-SMADs.

[4] Growth Differentiation Factors

[5] Müllerian Inhibiting Substance

from the Genetics Computer Group in Madison, Wisconsin (Chang, Brown, and Matzuk 788).

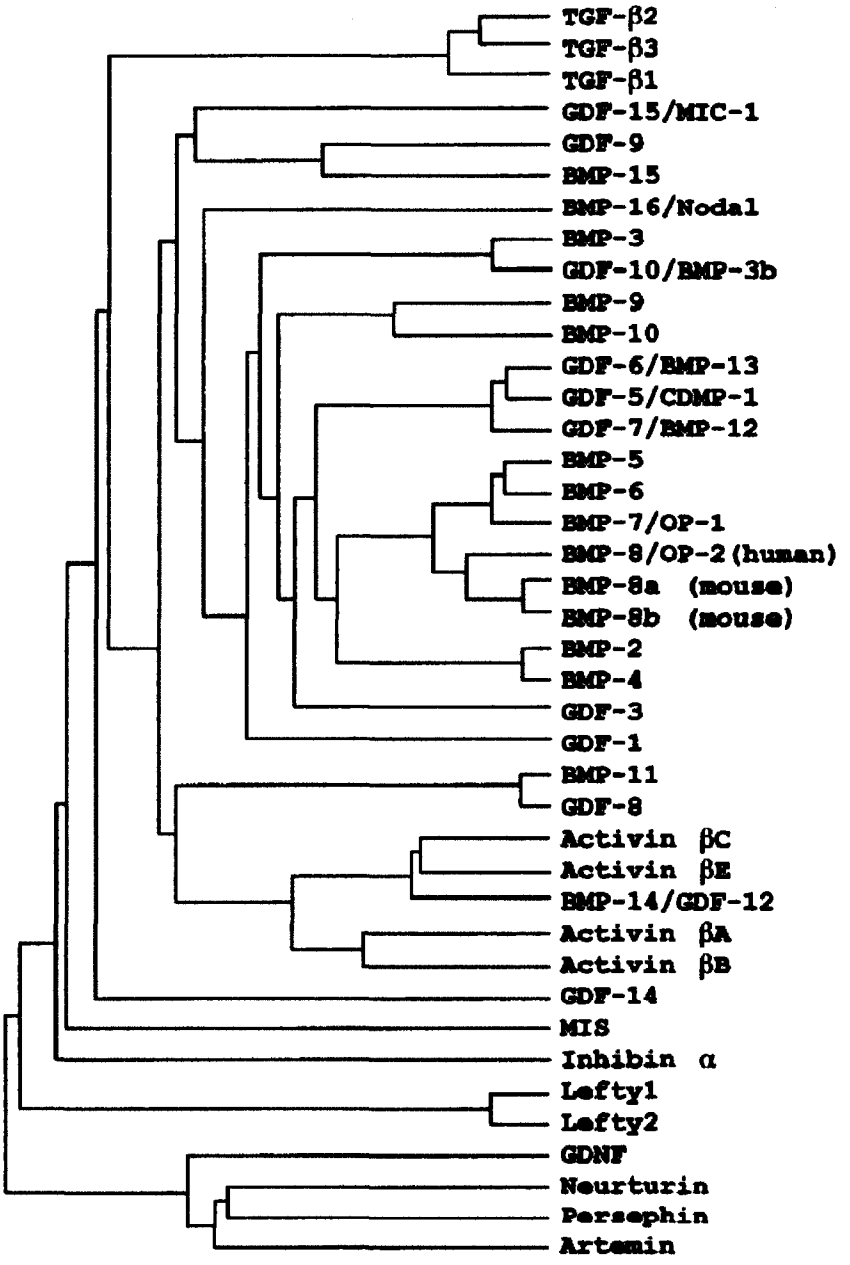

Figure 2. Different Subgroups of TGFβ Super-family: This figure was reprinted with permission of The Endocrine Society (Copyright 2002, The Endocrine Society). It is

important to note the relation different subgroups of BMPs have in the family tree above as we will later see that BMPs are also categorized according to their structure and functionality. The image above was compiled using amino acid sequence alignment programs such as PILEUP from the Genetics Computer Group in Madison, Wisconsin. .

Based on their amino acid structure and functionality, the BMPs can be categorized into as many as 20 subgroups. BMP-2 and BMP-4 have their own category, BMPs -5, -6, and -7 are in another category, and BMPs -8A and -8B are the third important subgroup (Miyazono et al. 251-63). These subgroups have close amino acid sequences (Fig. 2). Within each subgroup, individual members have approximately 90% amino acid identity, while different subgroups have only 60% identity (Dudley and Robertson 349-62). This categorization is important when interpreting spatiotemporal expression levels of BMPs in the chick heart, and which BMP ligands may share type I receptors.

BMP receptors are divided into two classes based on their amino acid structure and function: type I and type II receptors. Type I and type II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively. These receptors have about 150 amino acids in their extracellular region. Type I receptors have a highly conserved 30 amino acid stretch that is Gly-Ser rich due to its SGSGSG sequence. This portion is known as its GS domain, located intracellularly, and gets phosphorylated by type II receptors during ligand-induced signal transduction (Chang, Brown, and Matzuk 787-823). Due to the activation of signal through the phosphorylation of type I receptors, the specificity of the intracellular signal is mainly determined by these receptors. There are three type I receptors to which

BMPs bind: Alk2, Alk3, and Alk6[6] also known as ActRIIA, BMPR-IA, and BMPR-IB respectively. Alk3 and Alk6 are structurally more similar to each other; whereas Alk2 is less similar. BMP-2 and BMP-4 preferentially bind to Alk3 and Alk6, while BMP-5, BMP-6, and BMP-7 bind Alk2. Similarly, there are three types of type II receptors that BMPs bind to: BMPR-II, ActR-II,[7] and ActR-IIB (Miyazono et al. 251-63).

SMADs are the signal transducers for the TGFβ super-family. SMADs are divided into three categories based on their function: R-SMADs, Co-SMADs, and I-SMADs. R-SMADs are pathway specific signal transducing SMADs which get phosphorylated in order to associate with Co-SMADs. These positive BMP mediators are SMAD1, SMAD5, and SMAD8. SMAD4 binds to all pathway-specific R-SMADs to create the R-SMAD/Co-SMAD complex which translocates to the nucleus. Finally, the third type of SMADs is inhibitory and includes SMAD6 and SMAD7 which having an antagonizing influence on R-SMADs (Chang, Brown, and Matzuk 787-823; Miyazono et al. 251-63; Yamada et al. 48-61). SMAD1 and SMAD5 are efficiently activated by BMP-6 and BMP-7, whereas all other BMP related R-SMADs are activated by BMP-2 (Miyazono et al. 251-63).

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[6] Activin receptor-Like Kinase = Alk

[7] Activin type II receptor

1.3. Heart Development and BMPs

BMPs are vital regulators during cardiogenesis and early heart formation. A number of *in vitro* and genetic studies have shown that BMPs -2, -4, -5, -6, -7, -8, and -10 are involved in heart development. Mutations in BMP-2, BMP-4, Alk3, SMAD4, and SMAD5 have resulted in early mortality in many mouse models during embryogenesis. Single mutations in mice in one of the genes encoding BMPs -5, -6, or -7 typically have mild consequences in heart formation. Double mutations, however, in BMP-5/BMP-7 or BMP-6/BMP-7 can severely affect heart development (Delot 27-35). Table 1 presents a brief compilation of expression regions for various BMPs. To summarize, BMP-2 and -4 have important function throughout different development stages such that their expression pattern is seen in tissue that give rise to cardiac cushion, their components and myocardial regions (Table 1). More recent studies have shown that BMP-2 induces cell migration and periostin[8] expression during atrioventricular valvulogenesis (Inai et al. 383-96). BMP-5, -6, and -7 are also expressed widely and ubiquitously (Table 1).

These BMPs may be partially redundant in comparison to BMPs -2 and -4; since a single knockout gene allows the maintenance of heart function (Delot 27-35; Chang, Brown, and Matzuk 787-823). Finally, BMP-10 appears to be limited to the trabeculae (Chang, Brown, and Matzuk 797; Gaussin et al. 2878).

[8] Periostin is a novel factor responsible for ventricular dilation.

Table 1. Expression Regions of Various BMPs in the Heart

BMP-2	Expressed at H&H stages 4-5 in lateral mesoderm adjacent to heart forming region (1)	Chick
	H&H stages 6-9 in pharyngeal endoderm underlying cardiogenic mesoderm (1)	Chick
	H&H stages 10-11 in sino-atrial region but absent from tubular heart itself (1)	Chick
	Expressed in Myocardial layers (2)	Mouse
	Myocardium of endocardial cushion tissue-forming regions in spatiotemporally restricted manner (3)	Mouse
	Expressed in the AV/OT myocardium, but not in the endocardium or mesenchymal cells (3)	Chick
	Expressed in the atrial-ventricular (AV) canal (4)	Mouse
	In all stages, has always been expressed in muscularizing region, suggesting a role in late myocardium formation (5)	Chick
BMP-4	Myocardium of endocardial cushion tissue-forming regions in spatiotemporally restricted manner (3)	Mouse
	AV cushion (2)	Mouse
	AV cushion (4)	Mouse
	In all stages, has always been expressed in muscularizing region, suggesting a role in late myocardium formation (5)	Chick
BMP-5	Initially homogenous throughout heart (2)	Mouse
	Expressed homogenously in myocardium (4)	Mouse
	Involved in late myocardium formation (5)	Chick
BMP-6	Initially homogenous throughout heart (2)	Mouse
	Expressed homogenously in myocardium (4)	Mouse
	Mesenchymal cells (3)	Mouse
	Involved in late myocardium formation (5)	Chick
BMP-7	Initially homogenous throughout heart (2)	Mouse
	Expressed homogenously in myocardium (4)	Mouse
	Expressed at high levels ubiquitously throughout myocardium of mouse heart tube (6)	Mouse
	In all stages, expressed throughout the entire myocardium. Doesn't seem to be involved in muscularization and septation. (5)	Chick
BMP-10	Exclusively in trabeculae (2)	Mouse
	Expressed exclusively in trabeculae (4)	Mouse
References	1.(Schlange et al. 259-70) 2.(Gaussin et al. 2878-83) 3.(Nakajima et al. 119-27) 4.(Chang, Brown and Matzuk 787-823) 5.(Somi et al. 636-51) 6.(Delot 27-35)	

Somi et al. mapped the expression patterns of BMP isoforms -2, -4, -5, -6, and -7 using *in situ* hybridization techniques (636-51). Their experiments establish where expected expression domains for BMPs -2, -4, -5, -6, and -7 are present during HH stages 10, 16, 24, and 30 (Fig. 3).

The authors explain the spatial dispersion of expression as follows:

> In all stages analyzed BMP7 mRNA is expressed throughout the entire myocardium of the heart and therefore does not seem to be specifically involved in muscularization and septation. In vivo BMP2 and -4 mRNA are always present in the muscularizing regions, suggesting a role in late myocardium formation. However, in vitro BMP2 or BMP4 are not able to induce myocardium formation (van den Hoff et al., 2003), suggesting the involvement of additional factors. The fact that BMP5 mRNA is predominantly expressed in the cardiomyoctes that protrude into the BMP6 mRNA expressing cushion mesenchyme suggests that BMP5 and -6 are involved in late myocardium formation as well. (Somi et al. 650)

Thus far, the diversity of BMPs with their overlapping maps of expression suggests the utilization of BMP receptors to further complete and clarify the spatiotemporal expression domains of BMPs. Because BMP receptors are smaller in number and limited in valid heterodimeric permutations of type I and type II receptors, understanding their expression patterns during development may elucidate BMP roles in development. With their limited number, BMP receptors may interact with more than one ligand; and thus may have overlapping expression domains as well.

