Analysis of six Tbx Genes in Early Stages of Mouse Development

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ANALYSIS OF SIX Tbx GENES IN EARLY STAGES OF MOUSE DEVELOPMENT

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ABSTRACT

The Tbx family of transcription factors control many early cell fate decisions in a developing embryo. Mutations in these genes can result in devastating physical abnormalities. In order to determine the function of Tbx genes in early post-implantation mammalian development, RT-PCR techniques were used to analyze the expression of six Tbx genes: Tbx1, Tbx3, Tbx4, Tbx5, Tbx19 and Tbx20 in key stages of development in the mouse embryo. We determine that Tbx1, Tbx4, and Tbx20 were shown to be expressed from LVET to Streak stages - the later stages of development examined. Tbx3 was expressed at all stages examined, and Tbx5 and Tbx19 were not detected at any of the stages examined. Further analysis will determine their role in early mouse development.
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1 - BACKGROUND

1.1 INTRODUCTION TO MOUSE EMBRYOLOGY

All living organisms have some way to perpetuate themselves forward to the next generation. The process of developing an embryo from fertilization to birth is known as embryogenesis. Through the process of embryogenesis, an embryo is brought to term. In the murine species, this process is accomplished in approximately 20 days. In the course of mouse embryogenesis, there are a few landmark events that are essential to correct development. After fertilization, the embryo travels through the maternal oviduct and implants into the uterus. After implantation, the embryo undergoes morphological changes and cell differentiation that will eventually lead to gastrulation, an event giving rise to the three germ layers responsible for forming all structures of the embryo (Beddington and Robertson, 1999). The embryo then undergoes organogenesis and growth until birth. The first week of mouse development encompassing fertilization through gastrulation is outlined below.

1.1.2 FROM ZYGOTE TO BLASTOCYST

In the first 4 days post coitum (dpc) of mouse embryogenesis, the embryo establishes itself as a multi-cellular unit. After fertilization, the ovum of the female becomes a zygote. In the oviduct of the female, this single cell begins the process of cleavage as it passes down the female reproductive tract (Figure 1.) (Kaufman, 2005).
By 3.0 dpc an 8 cell stage embryo has formed a multi-cellular unit known as a compacted morula (Figure 2. D). The cells within a morula differentiate into two different types of cells; trophectoderm (TE) – which will give rise to the trophoblast, and the extraembryonic ectoderm, and the inner cell mass (ICM) – which gives rise to the fetus, extraembryonic mesoderm, and primitive endoderm (Beddington and Robertson, 1999). This stage is called a blastocyst (Nagy et al., 2003). The blastocyst is composed of three types of cells: the TE, primitive endoderm, and epiblast (Nagy et al., 2003). The primitive endoderm will then differentiate into the parietal and visceral endoderm, while the epiblast will eventually give rise to the entire fetus (Beddington and Robertson, 1999). These embryo stages can be visualized more closely in figure 2. At about 4.0 dpc.
the embryo will enter the uterus and hatch from its outer layer – the zona pellucida. It will then attach to the uterine wall in a process known as implantation. (Nagy et al., 2003).

1.1.3 PRE-PRIMITIVE STREAK STAGES

At 5.5 dpc, the mouse embryo is morphologically arranged as shown in figure 3. From the blastocyst at implantation, the TE gives rise to both the extraembryonic ectoderm, which projects into the blastocyst cavity, and the ectoplacental cone, which extends in the opposite direction (Hedrich et al., 2004). These tissues will form most of
the fetal parts of the placenta, eventually including the chorion and the allantois (the precursor to the umbilical cord) (Hedrich et al., 2004). The chorion and the allantois form the chorioallantoic placenta where gases and metabolites are exchanged between mother and fetus (Hedrich et al., 2004).

![Figure 3. Morphological Representation of Mouse Embryo 5.5 dpc. Figure taken from Rivera-Perez et al, 2003.](image)

After implantation, the visceral endoderm of the embryo plays an important role in development (Rivera-Perez et al., 2003). Around day 5.5, embryos can be morphologically staged due to a thickening of the visceral endoderm at the distal tip of the embryo (Rivera-Perez et al., 2003). The cells present at the distal tip of the visceral endoderm are twice as big as the other visceral endoderm cells, due to the presence of a taller epithelium at this location (Rivera-Perez et al., 2003). As the day continues, these cells will be found at the anterior-proximal side of the embryo at around 5.75 dpc.
(Rivera-Perez et al, 2003). Before the work of Rivera-Perez et al in 2003, a method of embryo staging at pre-gastrulation did not exist.

1.1.4_PRIMITIVE STREAK AND GASTRULATION

The process of gastrulation gives rise to the three germ layers of the embryo: the ectoderm, endoderm, and mesoderm (Hedrich et al., 2004). These three layers will eventually form all the tissues of the fetus. This process begins with epiblast cells undergoing an epithelial to mesenchymal transition at around 6.5 dpc, allowing them to move as a new tissue layer between the visceral endoderm and the epiblast, where they differentiate into mesodermal and edodermal cells (Hedrich et al., 2004). This region is known as the primitive streak (Figure 4), which can be visualized by staining the protein Brachyury which is expressed heavily in mesodermal cells. After the formation of the primitive streak, the three germ cell layers quickly differentiate.

Figure 4. Mouse Embryo Primitive Streak Highlighted by the Expression of Brachyury. Brachyury (dark stain) is expressed in the mesendodermal cells present in the primitive streak at 6.5 dpc. Figure from Rivera-Perez et al, 2005.
1.2 T-BOX TRANSCRIPTION FACTORS

T-box or Tbx genes encode a family of related transcription factors that function in early development of both vertebrates and invertebrates and help determine cell fate and the movement of cells during embryogenesis.

1.2.1 DISCOVERY

The discovery of the Tbx transcription factors began with the discovery of the Brachyury gene, a gene expressed in the primitive streak (Figure 4) and necessary for development of the posterior mesoderm (Herrmann, 1990). In the mouse, mutants homozygous for Brachyury eventually die at embryonic day 10.5 when the improperly formed allantois fails to fuse with the chorion (Gluecksohn-Schoenheimer, 1938). With the cloning of the Brachyury gene, a series of genes were found to encode transcription factors with homologous sequences in the DNA binding domain. These homologous DNA-binding domains were termed T-domains, and the series of genes were named the T-box genes. To date 18 different mammalian T-box genes have been elucidated (Showell et al, 2004). Figure 5 shows the structure of the two DNA-binding T-domains of transcription factor Xbra, the Brachyury homolog in Xenopus.
1.2.2 DEVELOPMENTAL EXPRESSION

The T-box gene family plays a significant role in the early development of vertebrates and invertebrates. Most decisions of cell fate and the movement of cells during embryogenesis rely on the correct expression of the T-box genes. Tbx genes 2-5 have been implicated in the development of limbs (Gibson-Brown et al, 1998). Tbx2 and Tbx3 are expressed in both sets of limbs, while Tbx4 and Tbx5 are expressed exclusively in the developmental stages of the hind limbs and fore limbs, respectively (Gibson-Brown et al, 1998). Due to the exclusive presence of these genes so early in limb development, some scientists have postulated that these genes are involved in the cell fate decisions in limb development. A number of studies have proven that this