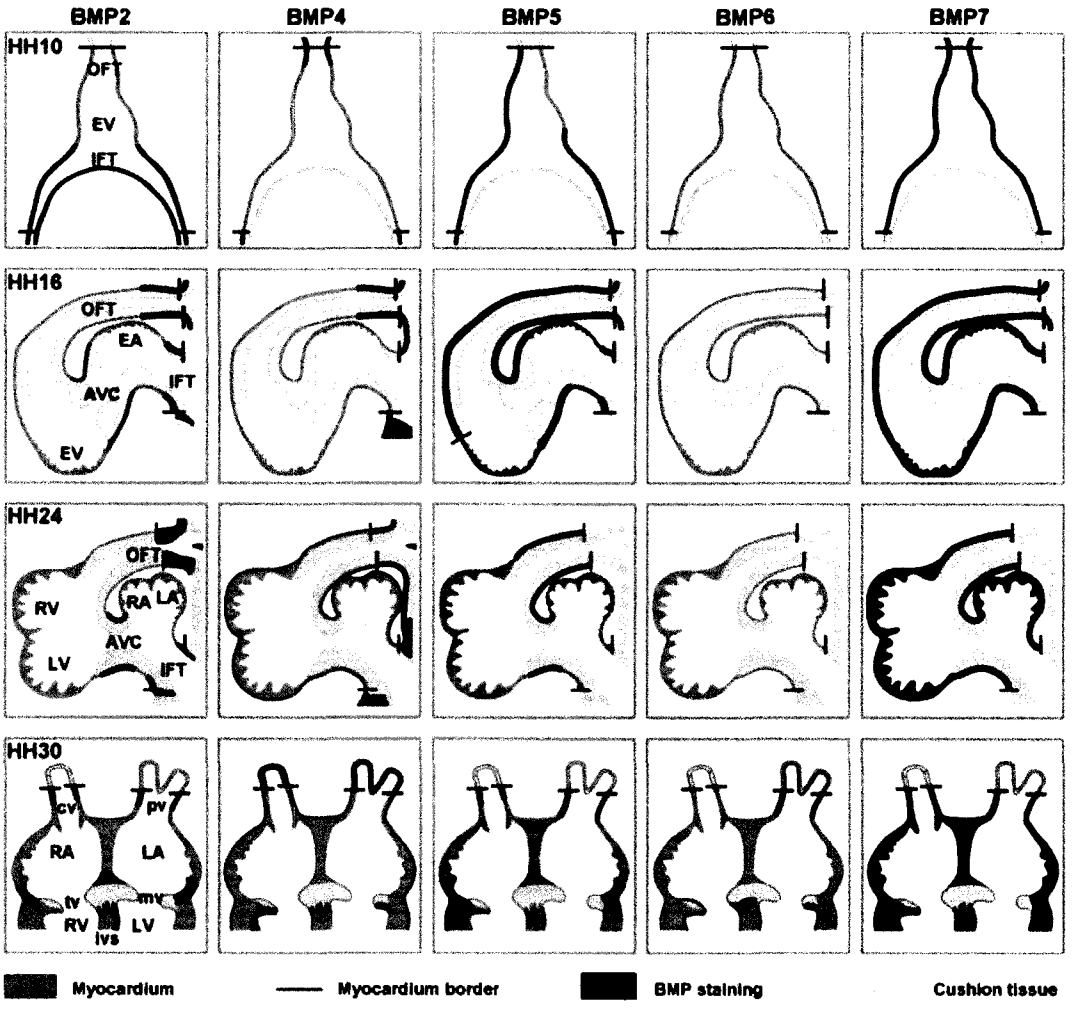

Figure 3. BMP-2, -4, -5, -6, and -7 Compared at Four Stages: This figure was reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. The black/darker areas are the regions of BMP expression. Ea, embryonic atrium; ev, embryonic ventricle; cv, caval vein; IFT, inflow tract; ivs, interventricular septum; LV, left ventricle; LA, left atrium; mv, mitral valve; OFT, outflow tract; os, outlet septum; pv, pulmonary vein; RA, right atrium; RV, right ventricle; tv, tricuspid valve (Somi et al. 2004). Note that BMP-7 is widely expressed in all stages, whereas BMP-6 is almost exclusively expressed in later stages of development. BMP-2 is mostly seen in myocardium throughout development. Chick embryonic Hamburger & Hamilton stages 10, 16, 24, and 30 are 33-38 hours, 51-56 hours, 4.5 days, and 6.5-7 days respectively.

1.4. BMP Receptors in the Heart

BMP receptors are limited in number, but there are over 15 types of known BMP ligands. It is more usual for receptors and their ligands to have a linearly corresponding relationship, such as insulin. It is likely that BMP receptors interact with more than one ligand and thus may have overlapping expression domains. Expression domains for BMP ligands in the chick heart are unique, but share regions in which they can overlap. How do corresponding BMP receptors correlate with respect to BMP expression domains?

The activation of signal through the phosphorylation of type I receptor suggests that the specificity of the intracellular signal is mainly determined by these receptors as well. Two type I receptors that have been implicated to play critical roles in cardiogenesis are Alk2 and Alk3. Alk3 is almost ubiquitously expressed (Delot 27-35; Gaussin et al. 2878-83) and knockout mice have defects in AV cushion formation, along with subsequent abnormalities in cardiac septa and valves (Chang, Brown, and Matzuk 787-823).

The Alk2 receptor, also called ActRI, SKRI, Tsk-7L, ActRIA, or ACVR1 (Macías-Silva et al. 25628-36; Chang, Brown, and Matzuk 787-823) has been reported to interact with BMPRII as its type II BMP receptor (Desgrosellier et al. 201-10) and has shown preference for BMP-7 as its ligand (Macías-Silva et al. 25628-36; Roelen et al. 541-9). In addition, Gaussin et al. have reported both BMP-6 and BMP-7 as the preferred ligands for Alk2 (2878-83).

The expression and role of Alk2 in the developing heart using genetic models or *in vitro* experiments has been well studied. Antibody against chick Alk2 reduced epithelial-to-mesenchymal transformation (EMT) in the atrioventricular cushions *in vitro* (Delot 27-

35). Targeted ectopic expression of dominant negative Alk2 has been seen to affect heart looping (Shi et al. 226-37). Chang et al. reported that "Mouse embryos...homozygous for an Alk2 null mutation are arrested at early gastrulation with abnormal visceral endoderm morphology and severe disruption of mesoderm formation..." (793-6).

Desgrosellier et al. concluded that Alk2 and SMAD6 regulate EMT during cardiac valve formation using *in vitro* and *in vivo* experiments (201-10). They have shown that in chicken embryos of HH stages 14-19, Alk2 mRNA is abundant in the OFT, ventricle, and AV cushion using *in situ* hybridization techniques:

> Expression was particularly abundant in endothelial cells comprising the endocardium of the heart. Expression of ALK2 was abundant in the OFT, ventricle, and AV cushion with a decrease in expression observed at the border between the AV cushion and the atria and between the OFT and the beginning of the aortic sac. Notably, little expression of ALK2 mRNA was observed in endothelial cells outside of the heart such as in the dorsal aorta. (203)

Wang et al. concluded that Alk2 expression in endothelial cells plays a critical role in the early phases of endocardial cushion formation during cardiac morphogenesis in the mouse heart (299-310). Others have reported that Alk2 can mediate AV cushion transformation in chicken embryos (Lai et al. 1-11). Radioactive *in situ* hybridization on mice E11.5 showed Alk2 expression by cardiac neural crest cells (Kaartinen et al. 3481-90).

1.5. Why Study the Chick Heart?

Historically, chick embryology dates back to 460-377 B.C. when Hippocrates used hens as incubators and cracked an egg open each day to check for change in development. William Harvery, 17th century physician and anatomist based some of his discoveries of heart formation and morphology changes on the chick's development *in ovo* (Bellairs and Osmond xxiii).

There are many reasons that the chick is ideal to use for investigating heart formation and differentiation. The chick has a four chambered heart similar to mammals and has a short period of heart development of only about 4 days. Fertile eggs are cheap and available almost year-round. The genetics of the chick is well known and the genome has been sequenced. The chick is the only species with a four-chambered heart with less physiological complications, such as the absence of a placenta and embryo development in the mother. For these reasons, chick is an appealing model to use for studying heart development (Bellairs and Osmond xxiii-45).

In this study, the mRNA for chick Alk2 was cloned, sequenced, and analyzed for splice sites. The Alk2 spatial expression pattern in the chick heart was determined using *in situ* hybridization during HH stage 18 (65-69 hours).

2. METHODOLOGY

2.1. Purchase, Storage, and Incubation of Eggs

Most initial work with embryos was from eggs purchased from our local Trader Joe's with an expiration date at least 3 weeks from the date of purchase to help ensure that the eggs were less aged. Older eggs would produce less reliable results. Detailed information about age of the eggs was not available; however, storage temperature for the eggs until purchase was noted to be at 7°C. Embryos were harvested and used throughout summer and fall. Unfortunately, abnormally cold temperatures in the winter had some sort of adverse affect on our egg fertility from Trader Joe's, resulting in no embryos from incubated eggs. Some embryos from Cal Cruz Hatchery in Santa Cruz, resulted in more reliable results of fertility and incubation periods.

All eggs were incubated at 37.5°C in an incubator that held constant temperature with an internal fan to create a forced draft for uniform temperature within the incubator as well. A reservoir of water at the bottom of the incubator provided a humid environment for the eggs. A motor helped turn the eggs 90 degrees about every hour.

2.2. Harvesting, Fixation, and Dehydration

After purchase, eggs were incubated at 37.5°C until desired stage of embryo was achieved (Fig. 4).[9] After appropriate incubation times, each embryo was aseptically isolated, the embryo's chorioallantoic membrane was removed in sterile PBS, and the amniotic sac was dissected away. Embryos were then fixed in 4% paraformaldehyde (PFA) for 4 hours at 4°C and then dehydrated in a methanol gradient (10%, 30%, 50%, 70%, and 90% for 10 minutes each) and stored in 100% methanol at 4°C until used for *in situ* hybridization. Embryos were roughly staged with respect to their morphology, gross anatomical features, and heart shape by two independent evaluators using a dissecting scope (8X) according to Hamburger & Hamilton.

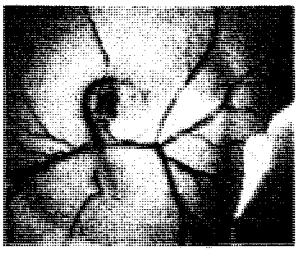

Figure 4. A 65-Hour Embryo prior to Isolation: Note that the heart is looped and numerous blood vessels have been established including the right and left omphalomesenteric arteries and the anterior and posterior omphalomesenteric veins.

[9] All embryos that were used for RNA isolation were 65-75 hours old. Embryos that were used for whole mount *in situ* hybridization were 65-69 hours. All figures reflect individual stages. Finally, embryos used for creating a serial map of sections of the chick heart were 72-80 hours old.

2.3. Whole Mount *In Situ* Hybridization

In situ hybridization was performed using the protocol of Dr. Maya Purisai (personal communication) with some modifications and changes to adapt to our tissue type. The modified protocol consists of rehydration, washes, prehybridization, hybridization, washes, block, antibody incubation, washes, and finally color reagent. The detailed protocol is included in Appendix A.

After dehydration, whole embryos were rehydrated in a methanol gradient (90%, 70%, 50%, 30%, 10% for 10 minutes each) to prepare tissue for *in situ* hybridization. Subsequently, embryos were washed in PBS-T and 2X SSC at room temperature on a shaker for 15 and 10 minutes respectively. Embryos were then placed in 48-well plates and left static in prehybridization buffer at 68°C for 1 hour. A maximum of three embryos per well was used, such that embryos were completely submerged in prehybridization buffer during incubation. Lastly, embryos were placed in hybridization buffer with probe concentration of 0.5ug/mL at 68°C for overnight static incubation. This high temperature helps ensure specificity of our probe to the target mRNA.

The following day, the embryos were removed from hybridization buffer and washed in 2X SSC and 1X SSC each twice for 15 minutes at 37°C, followed by two washes of 0.1X SSC at 37°C for 30 minutes each to reduce nonspecific reactions. These washes at high temperatures and salt concentration should allow for nonspecifically bound probes to become dislodged. All SSC washes were conducted on a shaker at 37°C, with 48-well plates covered and sealed with tape.

Next, the tissue was statically incubated in blocking solution (Appendix B) for 1 hour at room temperature, followed by 2 hours of incubation on a shaker at 37°C with anti-digoxygenin antibody. Subsequently, the tissue was washed for 10 minutes each in Buffer 1 and Buffer 2 (Appendix B) at room temperature on shaker.

The sudden increase in pH from 7.5 of Buffer 1 to 9.5 of Buffer 2 prepares the tissue for its final incubation in BCIP/NBT alkaline phosphatase substrate solution for color development. Experimental and negative controls were placed in this BCIP/NBT solution, and finally stop solution or Buffer 3 (Appendix B). Incubation times in BCIP/NBT alkaline phosphatase substrate solution were monitored carefully to ensure consistent exposure of experimental and control tissue.

Images were taken at 10X, 100X, and 200X using an Olympus CK40 compound inverted light microscope or dissecting microscope (8X). Embryos were then placed in 100% methanol, after going through a methanol gradient, so that each embryo's stage of development could be reconfirmed.

2.4. Probe Construction

Due to time constraints, the Alk2 receptor probe derived from a mouse sequence (ID # 6418954/Accession # BC058718) was ordered from Open Biosystems and sent for sequencing (Sequetech, DNA Sequencing) for clone verification purposes.[10]

Clones were provided as *E. coli* cultures in LB broth with 8% glycerol. Upon arrival, clones were allowed to grow in LB media at 37°C overnight and streaked on an LB/Amp plate until needed. One colony was picked from the plate and allowed to grow in 3ml of LB/Amp (100ug/mL of Ampicilin in LB) at 37°C at 230 rpm for 6 hours, and 150 ul of the culture was added to 100 mL of fresh LB/Amp and incubated at 37°C at 230 rpm for 16 hours. Cells were then pelleted by centrifuging for 10 minutes at 10,000 x g at 4°C. Cells were then resuspended in 3 ml of Cell Suspension Solution using the Wizard *Plus* Midipreps DNA Purification System (Promega). Plasmid DNA was extracted, purified, washed, eluted from column, and finally quantitated (Appendix C).

RNA/DNA measurements in all experiments presented in this thesis were made using a NanoDrop spectrophotometer. It is important to note that the absolute quantity of DIG-labeled probe may not be accurately measured with a NanoDrop. Since both our sense and antisense probes were measured using this spectrophotometer, however, it is sufficient to believe that our controls and experimental probes were subject to the same measurement device, and the small change in probe quantity does not adversely affect our *in situ* hybridization experiments.

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[10] IMAGE Consortium (Lennon et al., 1996) [LLNL] cDNA clones: http://image.llnl.gov

EcoR1 and Not1 restriction enzymes were used for linearizing at the 5' and 3' ends respectively to make the antisense and sense probes (Fig5; Appendix D).

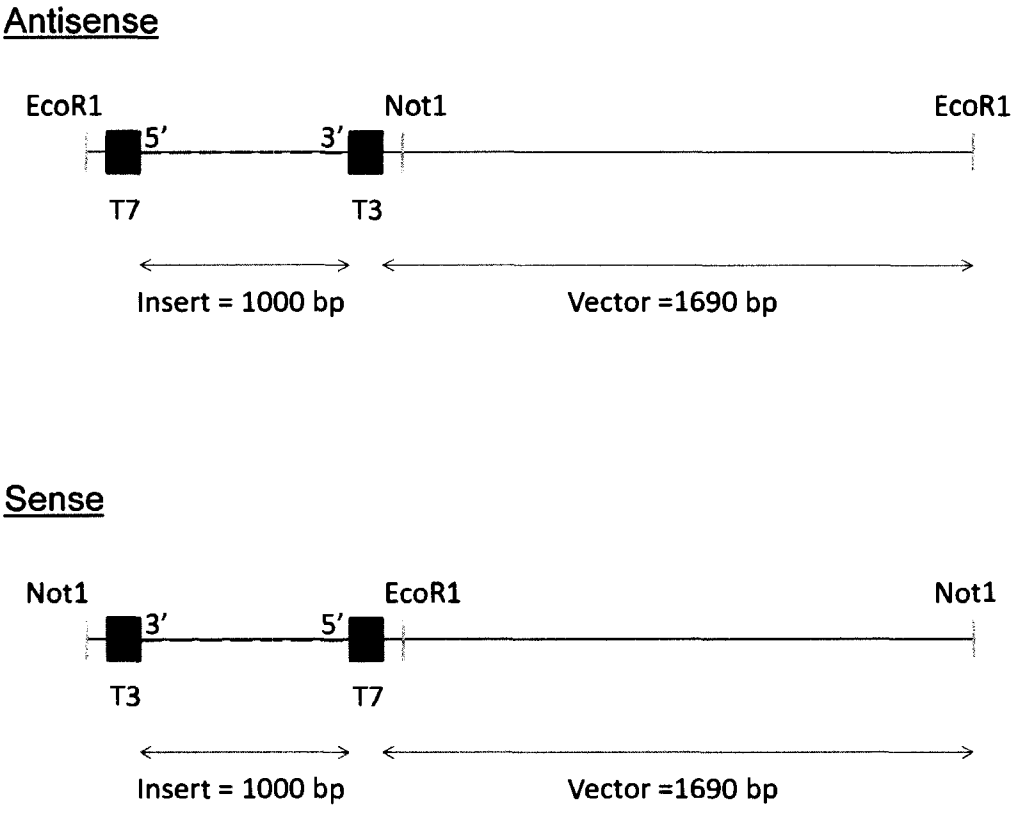

Figure 5. Template Linearization: Restriction enzyme digest using EcoR1 (top) and Not1 (bottom). These images help depict why T3 and T7 promoters were used for *in vitro* transcription of antisense and sense probes respectively. Note position of the insert with respect to its upstream and downstream promoters and restriction enzymes.

Based on the plasmid map (Fig. 5) the appropriate enzymes, buffers, BSA, water, and plasmid DNA were assembled (Appendix E), mixed, and incubated at 37°C for 3 hours, before placing on ice to inactivate the enzyme. After linearization of plasmid, DNA was

precipitated and quantitated in order to proceed to *in vitro* transcription. Briefly, the procedure involved: addition of an equal volume of isopropanol, chilling at -20°C for 16 hours, pelleting by centrifugation, washing pellet with 70% ethanol, pelleting, and finally air-drying. Pellet was resuspended in TE buffer to yield a concentration of 0.786 ug/ul of antisense and 0.24 ug/ul of sense linearized probe (Appendix E). Linearization of plasmid DNA was confirmed before proceeding to *in vitro* transcription on a 2% TAE based native agarose gel (Fig. 6). T3 and T7 promoters were selected for our antisense and sense probes for *in vitro* transcription. The appropriate enzymes, buffers, dNTPs, linearized template DNA, and water were assembled (Appendix E) and mixed. After 1 hour of incubation of the *in vitro* transcription reaction at 37°C, DNase1 was added to the reaction to remove any template DNA and allowed to incubate at 37°C for 15 minutes. The RNA is then precipitated by addition of ½ volume of lithium chloride and incubated at -20°C for 1 hour. Precipitated RNA is pelleted by centrifugation, washed with 70% ethanol, pelleted, and finally air-dried. Pellet was then resuspended in DEPC-treated water to yield 0.493 ug/ul and 0.445 ug/ul of antisense and sense Dig-UTP labeled probes respectively. Aliquots of Dig-UTP labeled probes in prehybridization buffer were made at a 0.5ug/mL and stored at -80°C until needed (hybridization buffer).

2.5. Serial Sectioning

Embryos were surgically isolated and fixed in 4% PFA at 4°C overnight. The next day, embryos were immersed in a phosphate buffered 10% sucrose solution (Appendix F) at 4°C for 1 day followed by an overnight static incubation in a phosphate buffered 30% sucrose solution (Appendix F) at 4°C. The following day, embryos were frozen on aluminum foil placed on dry ice and stored at -80°C until sectioned. The immersion in sucrose helps cryo-protect the tissue so that once frozen, the crystalline structures created by water do not damage the tissue, and it improves sectioning.

Once the chamber temperature had been optimized to -30°C, serial sections were cut at 100 um and 50 um for free floating *in situ* hybridization.
Sections were then cut at 30 um at -30°C and gently placed on the warm surface of room-temperature slides to enhance initial attachment. Different coated slides were used to test attachment including VWR superfrost slides, charged slides, poly-l-lysine coated slides, and silane coated slides.[11]

Some embryos were surgically extracted, fixed in 4% PFA, dehydrated in a methanol gradient, and embedded in paraffin to create sections as thin as 10 um. Sections were then carried through a xylene gradient with methanol, followed by a paraffin gradient with xylene, until 100% paraffin was achieved (Appendix G). Tissue was cut at 10 um using a microtome and placed on a coated slide with Haupt's adhesive.[12]

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[11] These slides are made with 3-aminopropyltriethoxysilane and are recommended for frozen sections. Manufacturer: Erie Scientific: (http://www.eriescientific.com, part number ER-298B, lot #199971).

[12] This is a glycerine adhesive. Please refer to Dr. David Bruck in the Biological Sciences Department at San Jose State University for more detailed information on this adhesive.

2.6. Alk2 mRNA Sequence

Embryos used for RNA isolation were 65-75 hours old. Embryos were collected and preserved in RNA*later* until used. RNA was extracted from the embryos by homogenization of the tissue in a chaotropic denaturation solution, deproteination by extraction with Phenol:Chloroform:IAA followed by a second extraction in acid phenol to remove any contaminating genomic DNA (ToTALLY RNA Ambion kit; cat#1910).[13] RNA was precipitated by isopropanol, concentrated, washed, dried, resuspended, and finally quantified to yield 1.4 ug/ul. The integrity and overall quality of the RNA was evaluated by gel electrophoresis. After optimizing the RT-PCR by using the QIAGEN one-step RT-PCR kit (Appendix I), a step-down PCR program was used to increase performance and specificity (Appendix I). To gel purify PCR products, 5 ul of PCR product was electrophoresed on a 0.8% agarose gel and band-isolated using the QIAGEN MiniElute Gel Extraction kit (Appendix H). Briefly, the procedure involves band excision, addition of buffer QG based on gel weight, incubation at 50°C to dissolve gel, precipitation by isopropanol, washes with buffers QG and PE, and finally collection of DNA through elution in buffer EB and centrifugation on column. DNA was then quantified to yield a concentration of 16.4 ng/ul.

The Alk2 chick cDNA was then cloned into a pCR®8/GW/TOPO vector from Invitrogen's pCR®8/GW/TOPO® TA Cloning® Kit (www.invitrogen.com). The procedure involved assembling a mixture of PCR product (4ul), Salt Solution (1ul), and

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[13] Please refer to Ambion's website for detailed protocol (www.ambion.com).

TOPO® Vector (1ul). This cloning reaction was incubated at room temperature for 25 minutes and 2 ul from the cloning reaction was added into a vial of One Shot® chemically competent *E. coli* and allowed to incubate on ice for 10 minutes. Cells were heat-shocked for 30 seconds at 42°C and placed on ice until 250 ul of S.O.C. medium (pCR®8/GW/TOPO® TA Cloning® Kit) was added to the mixture. The cells were incubated at 37°C for 1 hour with shaking. The bacterial culture was then spread on pre-warmed LB agar plates containing 100 ug/mL spectinomycin and allowed to incubate overnight at 37°C.

Thirteen colonies from the transformed cells were picked and grown in individual flasks of LB containing 100 ug/mL of spectinomycin. The QIAprep Spin Miniprep kit was used to isolate plasmid (http://www1.qiagen.com/; cat# 27104). Using their protocol, plasmid DNA was extracted, purified, washed, eluted from column, and finally quantitated. After quantification of plasmid DNA, the four best quality clones were sent for sequencing (Sequetech, DNA Sequencing) to find a distinct cDNA sequence at this developmental stage and compare it to chick genomic DNA for selection of unique splice sites.

3. RESULTS

3.1. Probe Construction

After probe linearization, a 1% agarose gel was run for confirmation (Fig. 6).

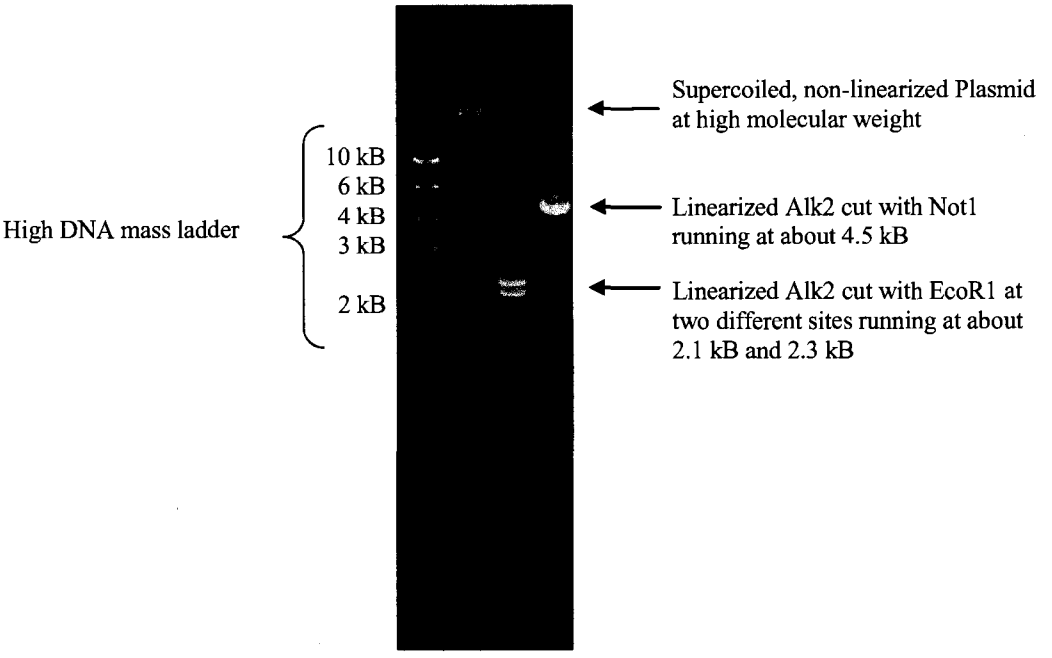


Figure 6. Gel Confirming Linearization of Plasmid DNA: Lane 1: high DNA mass ladder, lane 2: super-coiled uncut plasmid DNA, lane 3: linearized Alk2 plasmid cut with EcoR1, lane 4: linearized plasmid cut with Not1 restriction enzyme. Super-coiled plasmid DNA usually migrates faster than linearized plasmid, however, the results here have been found reproducible and consistent.

The Alk2 plasmid (from Open Biosystems) sequence was compared with the chicken Alk2 from NCBI by a BLAST and was found to be 99% similar (Fig. 8). Alignment of the chicken CDS from NCBI (presented in blue font) and the sequenced results of the Open Biosystems mouse probe (shown in orange font) show that the two sequences are almost

identical in overlapping regions (Fig. 7). The alignment indicates that the mouse probe is almost identical to the chicken cDNA, and should be suitable as a probe for *in situ* hybridization.

It is important to note that only a portion of the entire Alk2 CDS region from mouse BC058718 was incorporated into the plasmid from Open Biosystems. This segment was 1000 base pairs which should have sufficient permeability and specificity in our probing system.

In the multiple sequence alignment presented here, the stars represent identity in the two sequences. This alignment also shows potential nucleotide gaps, point mutations, deletions, or insertions.

```
NCBI_Chicken_Alk2              ATGGCTCTCCCCGTGCTGCTGCTGCTGCTGGCCCTGCCATCCCGCAGTGT
OpenBiosystems_Mouse_Alk2      ----------------------GGGATGGGGTACGCCATGCTGCAG-AC
                                                     *     **    ***** * ****

NCBI_Chicken_Alk2              GCAAGATGAGGAGCTGAAGCTAAATGAATGTGTGTGTGAAGGCATGTCAT
OpenBiosystems_Mouse_Alk2      GC---GTTACGTATCGGATCCAGA-------ATTCGTGAT----TGTCAT
                               **    * * *    * * * * *       *  ****     ******

NCBI_Chicken_Alk2              GTGGGAATGGAGACCGCTGCCAGGGCCAGCAGTGCTTCGCCTCCCTGAGC
OpenBiosystems_Mouse_Alk2      GTGGGAATGGAGACCGCTGCCAGGGCCAGCAGTGCTTCGCCTCCCTGAGC
                               **************************************************

NCBI_Chicken_Alk2              ATTAACGATGGTGCTAAGGTTTACCAGAAAGGCTGCTTCCAAGTCTATGA
OpenBiosystems_Mouse_Alk2      ATTAGCGATGGTGCTAAGGTTTACCAGAAAGGCTGCTTCCAAGTCTATGA
                               **** ***********************************************

NCBI_Chicken_Alk2              ACAAGGGAAAATGACGTGCAAAACTCCGCCATCTCCTGACCAAGCTGTGG
OpenBiosystems_Mouse_Alk2      ACAAGGGAAAATGACGTGCAAAACTCCGCCATCTCCTGACCAAGCTGTGG
                               **************************************************

NCBI_Chicken_Alk2              AGTGCTGCCAAGGATACCTTTGCAACATGAATATCACTGCGAAGTTGCCC
OpenBiosystems_Mouse_Alk2      AGTGCTGCCAAGGATACCTTTGCAACATGAATATCACTGTGAAGTTGCCC
                               ************************************ **********

NCBI_Chicken_Alk2              TCTTCTAAAGGGCAAACCCTGCAAGGGGAAGCTGCAGGTTACAGCATGGA
OpenBiosystems_Mouse_Alk2      TCTTCTAAAGGGCAAACCCTGCAAGGGGAAGCTGCAGGTTACAGCATGGA
                               **************************************************

NCBI_Chicken_Alk2              AACACTAATCATCGTTATACTGGCTCCTGTAGTAGTGCTGGTAATTTTCT
OpenBiosystems_Mouse_Alk2      AACACTAATCATCGTTATACTGGCTCCTGTAGTAGTGCTGGTAATTTTCT
                               **************************************************

NCBI_Chicken_Alk2              CTGTGGTAGCTGTGCTCATCATCCGGAGGATACAGAAGAACCACATGGAG
OpenBiosystems_Mouse_Alk2      CTGTGGTAGCTGTGCTCATCATCCGGAGGATACAGAAGAACCACATGGAG
                               **************************************************

NCBI_Chicken_Alk2              AGACTCAATTCTAGAGATGCAGAATATGGCACAATTGAGGGACTCATTGC
OpenBiosystems_Mouse_Alk2      AGACTCAATTCTAGAGATGCAGAATATGGCACAATTGAGGGACTCATTGC
                               **************************************************

NCBI_Chicken_Alk2              TTCAAATGTTGGAGACAGTACATTGGCAGATTTATTGGACCATTCCTGCA
OpenBiosystems_Mouse_Alk2      TTCAAATGTTGGAGACAGTACATTGGCAGATTTATTGGACCATTCCTGCA
                               **************************************************

NCBI_Chicken_Alk2              CATCTGGAAGTGGTTCTGGACTTCCATTCTTGGTGCAAAGAACAGTGGCT
OpenBiosystems_Mouse_Alk2      CATCTGGAAGTGGTTCTGGACTTCCATTCTTGGTGCAAAGAACAGTGGCT
                               **************************************************

NCBI_Chicken_Alk2              CGCCAGATCACGCTTGTGGAGTGTGTAGGAAAGGGCCGTTATGGAGAAGT
OpenBiosystems_Mouse_Alk2      CGCCAGATCACGCTTGTGGAGTGTGTAGGAAAGGGCCGTTATGGAGAAGT
                               **************************************************

NCBI_Chicken_Alk2              CTGGAGGGGTCAGTGGCAAGGAGAGAATGTTGCTGTGAAGATCTTCTCTT
OpenBiosystems_Mouse_Alk2      CTGGAGGGGTCAGTGGCAAGGAGAGAATGTTGCTGTGAAGATCTTCTCTT
                               **************************************************

NCBI_Chicken_Alk2              CTAGGGATGAAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTG
OpenBiosystems_Mouse_Alk2      CTAGGGATGAAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTG
                               **************************************************

NCBI_Chicken_Alk2              TTGCTGCGGCATGAAAACATTTTAGGTTTTATTGCATCCGATATGACTTC
OpenBiosystems_Mouse_Alk2      TTGCTGCGGCATGAAAACACTTTAGGTTTTATTGCATCCGATATGACTTC
                               ****************** ******************************
```

```
NCBI_Chicken_Alk2              CAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCACGAGATGG
OpenBiosystems_Mouse_Alk2      CAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCACGAGATGG
                               **************************************************

NCBI_Chicken_Alk2              GGTCTTTGTATGACTATCTGCAGCTCACCACCCTGGACACTGTCAGCTGC
OpenBiosystems_Mouse_Alk2      GGTCTTTGTATGACTATCTGCAGCTCACCACCCTGGACACTGTCAGCTGC
                               **************************************************

NCBI_Chicken_Alk2              CTGCGGATAGTACTGTCCATAGCCAGCGGCCTTGCACATTTGCACATAGA
OpenBiosystems_Mouse_Alk2      CTGCGGATAGTACTGTC-ATAGCCAGCGGCCTTGCACATTTGCACATAGA
                               ***************** ********************************

NCBI_Chicken_Alk2              GATATTTGGAACGCAGGGGAAGCCTGCCATTTCTCATCGGGACTTGAAGA
OpenBiosystems_Mouse_Alk2      GATATTTGGA-CGCAGGG--AGCCTGC-ATTTCTCATCGGGACTTGA-GA
                               ********** *******  ******* ******************** **

NCBI_Chicken_Alk2              GCAAAAATATCCTTGTTAAGAAAAATGGACAGTGCTGCATAGCAGACTTA
OpenBiosystems_Mouse_Alk2      GCAAAA-TATCTTT------AAGAAATGACAGTGCTGCATAGCAGACTAG
                               ****** **** **       ** **  ********************

NCBI_Chicken_Alk2              GGCCTCGCAGTCATGCACTCCCAAAGCACGAACCAGTTGGATGTGGGGAA
OpenBiosystems_Mouse_Alk2      C-----------------------------------------------------

NCBI_Chicken_Alk2              CAACCCCCGAGTGGGCACCAAACGCTACATGGCTCCGGAGGTCTTGGACG
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              AAACCATCCAGGCAGACTGCTTCGACTCCTACAAGAGGGTCGATATCTGG
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              GCCTTCGGGCTGGTCCTGTGGGAGGTAGCTAGGCGCATGGTTAGCAATGG
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              TATTGTAGAAGACTATAAACCACCATTTTATGACCTGGTTCCAAATGATC
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              CCAGTTTTGAAGACATGAGGAAAGTGGTCTGTGTAGATCAGCAAAGGCCA
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              AACATTCCCAACAGGTGGTTCTCAGACCCTACATTAACATCTCTTGCCAA
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              GTTGATGAAAGAATGCTGGTATCAGAATCCATCAGCAAGACTAACAGCCC
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

NCBI_Chicken_Alk2              TGCGAATCAAAAAGACTTTGACCAAAATTGATAATTCCTTAGATAAACTG
OpenBiosystems_Mouse_Alk2      ----------------------------------------------------

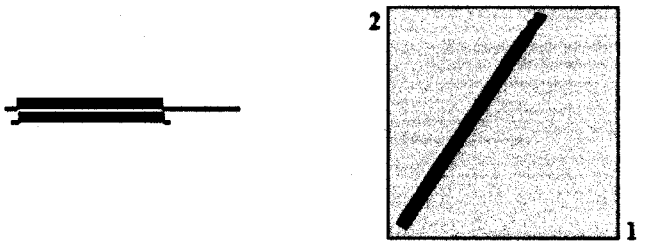
NCBI_Chicken_Alk2              AAGGCTGACTGTTGA
OpenBiosystems_Mouse_Alk2      ---------------
```

Figure 7. Multiple Sequence Alignment of Alk2 in Chick and Mouse: CLUSTAL W (1.81) of chicken Alk2 from NCBI with mouse Alk2 from Open Biosystems using an alignment program (http://align.genome.jp/).

The chicken CDS and sequenced mouse Open Biosystems probe are 99% similar (NCBI BLAST).[14]

Sequence 1: lcl|1
Length = 1515 (1 .. 1515)

Sequence 2: lcl|65536
Length = 1000 (1 .. 1000)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
Score = 1654 bits (860),  Expect = 0.0
Identities = 903/912 (99%), Gaps = 6/912 (0%)
Strand=Plus/Plus
```

Figure 8. Comparison of Chick and Mouse Alk2 Using BLAST: Comparison of chicken Alk2 cDNA from NCBI and mouse Alk2 cDNA from Open Biosystems. The BLAST result indicates 99% identity between the mouse and chicken Alk2 cDNA sequences. This confirms that the mouse probe from Open Biosystems is a reasonable substitute for the chick Alk2.

[14] NCBI BLAST software can be found at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi.

3.2. Serial Sectioning

Frozen sections were cut in a cold cryostat at 100um and 50 um thicknesses for free floating *in situ* hybridization. Due to the watery nature of tissue, however, these sections were all torn during the first initial washes before *in situ* hybridization. The 30 um sections that were similarly made and attached to VWR superfrost slides, charged slides, poly-l-lysine coated slides, and silane coated slides were ineffective as well. All sections were detached from the coated slide during the initial wash steps before *in situ* hybridization.

Paraffin embedded embryos were also inadequate as after hybridization at high temperatures such as 68°C. Since all these sections detached from the slides, whole mount *in situ* hybridization was pursued, and the remaining unused sectioned embryos were used to create a reference map for the differentiating heart, resulting in 45 sagittal sections that are nearly consecutive (Fig. 9).

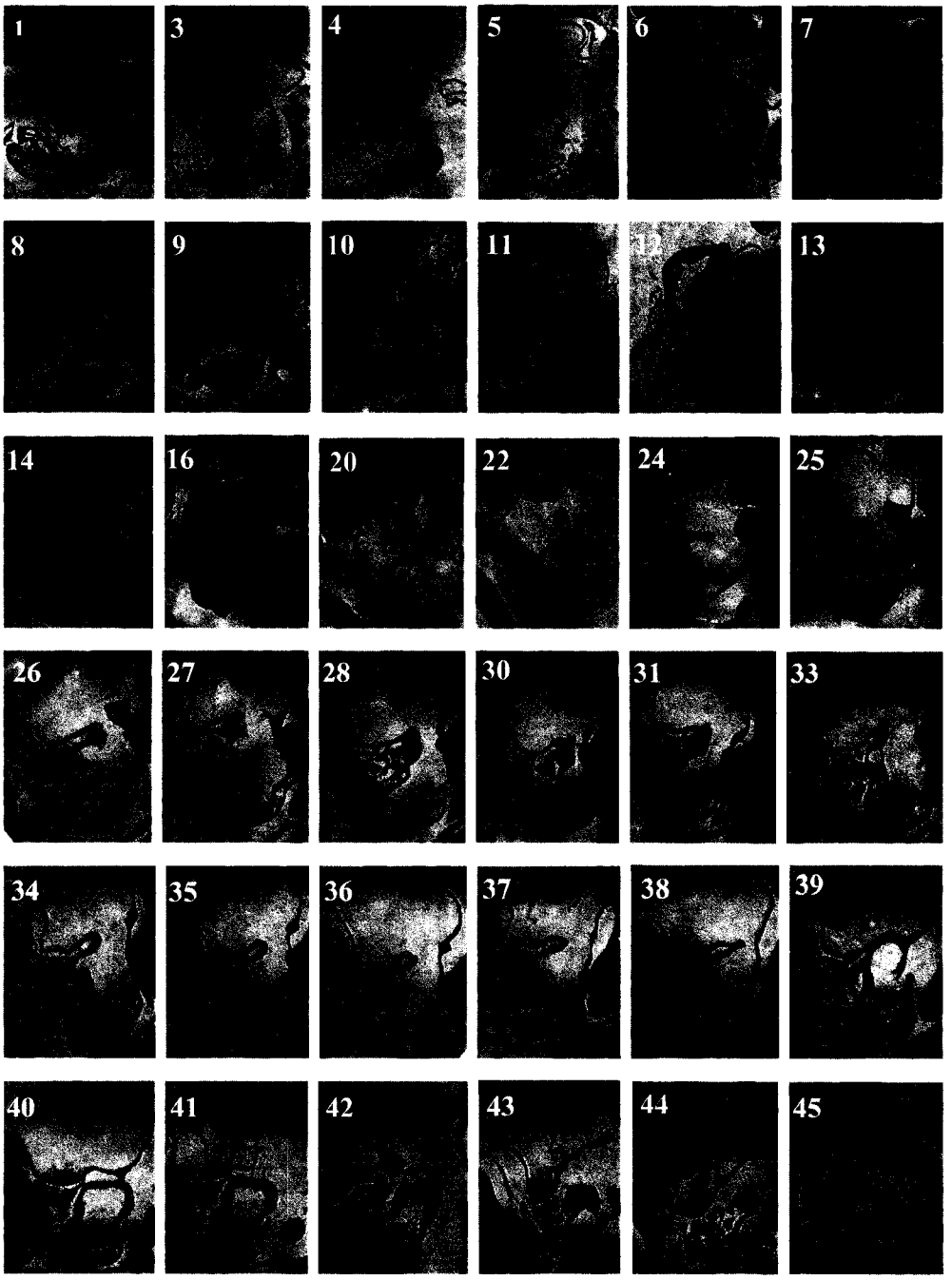


Figure 9. Serial Sections of the Chick Heart: Taken from a 72-80-hour embryo as a reference map for the differentiating heart. Sections were cut 30 um in thickness, stained with cresyl violet, and imaged with 10X dissecting scope. Reference numbers show section ordering as some sections were lost due to detachment during staining.

3.3. Whole Mount *In Situ* Hybridization

Whole mount *in situ* hybridization was conducted using the techniques described in Methods. Tissue was probed with DIG-labeled sense or antisense Alk2 receptor RNA (Fig. 10 and Fig. 11). No hybridization occurred in control tissue probed with sense RNA (Fig. 10C, 10D, 11I, and 11J). In addition, the specificity of the anti-DIG antibody was confirmed using hybridizations lacking probe (Fig. 10A and Fig. 10B).

Antisense probes produce and intense signal that indicates the presence of Alk2 receptor mRNA in heart tissue (Fig. 10E-10H and Fig. 11K-11P).

Note that in all antisense embryos (Fig. 10E-10H and Fig. 11K-11P) the hybridization seems to be distinctly occurring in the developing interventricular septa and/or in the membraneous septa of the outflow tracks.

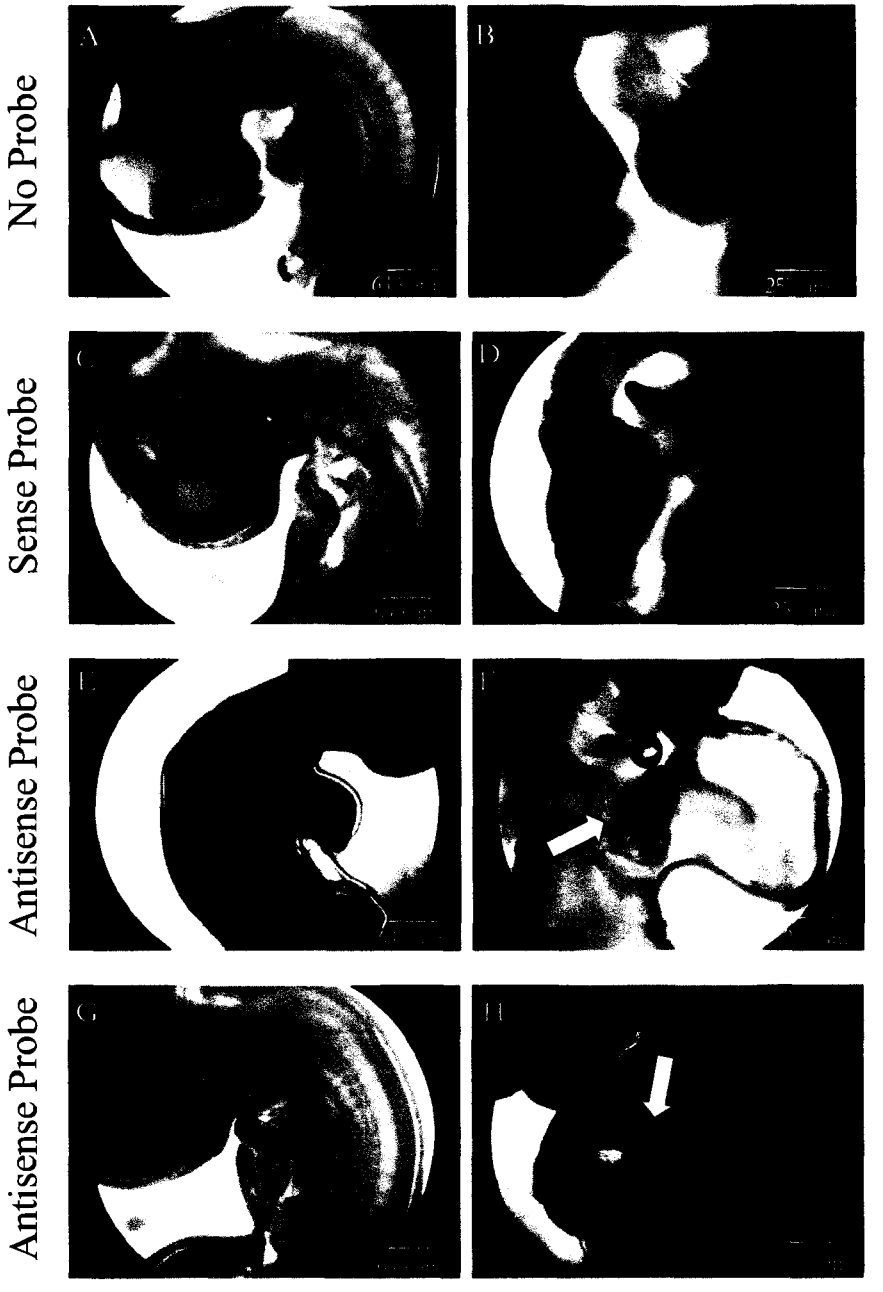


Figure 10. Whole Mount *In Situ* Hybridization on Chick Embryos (Part 1): Panels A, B, C, and D are negative controls showing the lack of non-specific hybridization of the anti-DIG antibody and the specific binding of the Alk2 receptor probe to the mRNA and not the genomic DNA. Panels E, F, G, and H are examples of specific hybridization of the Alk2 receptor probe to its mRNA complement in regions such as the outflow tract and atrium. Note that these embryos are at H&H stage 18.

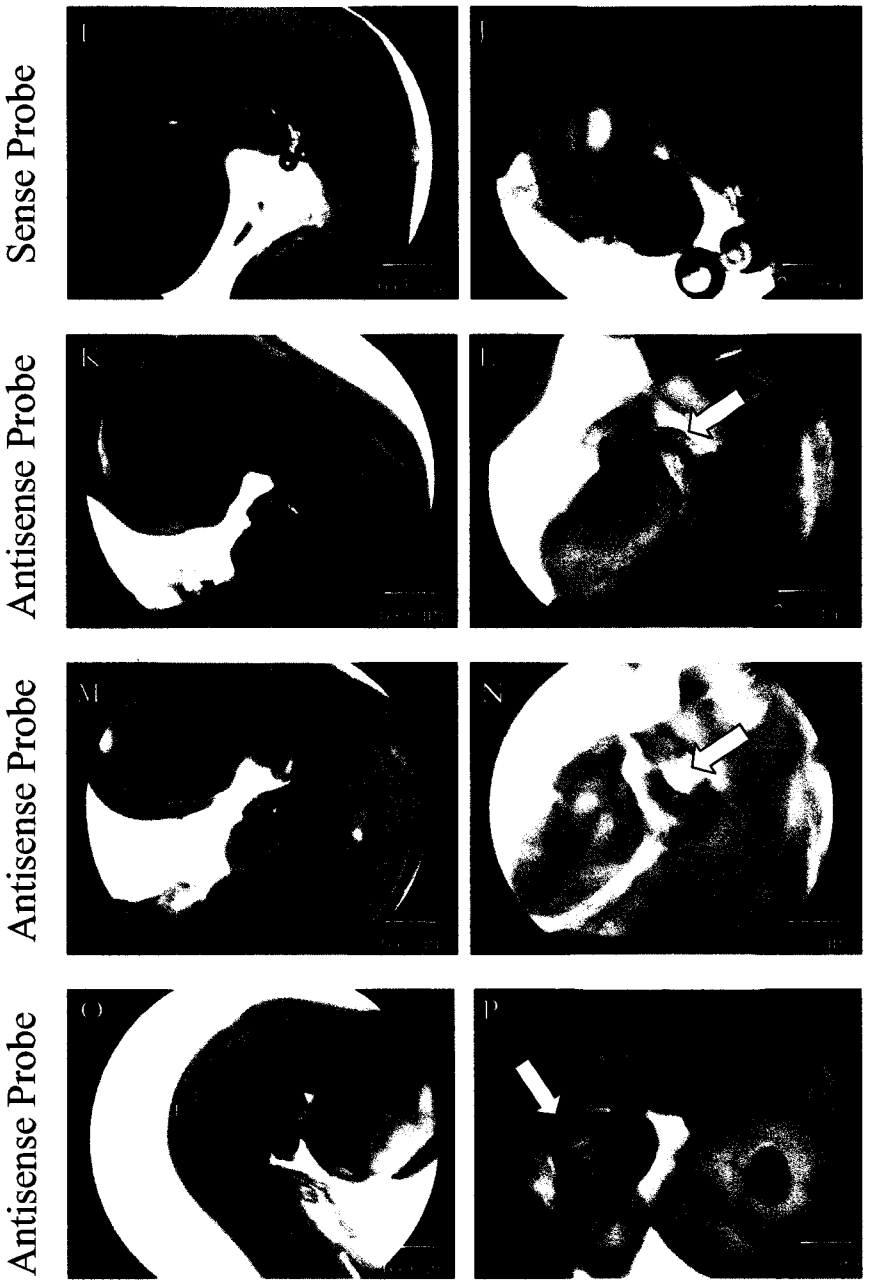


Figure 11. Whole Mount *In Situ* Hybridization on Chick Embryos (Part 2): Panels I and J depict another embryo probed with sense RNA as a negative control. Similar to figures 10C and 10D, no hybridization is seen in any of the heart regions; indicating that the Alk2 receptor probe is hybridizing to the mRNA in the target tissue. Panels K, L, M, N, O, and P depict embryos that were probed with the antisense Alk2 receptor DIG-labeled RNA. As shown by the white arrows, panels L and N show hybridization in regions such as the

outflow tract and atrium. Panel P depicts hybridization to ventricular regions. There also seems to be some signal depicted in the eye in panel P. This phenomenon was also seen in other embryos, such that 20% of all antisense embryos had some sort of signal in their eye. Note that these embryos are at H&H stage 18. Appendix A was followed for *in situ* hybridization protocol.

3.4. Alk2 mRNA Sequence

The image below is a 1% native agarose gel to confirm RNA quality before RT-PCR. It is important to note that the 28S/18S bands are too broad to rule out degradation.[15]

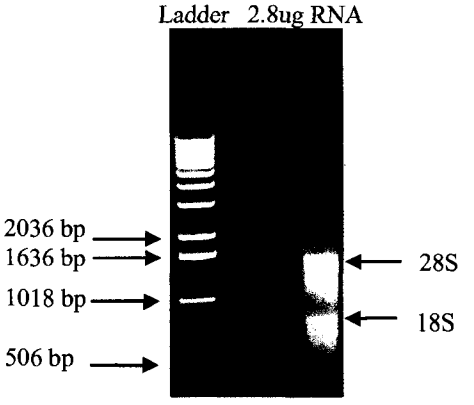


Figure 12. Native Agarose Gel on Chick Total RNA: A 1% native agarose gel was used to assess quality and location of 18S/28S RNA bands. For better depiction, a formaldehyde gel should be employed as a native gel cannot clearly depict the true molecular weight of an RNA structure due to its double stranded secondary structure. The apparent molecular weight of the 28S and 18S bands are 1.5 kB and 700 bp respectively. This is a good correlation to what Kim et al. have stated.[16]

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[15] Due to time constraints, the Open Biosystems mouse Alk2 cDNA was utilized as the probe for *in situ* hybridization. Once the chick RNA was isolated, we decided to examine the uniqueness of the cDNA sequence.

[16] This article (Kim et al. 1473-80) shows that for chicken, the 28S and 18S bands are at 2.6 kB and 1.8 kB respectively. These numbers can be correlated to the apparent molecular weight of the 28S/18S bands (Figure 12). The displacement in these bands could be due to the migration differences in a denaturing formaldehyde gel to a native agarose gel.

Optimized RT-PCR amplified 3 bands on gel electrophoresis (Fig. 13). The circled band was extracted and gel purified.

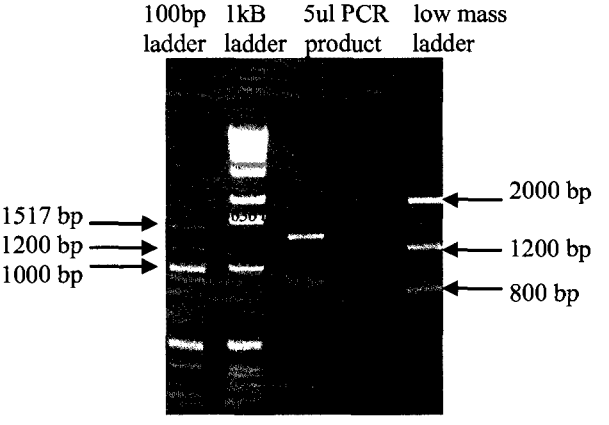

Figure 13. Native Agarose Gel on Alk2 PCR Product: The 0.8% agarose gel with corresponding standards indicates a 1361bp band in the PCR product lane at the expected length for excision.

The resulting band was cloned into the TOPO vector and sent for sequence analysis (Appendix J). The resulting sequence was aligned[17] with chicken CDS sequence from NCBI.[18] Alignment results indicate that other than small anomalies such as two bases (which are likely due to sequencing errors) the two sequences are identical (Fig. 14). Since our cDNA sequence matched exactly with that of NCBI, it is not a novel cDNA isoform; and therefore it's unlikely that we can find novel splice sites.

[17] http://align.genome.jp/

[18] NM_204560

```
NCBI_Chicken_Alk2_CDS           ATGGCTCTCCCCGTGCTGCTGCTGCTGGCCCTGCCATCCCGCAGTGT
RT-PCR_Sequenced_Chicken_Alk2   ------------------------------------------------

NCBI_Chicken_Alk2_CDS           GCAAGATGAGGAGCTGAAGCTAAATGAATGTGTGTGTGAAGGCATGTCAT
RT-PCR_Sequenced_Chicken_Alk2   ------------------------------------------GTCAT
                                                                          *****

NCBI_Chicken_Alk2_CDS           GTGGGAATGGAGACCGCTGCCAGGGCCAGCAGTGCTTCGCCTCCCTGAGC
RT-PCR_Sequenced_Chicken_Alk2   GTGGGAATGGAGACCGCTGCCAGGGCCAGCAGTGCTTCGCCTCCCTGAGC
                                **************************************************

NCBI_Chicken_Alk2_CDS           ATTAACGATGGTGCTAAGGTTTACCAGAAAGGCTGCTTCCAAGTCTATGA
RT-PCR_Sequenced_Chicken_Alk2   ATTAACGATGGTGCTAAGGTTTACCAGAAAGGCTGCTTCCAAGTCTATGA
                                **************************************************

NCBI_Chicken_Alk2_CDS           ACAAGGGAAAATGACGTGCAAAACTCCGCCATCTCCTGACCAAGCTGTGG
RT-PCR_Sequenced_Chicken_Alk2   ACAAGGGAAAATGACGTGCAAAACTCCGCCATCTCCTGACCAAGCTGTGG
                                **************************************************

NCBI_Chicken_Alk2_CDS           AGTGCTGCCAAGGATACCTTTGCAACATGAATATCACTGCGAAGTTGCCC
RT-PCR_Sequenced_Chicken_Alk2   AGTGCTGCCAAGGATACCTTTGCAACATGAATATCACTGCGAAGTTGCCC
                                **************************************************

NCBI_Chicken_Alk2_CDS           TCTTCTAAAGGGCAAACCCTGCAAGGGGAAGCTGCAGGTTACAGCATGGA
RT-PCR_Sequenced_Chicken_Alk2   TCTTCTAAAGGGCAAACCCTGCAAGGGGAAGCTGCAGCTTACAGCATGGA
                                **************************************************

NCBI_Chicken_Alk2_CDS           AACACTAATCATCGTTATACTGGCTCCTGTAGTAGTGCTGGTAATTTTCT
RT-PCR_Sequenced_Chicken_Alk2   AACACTAATCATCGTTATACTGGCTCCTGTAGTAGTGCTGGTAATTTTCT
                                **************************************************

NCBI_Chicken_Alk2_CDS           CTGTGGTAGCTGTGCTCATCATCCGGAGGATACAGAAGAACCACATGGAG
RT-PCR_Sequenced_Chicken_Alk2   CTGTGGTAGCTGTGCTCATCATCCGGAGGATACAGAAGAACCACATGGAG
                                **************************************************

NCBI_Chicken_Alk2_CDS           AGACTCAATTCTAGAGATGCAGAATATGGCACAATTGAGGGACTCATTGC
RT-PCR_Sequenced_Chicken_Alk2   AGACTCAATTCTAGAGATGCAGAATATGGCACAATTGAGGGACTCATTGC
                                **************************************************

NCBI_Chicken_Alk2_CDS           TTCAAATGTTGGAGACAGTACATTGGCAGATTTATTGGACCATTCCTGCA
RT-PCR_Sequenced_Chicken_Alk2   TTCAAATGTTGGAGACAGTACATTGGCAGATTTATTGGACCATTCCTGCA
                                **************************************************

NCBI_Chicken_Alk2_CDS           CATCTGGAAGTGGTTCTGGACTTCCATTCTTGGTGCAAAGAACAGTGGCT
RT-PCR_Sequenced_Chicken_Alk2   CATCTGGAAGTGGTTCTGGACTTCCATTCTTGGTGCAAAGAACAGTGGCT
                                **************************************************

NCBI_Chicken_Alk2_CDS           CGCCAGATCACGCTTGTGGAGTGTGTAGGAAAGGGCCGTTATGGAGAAGT
RT-PCR_Sequenced_Chicken_Alk2   CGCCAGATCACGCTTGTGGAGTGTGTAGGAAAGGGCCGTTATGGAGAAGT
                                **************************************************

NCBI_Chicken_Alk2_CDS           CTGGAGGGGTCAGTGGCAAGGAGAGAATGTTGCTGTGAAGATCTTCTCTT
RT-PCR_Sequenced_Chicken_Alk2   CTGGAGGGGTCAGTGGCAAGGAGAGAATGTTGCTGTGAAGATCTTCTCTT
                                **************************************************

NCBI_Chicken_Alk2_CDS           CTAGGGATGAAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTG
RT-PCR_Sequenced_Chicken_Alk2   CTAGGGATGAAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTG
                                **************************************************

NCBI_Chicken_Alk2_CDS           TTGCTGCGGCATGAAAACATTTTAGGTTTTATTGCATCCGATATGACTTC
RT-PCR_Sequenced_Chicken_Alk2   TTGCTGCGGCATGAAAACATTTTAGGTTTTATTGCATCCGATATGACTTC
                                **************************************************
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```
NCBI_Chicken_Alk2_CDS           CAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCACGAGATGG
RT-PCR_Sequenced_Chicken_Alk2   CAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCAG-AGATGG
                                ****************************************  ******

NCBI_Chicken_Alk2_CDS           GGTCTTTGTATGACTATCTGCAGCTCACCACCCTGGACACTGTCAGCTGC
RT-PCR_Sequenced_Chicken_Alk2   GGTCTTTGTATGACTATCTGCAGCTCACCACCCTGGACACTGTCAGCTGC
                                **************************************************

NCBI_Chicken_Alk2_CDS           CTGCGGATAGTACTGTCCATAGCCAGCGGCCTTGCACATTTGCACATAGA
RT-PCR_Sequenced_Chicken_Alk2   CTGCGGATAGTACTGTCCATAGCCAGCGGCCTTGCACATTTGCACATAGA
                                **************************************************

NCBI_Chicken_Alk2_CDS           GATATTTGGAACGCAGGGGAAGCCTGCCATTTCTCATCGGGACTTGAAGA
RT-PCR_Sequenced_Chicken_Alk2   GATATTTGGAACGCAGGGGAAGCCTGCCATTTCTCATCGGGACTTGAAGA
                                **************************************************

NCBI_Chicken_Alk2_CDS           GCAAAAATATCCTTGTTAAGAAAAATGGACAGTGCTGCATAGCAGACTTA
RT-PCR_Sequenced_Chicken_Alk2   GCAAAAATATCCTTGTTAAGAAAAATGGACAGTGCTGCATAGCAGACTTA
                                **************************************************

NCBI_Chicken_Alk2_CDS           GGCCTCGCAGTCATGCACTCCCAAAGCACGAACCAGTTGGATGTGGGGAA
RT-PCR_Sequenced_Chicken_Alk2   GGCCTCGCAGTCATGCACTCCCAAAGCACGAACCAGTTGGATGTGGGGAA
                                **************************************************

NCBI_Chicken_Alk2_CDS           CAACCCCCGAGTGGGCACCAAACGCTACATGGCTCCGGAGGTCTTGGACG
RT-PCR_Sequenced_Chicken_Alk2   CAACCCCCGAGTGGGCACCAAACGCTACATGGCTCCGGAGGTCTTGGACG
                                **************************************************

NCBI_Chicken_Alk2_CDS           AAACCATCCAGGCAGACTGCTTCGACTCCTACAAGAGGGTCGATATCTGG
RT-PCR_Sequenced_Chicken_Alk2   AAACCATCCAGGCAGACTGCTTCGACTCCTACAAGAGGGTCGATATCTGG
                                **************************************************

NCBI_Chicken_Alk2_CDS           GCCTTCGGGCTGGTCCTGTGGGAGGTAGCTAGGCGCATGGTTAGCAATGG
RT-PCR_Sequenced_Chicken_Alk2   GCCTTCGGGCTGGTCCTGTGGGAGGTAGCTAGGCGCATGGTTAGCAATGG
                                **************************************************

NCBI_Chicken_Alk2_CDS           TATTGTAGAAGACTATAAACCACCATTTTATGACCTGGTTCCAAATGATC
RT-PCR_Sequenced_Chicken_Alk2   TATTGTAGAAGACTATAAACCACCATTTTATGACCTGGTTCCAAATGATC
                                **************************************************

NCBI_Chicken_Alk2_CDS           CCAGTTTTGAAGACATGAGGAAAGTGGTCTGTGTAGATCAGCAAAGGCCA
RT-PCR_Sequenced_Chicken_Alk2   CCAGTTTTGAAGACATGAGGAAAGTGGTCTGTGTAGATCAGCAAAGGCCA
                                **************************************************

NCBI_Chicken_Alk2_CDS           AACATTCCCAACAGGTGGTTCTCAGACCCTACATTAACATCTCTTGCCAA
RT-PCR_Sequenced_Chicken_Alk2   AACATTCCCAACAGGTGGTTCTCAGACCCTACATTAACATCTCTTGCCAA
                                **************************************************

NCBI_Chicken_Alk2_CDS           GTTGATGAAAGAATGCTGGTATCAGAATCCATCAGCAAGACTAACAGCCC
RT-PCR_Sequenced_Chicken_Alk2   GTTGATGAAAGAATGCTGGTATCAGAATCCATCAGCAAGACTAACAGCCC
                                **************************************************

NCBI_Chicken_Alk2_CDS           TGCGAATCAAAAAGACTTTGACCAAAATTGATAATTCCTTAGATAAACTG
RT-PCR_Sequenced_Chicken_Alk2   TGCGAA----------------------------------------------
                                ******

NCBI_Chicken_Alk2_CDS           AAGGCTGACTGTTGA
RT-PCR_Sequenced_Chicken_Alk2   ---------------
```

Figure 14. Multiple Sequence Alignment of Alk2 Sequences: CLUSTAL W (1.81) of chicken Alk2 from NCBI with Alk2 cDNA from stage 18 embryos using an alignment program (http://align.genome.jp).

4. DISCUSSION

Since the ability of BMP to transmit an intracellular signal ultimately occurs through the phosphorylation of type I receptors, the specificity of the intracellular signal is mainly determined by these receptors. Alk2 is a type I receptor that has been suggested to be present in the OFT, ventricles, and AV cushion (Desgrosellier 201-10). In spite of the many problems associated with whole mount early stage embryo *in situ* hybridization (the intrinsic fragility of the embryo, the difficulty in removing closely associated membranes, optimization of the hybridization conditions, non-specific entrapment of the probe and/or antibody conjugates in tissue mass, the optimization of treatments that increase tissue permeability while still preserving tissue morphology etc), we were able to detect consistent signal in the outflow tract and regions of the atria and ventricles.

We are struck, however, by the apparent discrepancies between the BMP expression domain models proposed by Somi et al. and Alk2 expression domains we identified here (648). At embryonic stage HH16, for example, the expression model proposed by Somi et al. indicates that BMP5 and 7 are expressed essentially throughout the entire myocardium (Fig. 3). The embryos we examined in the stage HH16-18 range displayed a more restricted expression domain for Alk2. BMP7 is widely believed to be a ligand that preferentially binds to Alk2 (Macías-Silva et al. 25628-36; Roelen et al. 541-9). Why then is the entire heart bathed in this BMP while so little of the heart able to respond to it? This difference in expression domains presents a number of interesting questions. Are there other Alk2 isoforms expressed throughout the heart that our probe did not detect? Those different Alk2 isoforms might then confer BMP responsiveness for those regions of the

heart outside of the Alk2 expression domain we identified. Are other non-Alk2 BMP receptors expressed in those BMP domains that might confer responsiveness? Is it possible that large sections of the heart do not express BMP receptors and really are unresponsive to these BMPs, despite producing them? Are the broad expression domains presented by Somi et al. misleading due to non-specific hybridization and/or probe entrapment? Are the more restrictive expression domains we have found for Alk2 themselves artifactually small due to limitations in probe accessibility during hybridization and/or the use of overly stringent hybridization conditions?

Isoforms of Alk2 (and possibly other BMP receptors) might be present and abundant enough to amplify out from total chick heart mRNA using our primers and RT-PCR protocol. While we were successful in amplifying and cloning one Alk2 cDNA, it turned out to be the same isoform in the NCBI database. This does not rule out, however, the possibility that other isoforms exist. We noted 3 different amplified products present which could be different isoforms of Alk2 (Fig. 13, lane 3). Due to its size, and higher abundance, we only cloned the one PCR product (Fig. 13). Although it is likely that these extra bands may be reaction by-products, due to the RNA's secondary structure when the polymerase reached regions of non-linearity, their importance was not evaluated. Undescribed isoforms could be elucidated any number of ways, including modifications to our primer design, changing the RT-PCR cycling conditions, and screening Northern blots of chick heart mRNA under conditions of low stringency.

In addition, there is a discrepancy in the literature regarding which BMPs interact with Alk2. Some have found that BMPs -2, -4, and -7 (Kishigami and Mishina 265-78) are the

interacting ligands, while others have reported BMPs -6 and -7 (Gaussin et al. 2878-83). Some references have referred to BMP-7 as the only Alk2 ligand (Macías-Silva et al. 25628-36; Roelen et al. 541-9). Therefore, our results may be interpreted differently, based on which BMPs actually interacts with Alk2. For example, Somi et al. found (Fig. 3) that during HH16, BMPs -5 and -7 are widely expressed, while, BMPs -2 and -4 are only expressed in small regions of the outflow tract and AV cushion (only BMP-2). BMP-6 expression is not present during this stage of development (648). If Alk2 is indeed the receptor for BMPs -2 and -4, then based on our results, why is its expression seen more abundantly (atrium, ventricle, and outflow tract) than its corresponding ligands? Similarly, if BMPs -6 or -7 are so widely expressed, then why are its receptors so restricted?

Finding reliable expression domains is experimentally difficult to address. In order to provide convincing evidence that any signal is in fact specific one must establish a large group of experimental replicates and, ideally, hybridize using a family of target-specific probes of various lengths. The latter approach would help define both the maximum probe length capable of ensuring probe permeability while still maintaining adequate signal strength, and the minimum probe length required to maintain signal specificity. Examination of the probe sequence through bioinformatics programs will ensure that cross-hybridization to mRNAs of other family members does not occur. Whole mount *in situ* hybridization has limitations. The presence of heavy cartilage, abundant extracellular matrix or thick basement membranes, may limit the ability of the hybridization probe (or subsequent antibodies) to gain access to the target mRNAs. A number of techniques can be used to increase permeability such as incubation times, proteinase K digestion, and smaller

probe sizes. The use of cross-linking fixatives such as PFA, methanol dehydration steps, and incubation at high temperatures with 50% formamide solutions may have the ability to damage the tissue or render at least some fraction of the target mRNA molecules unavailable for hybridization (via degradation, aggregation and/or complex formation with proteins or other macromolecules). These conditions may then produce some reduction in signal strength. In order to determine the extent of signal reduction, one can surgically isolate embryos and proceed to *in situ* hybridization on the same day, so as to remove the dehydration steps. This task will be labor intensive, but may yield informative results.

Quantitative RT-PCR could be used to determine the presence and relative abundance of Alk2 mRNA in various regions of the heart tube and compared to *in situ* data. Interpreting staining patterns was sometimes difficult due to tissue damage during embryo manipulation. Enhancing signal strength a variety of modifications can be made to our current protocol. Tissue digestion with proteinase K will increase permeability of probe, but also make tissue more fragile. Increasing the probe concentration from 0.5 ug/mL to 1 or 2 ug/mL and overnight incubation of the color reaction will increase signal strength, but may cause over-staining and non-specific reactions.

Probe size must also be carefully considered. Longer probes provide increased specificity, but have more permeability problems. Our probe from Open Biosystems was 1000 base pairs compared to published methods that recommend a range of 300 to 750 base pairs for *in situ* hybridization probes. It is plausible that a smaller probe would more easily hybridize to less abundant message, and this can be tested by hydrolyzing probe into smaller fragments. Increasing hybridization temperature can also help increase specificity

of signal, and decrease nonspecific hybridization. Moorman et al. found that increasing hybridization temperature also helps increase permeability of tissue (1-8). Some protocols call for more washes at higher temperatures post-hybridization to decrease non-specificity.

Using sections instead of whole embryos will inherently help the signal quality, as sections will not entrap probe or antibody as membranous tissue might. Radioactive *in situ* hybridization allows for more specific signal, as radioactive probes are directly responsible for signal, but increases protocol complexity.

We also tried sectioning embryos after whole mount *in situ* hybridization; so that we could interpret the signal from two different viewpoints. After whole mount *in situ* hybridization, tissue was dehydrated and paraffin embedded as outlined in Appendix G. Upon sectioning with microtome, however, we found that the tissue shattered and did not remain intact. This could be due to residual formamide still trapped in the tissue from the prehybridization and hybridization steps.

The native agarose gel (Fig. 12) used to estimate RNA quality showed some RNA degradation due indicated by the breadth of the 28S and 18S bands. The quality of this RNA that was used in a one-step RT-PCR reaction, may imply the need for RT-PCR optimization. There was discrepancy of 2 base pairs between the chick cDNA sequence that we extracted from RT-PCR and sent for sequencing and NCBI's chick CDS. This discrepancy may be from small base-pair sequencing errors.

Finally, one of the most challenging tasks was finding optimum attachment conditions such that the watery tissue could be sectioned and completely attached to its microscope slide. We found that VWR superfrost slides, charged slides, poly-l-lysine coated slides,

and silane coated slides would not sufficiently attach cryo-protected, frozen, fixed tissue that were cut at 30, 50, or 100 um thicknesses. Even paraffin embedded sections detach at high incubation temperatures during the initial steps of *in situ* hybridization. Fortunately, the tissue remains adhered to microscope slides long enough to stain them with cresyl violet and allowed us to generate a small atlas of 45 sagittal sections from the developing chick heart (72-80 hours). Despite our lab having a rather extensive collection of chick developmental texts and papers, it is extremely difficult to assign a specific developmental stage to any whole mount unstained embryo, as evidenced by the numerous and obvious anatomical discrepancies present throughout these developmental textbooks and the literature at large. Even upon staining, various texts and papers ascribe a different developmental stage to embryos that appear identical, and the same developmental stage to embryos that exhibit subtle but definable and important anatomical differences. Construction of a developmental atlas for the chick using both whole mount and sectioned embryos at a variety of planes for each "HH" stage will provide useful data for developmental biologists.

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APPENDIX A

In Situ Hybridization Protocol

Day 1: Fixation and Dehydration

1. Fix embryos in 4% PFA in PBS for 4 hours in 4°C in liquid scintillation vials with tightly fitting screw caps.
2. Dehydrate embryos in methanol gradient: 10%, 30%, 50%, 70%, 90%, and 100% for 10 minutes each at RT on shaker. A maximum of 10 embryos per vial and 10 mL of methanol gradient per vial can be used. Methanol gradient is made with ddH2O. Solutions can gently be decanted from one gradient to the next such that embryos are not lost or damaged during this process. Embryos can be stored in 100% methanol in 4°C, or you can proceed to the next step. It is best to leave the embryos in fresh 100% MeOH if you will be storing them.

Day 2: Rehydration and Hybridization

1. Rehydrate embryos in methanol gradient: 90%, 70%, 50%, 30%, and 10% for 10 minutes each at RT on shaker. Similar methodology as dehydration step.
2. Wash in **PBS-T** in liquid scintillation vials for 15 minutes at RT on shaker. Use about 15 mL of PBS-T per vial.
3. Gently transfer embryos to 48-well plate using a sterile spatula. Embryos will remain in plates throughout protocol and will be moved from well to well with a soft sable brush in each step. A maximum of 3 embryos per well should be used. Wash in **2X SSC** for 10 min at RT on shaker.
4. Statically incubate for 1 hour in **pre-hybridization** buffer at 68°C.
5. Statically incubate in **hybridization** buffer (pre-hybridization buffer containing DIG-labeled RNA probe at 0.5 ug/mL concentration) at 68°C overnight. The user must use his/her discretion for the adequate amount of hybridization buffer per well such that all embryos are submerged in buffer, but excessive buffer is not wasted (as it is costly). Seal the plate with tape and adjust humidity of chamber by leaving a beaker of water in the hybridization oven.

Day 3: Washes, Labeling and Color

As noted before, embryos are transferred from well to well with a soft sable brush in each step. On step 7, 8, and 9 a separate brush must be used as this brush will be in contact with the NBT/BCIP substrate solution and must not be used for other steps of this protocol.

1. Wash 2 x 15 minutes at 37°C with **2 X SSC** on shaker.
2. Wash 2 x 15 minutes at 37°C with **1 X SSC** on shaker.
3. Wash 2 x 30 minutes at 37°C with **0.1 X SSC** on shaker. These incubation times are a good chance to prepare the Block and Antibody Solution. Do not add the antibody until ready for step 5.
4. Statically incubate in **Blocking Solution**[19] for 1 hour at RT. (At this time, make fresh Buffer 2 & Color Solution.)[16]
5. Incubate in **Antibody Solution** in humid shaking chamber for 2 hours at 37°C.
6. Wash for 10 minutes at RT with **Buffer # 1** on shaker.
7. Wash for 10 minutes at RT with **Buffer # 2** on shaker.
8. Incubate tissue with **NBT/BCIP substrate solution** until you see signal. At this point, cover 48-well plate with aluminum foil and intermittently watch for signal development. Use a dissecting scope as needed.
9. Stop color reaction with **Buffer # 3**.

[19] See Appendix B for how to make Block, Antibody Solution, Buffer 1, Buffer 2, Buffer 3, and Color Solution.

APPENDIX B

Formulation of Buffers and Chemicals

PBS-T
1 X PBS and 0.1% Tween 20

Pre-Hybridization buffer

Formamide	2.5	3	5	7.5	10	12.5	17.5
20X SSC	1	1.2	2	3	4	5	7
50% Dextran Sulfate	1	1.2	2	3	4	5	7
50X Denhardt's	0.1	0.12	0.2	0.3	0.4	0.5	0.7
Salmon DNA	0.25	0.3	0.5	0.75	1	1.25	1.75
Yeast tRNA	0.125	0.15	0.25	0.375	0.513	0.65	0.9
Total Volume **(mL)**	4.975	5.97	9.95	14.925	19.913	24.9	34.85

Hybridization buffer
0.5 ug/mL of DIG labeled RNA probe in pre-hybridization buffer.

0.1X SSC, 1X SSC, 2X SSC
All made from manufactured 20X SSC and DEPC treated water.

Block
Scale to need:

Buffer #1	3 mL
10% Triton X	30 ul
Normal Sheep Serum	60 ul

Antibody
Scale to need:

Buffer #1	2 mL
10% Triton X	20 ul
Normal Sheep Serum	20 ul
Anti-Dig Antibody	10 ul

Buffer # 1: (100mM Tris-HCl; 150mM NaCl; pH 7.5)

Tris-HCl	7.875 g
NaCl	4.375 g
DEPC H_2O	500 mL

Filter the above mix.

Buffer # 2: (100mM Tris-HCl; 100mM NaCl; 50mM $MgCl_2$; pH 9.5)
ALWAYS MAKE FRESH

Tris-HCl	0.790 g
NaCl	0.292 g
$MgCl_2$	0.508 g
DEPC H_2O	50 mL

Filter the above mix.

Buffer # 3: (10mM Tris-HCl; 1mM EDTA; pH 8.0)

Tris-HCl	0.79 g
EDTA	0.184 g
DEPC H_2O	500 mL

Filter the above mix.

Color Solution
*Add the following to freshly made Buffer #2.

NBT	22.5 ul
BCIP	17.5 ul
Levamisole	1.2 mg
Buffer #2	5 mL

APPENDIX C

Promega Wizard® *Plus* Midipreps DNA Purification System

Instructions for Use of Products A7640, A7651 AND A7701, Protocol

Preparation of Cleared Lysate

1. Pellet cells at 10,000xg for 10 minutes at 4° C.
2. Suspend pellet in 3 ml Cell Resuspension Solution.
3. Add 3ml Cell Lysis Solution. Invert to mix.*
4. Add 3ml of Neutralization Solution. Invert to mix.
5. Centrifuge at 14,000 Xg for 15 minutes at 4°C. Decant supernatant containing DNA.

Plasmid Purification

6. Resuspend resin. Add 10ml resin to DNA from Step 5. Swirl to mix.
7. Attach Midicolumn to vacuum manifold Transfer resin/DNA mixture to Midicolumn. Apply vacuum, releasing when all liquid has passed through the column.*

Washing

8. Add 15 ml Column Wash Solution containing ethanol. Apply vacuum, pulling liquid through column. Release vacuum.
9. Repeat Step 8. After second wash, continue vacuum for 30 seconds after liquid has passed through the Midicolumn. **Do not dry** longer than 30 seconds.
10. Separate Midicolumn and Reservoir. Place Midicolumn in 1.5 mL microcentrifuge tube. Centrifuge at 10,000 x g for 2 minutes.

Elution

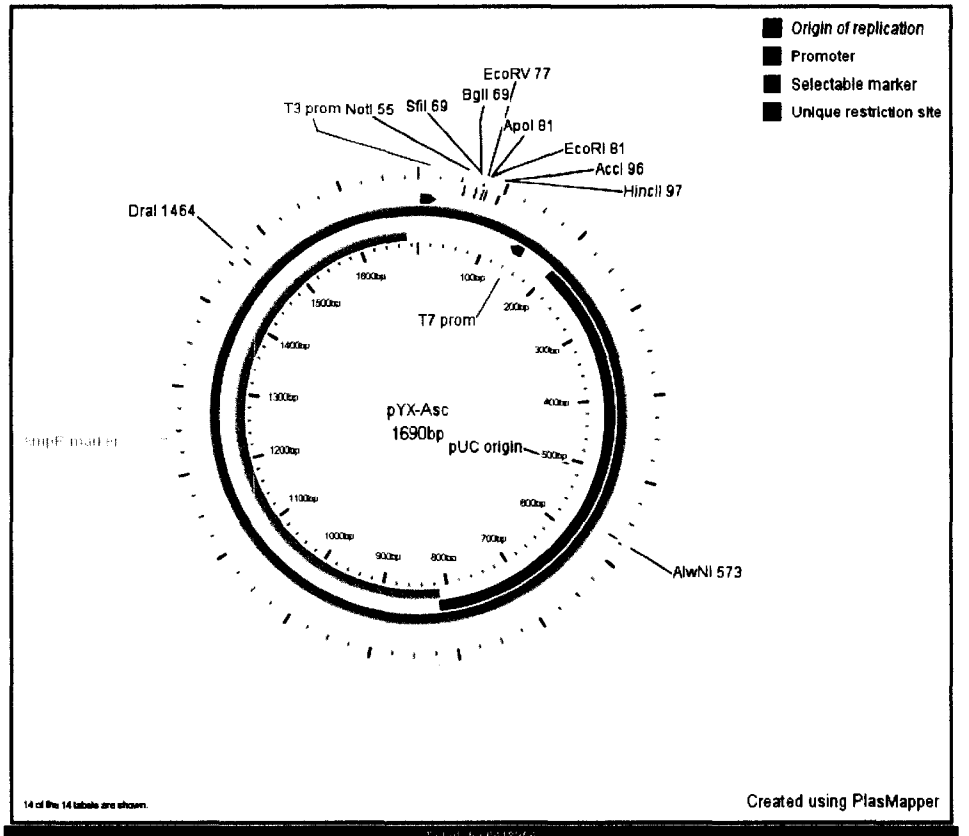
11. Place Midicolumn in new 1.5 mL tube. Add 300 ul preheated water. Wait 1 minute, then centrifuge at 10,000 x g for 20 seconds to elute DNA. For plasmids ≥ 10kb use water preheated to 70°C; for plasmids ≥ 20 kb use water preheated to 80°C.
12. Centrifuge eluate an additional 5 minutes at 10,000 x g to pellet resin fines.
13. Transfer supernatant containing DNA to a new centrifuge tube. Store at -20°C or below.

*For $endA^+$ stains and other modifications, see additional protocol information in Technical Bulletin #TB173, available upon request from Promega or online at www.promega.com

APPENDIX D

Alk2 Vector Map from Open Biosystems

This image was reproduced with permission from Open Biosystems using 6418954 or BC058718 as search query.



Cloning Details	
Product Page	(click to open)
Catalog Number	MMM1013-9498171
Clone Id	6418954
Cluster	Mm.689
Description	Mouse MGC Verified FL cDNA (IRAV+IRAW)
Detailed Description	NIH_BMAP_EW0
Accessions	BC058718,BC058718.1,CO041650,CO041650.1
Host Strain	DH10B (phage-resistant)
Tissue	brain/CNS
Species	Mus musculus
Location	129-a-10
3' Restriction Site	NotI
5' Restriction Site	EcoRI
Vector Name	pYX-Asc
Vector Type	Non Expression
Antibiotic Information	Ampicillin (Concentration: 100 µg/ml, Resistant Range: 50-200 µg/ml)
Sequencing Primers	T3, T7

APPENDIX E

Probe Construction

E.1. Probe Linearization and *In Vitro* Transcription

Probe Linearization

The following buffers and enzymes were obtained from Promega.

	Alk2 antisense	Alk2 sense
Deionized water	319 ul	31.7 ul
10 X buffer type	H	D
10 X buffer Vol.	40.0 ul	4.0 ul
BSA (acetylated)	4.0 ul	0.4 ul
Plasmid DNA	(0.75ug/ul) 27 ul	(0.7ug/ul) 2.9 ul
Enzyme (10u/ul)	(EcoR1) 10 ul	(Not1) 1.0 ul
Total	400 ul	40.0 ul

***In Vitro* Transcription**

The following buffers and dNTPs were obtained from Ambion's Maxiscript. Digoxigenin-labeled-UTP was purchased from Roche.

	Alk2 antisense	Alk2 sense
Nuclease-free water	107.5 ul	11.75 ul
Linearized DNA template	(0.8 ug/ul) 12.5 ul	(0.24 ug/ul) 6.25 ul
10 X Buffer for enzyme	20.0 ul	3.0 ul
10 mM ATP	10.0 ul	1.5 ul
10 mM GTP	10.0 ul	1.5 ul
10 mM CTP	10.0 ul	1.5 ul
10 mM UTP	6.0 ul	0.9 ul
Dig-labeled UTP	4.0 ul	0.6 ul
Enzyme mix	(T3) 20.0 ul	(T7) 3.0 ul
Total	200.0 ul	30.0 ul

E.2. Precipitation of Linearized DNA

1. Add equal volume of isopropanol.
2. Chill at -20°C for at least 1 hour to overnight.
3. Centrifuge at full speed for 15 minutes at 4°C.
4. Aspirate the supernatant.
5. Wash pellet with chilled 70% ethanol.
6. Re-centrifuge for at full speed for 10 minutes at 4°C.
7. Discard supernatant.
8. Air-dry the pellet for 5 minutes.
9. Re-suspend the DNA in the appropriate volume of TE buffer.
10. Quantitate DNA by spectrophotometry. (Alk2 antisense =0.786ug/ul & sense =0.24ug/ul)

APPENDIX F

Recipe for Phosphate Buffered Sucrose

Phosphate buffered solutions of 10% and 30% sucrose were used for removing water from the embryo tissue so that once frozen; water crystals do not damage the tissue. Furthermore, this process helps cryo-protect the tissue as well.

10% sucrose

Sucrose	10 g
Solution A	20.25 mL
Solution B	4.75 mL
NaCl	0.8 g
KCl	0.02 g
H_2O	to 100 mL

30% sucrose

Sucrose	30 g
Solution A	20.25 mL
Solution B	4.75 mL
NaCl	0.8 g
KCl	0.02 g
H_2O	to 100 mL

Solution A

0.4 M solution:

Sodium phosphate dibasic*$7H_2O$	107.228 g in 1L H_2O

Solution B

0.4 M solution:

Sodium phosphate monobasic monohydrate	55.2 g in 1L of H_2O

APPENDIX G

Paraffin embedding[20]

After harvesting embryos, and fixation in 4 % PFA for 4 hours at room temperature, the following protocol was followed for paraffin embedding.

1. Dehydrate embryos in methanol gradient: 25%, 50%, 75%, 85%, 95%, 100%, and 100% for 15 minutes each at RT on shaker. Embryos can be stored in 100% methanol in 4°C, or you can proceed to the next step.
2. Incubate tissue in 50% xylene/50% methanol on shaker at room temperature for 5 minutes.
3. Incubate tissue in 100% xylene on shaker at room temperature for 10 minutes.
4. Incubate tissue in 100% xylene for 5 minutes.
5. Incubate tissue in 50% paraffin/50% xylene at 60°C for 1.5 hours.
6. Incubate tissue in 75% paraffin/25% xylene at 60°C for 30 minutes.
7. Incubate tissue in 100% paraffin at 60°C for 1 hour.
8. Incubate in 100% paraffin at 60°C for 4 hours.
9. Incubate in 100% paraffin at 60°C overnight.
10. Next morning, replace paraffin and let incubate at 60°C for 10 minutes.
11. Add to molding trays and let solidification to occur.

[20] The protocol for paraffin embedding has been optimized and improved by Ricardo Leitáo until this protocol was established. Please refer to his graduate research thesis for more information on this topic.

APPENDIX H

QIAGEN MiniElute Gel Extraction Kit Protocol

1. Carefully excise bands.
2. Weigh slices. (Cannot use more than 400mg)
3. Add 3 vol. of buffer **QG** for 2% gels. (i.e. 300ul to 100mg or 6 vol. for higher % gels)
4. Incubate at 50°C for 10 minutes. (Until gel dissolves completely. The color of the solution should be yellow at this point.)
5. Add 1 gel volume isopropanol (i.e. 100 ul to 100 mg) and mix by inverting.
6. Apply sample to column. Centrifuge at max speed for 1 minute. Discard flow-through.
7. Add 500 ul of buffer **QG**. Centrifuge at max speed for 1 minute. Discard flow-through.
8. Add 750 ul of buffer **PE**.
9. Let stand for 5 minutes.
10. Centrifuge at max speed for 1 minute. Discard flow-through.
11. Centrifuge again at max speed for 1 minute.
12. Place column in new 1.5 mL tube.
13. Elute DNA by adding 10 ul of buffer **EB**.
14. Let stand for 1 minute.
15. Centrifuge at maximum speed for 1 minute.
16. Quantitate DNA by spectrophotometry.

The band excised was about 50 mg in weight. This resulted in using 150 ul of buffer QG for step 3 and 50 ul of isopropanol in step 5. Finally, for step 13, 15 ul of buffer EB was added to the column resulting in a DNA concentration of 16.4 ng/ul.

APPENDIX I

RT-PCR Reaction and Program

The following RT-PCR reaction mix was assembled on ice using QIAGEN's one-step RT-PCR kit.

	½ Volume/Rxn
RNase-free Water	11.5 ul
5X Qiagen OneStep RT-PCR Buffer	5 ul
dNTP Mix (containing 10 uM of each dNTP)	1 ul
5X Q Solution	5 ul
Alk2 forward (10 uM)	0.5 ul
Alk2 reverse (10 uM)	0.5 ul
QIAGEN OneStep RT-PCR Enzyme mix	1 ul
RNA (1.4 ug/ul)	0.5 ul
Total	25 ul

After reaction was prepared, the following step-down one-step RT-PCR program was used. Annealing temperature was based on average T_ms of primers which was an average of 56°C. A step-down annealing reaction will increase specificity during initial cycling as higher temperatures will limit nonspecificty.

Reverse Transcription	50°C	30 minutes
Initial PCR activation step	95°C	5 minutes
3-step cycle:		
Denaturation	94°C	45 seconds
Annealing	66°C to 51°C	45 seconds
Extenstion	72°C	1.5 minutes
Each cycle, the annealing temperature drops 0.5°C (ranging from 66°C to 51°C)		30 cycles
Annealing temperature at 51°C		10 cycles
Final extension	72°C	10 minutes

APPENDIX J

Raw Data from Sequetech Corporation

The insert was sequenced from both directions resulting in a forward and reverse strand (Sequetech, DNA Sequencing). In order to compare the two strands, a reverse complement was made from the reverse strand. Subsequently, M13_For was compared to the reverse complement. The green areas are plasmid DNA and the red regions are the overlapping regions between the forward and reverse. Since the insert was about 1300 base pairs, the forward and reverse bands were only able to provide half of the equation. Therefore, these two pieces were combined together to give the final sequence shown in the results section.

>#10;M13_For
CCGTGGGCATATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCC
AACTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTGTCATGTGGGAATGGAGACCGCTGCCAGGGCCAGCAGTGCTTC
GCCTCCCTGAGCATTAACGATGGTGCTAAGGTTTACCAGAAAGGCTGCTTCCAAGTCTATGAACAAGGGAAAATGACGTG
CAAAACTCCGCCATCTCCTGACCAAGCTGTGGAGTGCTGCCAAGGATACCTTTGCAACATGAATATCACTGCGAAGTTGC
CCTCTTCTAAAGGGCAAACCCTGCAAGGGGAAGCTGCAGGTTACAGCATGGAAACACTAATCATCGTTATACTGGCTCCT
GTAGTAGTGCTGGTAATTTTCTCTGTGGTAGCTGTGCTCATCATCCGGAGGATACAGAAGAACCACATGGAGAGACTCAA
TTCTAGAGATGCAGAATATGGCACAATTGAGGGACTCATTGCTTCAAATGTTGGAGACAGTACATTGGCAGATTTATTGG
ACCATTCCTGCACATCTGGAAGTGGTTCTGGACTTCCATTCTTGGTGCAAAGAACAGTGGCTCGCCAGATCACGCTTGTGG
AGTGTGTAGGAAAGGGCCGTTATGGAGAAGTCTGGAGGGGTCAGTGGCAAGGAGAGAATGTTGCTGTGAAGATCTTCTC
TTCTAGGGATGAAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTGTTGCTGCGGCATGAAAACATTTTAGGTTT
TATTGCATCCGATATGACTTCCAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCATGAGATGGGGTCTTTGTA
TGACTATCTGCAGCTCACACCCTGGACACTGTCAGCTGCCTGCGGATAGTACTGTCATAGCAGCGGCCTGCACATTT
GCACATAGAGATATTTGGACGCAGGGAAGCTTCCATTCTC

>#10;M13_Rev
TGTTCGACTATATAGGGGAATCAGCTGGATGGCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAA
TTGATGAGCAATTATTTTTTATAATGCCAACTTTGTACAAGAAAGCTGGGTCGAATTCGCCCTTTTCGCAGGGCTGTTAGT
CTTGCTGATGGATTCTGATACCAGCATTCTTTCATCAACTTGGCAAGAGATGTTAATGTAGGGTCTGAGAACCACCTGTTG
GGAATGTTTGGCCTTTGCTGATCTACACAGACCACTTTCCTCATGTCTTCAAAACTGGGATCATTTGGAACCAGGTCATAA
AATGGTGGTTTATAGTCTTCTACAATACCATTGCTAACCATGCGCCTAGCTACCTCCCACAGGACCAGCCCGAAGGCCCA
GATATCGACCCTCTTGTAGGAGTCGAAGCAGTCTGCCTGGATGGTTTCGTCCAAGACCTCCGGAGCCATGTAGCGTTTGGT
GCCCACTCGGGGGTTGTTCCCCACATCCAACTGGTTCGTGCTTTGGGAGTGCATGACTGCGAGGCCTAAGTCTGCTATGCA
GCACTGTCCATTTTTCTTAACAAGGATATTTTTGCTCTTCAAGTCCCGATGAGAAATGGCAGGCTTCCCCTGCGTTCCAAA
TATCTCTATGTGCAAATGTGCAAGGCCGCTGGCTATGGACAGTACTATCCGCAGGCAGCTGACCAGTGTCCAGGGTGGTGA
GCTGCAGATAGTCATACAAAGACCCCATCTCATGGTAGTGAGTAATTAGCCACAGCTGGGTGCTAGAGTTTCTGGAAGTC
ATATCGGATGCAATAAAACCTAAAATGTTTTCATGCCGCAGCAACACAGTGTTATACAATTCAGTTTCCCTGAACCAGGA
TTTTCATCCCTAGAAGAGAAGATCTTCACAGCACATTCTCTCTTGCCACTGACCCCCCAGACTCTCCATAACG
GCCTTTCTAACCCTCCACAAGCGTGATCTGGCGAGCACTG

>REVERSE COMPLEMENT of M13_Rev
CAGTGCTCGCCAGATCACGCTTGTGGAGGGTTAGAAAGGCCGTTATGGAGAGTCTGGGGGGTCAGTGGCAAGAGAGAAT
GTGCTGTGAAGATCTTCTCTTCTAGGGATGAAAATCCTGGTTCAGGGAAACTGAATTGTATAACACTGTGTTGCTGCGGCA
TGAAAACATTTTAGGTTTTATTGCATCCGATATGACTTCCAGAAACTCTAGCACCCAGCTGTGGCTAATTACTCACTACCA
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CAGTTGGATGTGGGGAACAACCCCCGAGTGGGCACCAAACGCTACATGGCTCCGGAGGTCTTGGACGAAACCATCCAGG
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GATGAAAGAATGCTGGTATCAGAATCCATCAGCAAGACTAACAGCCCTGCGAAAAGGGCGAATTCGACCCAGCTTTCTTG
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TATTTGCCATCCAGCTGATTCCCCTATAGTCGAACA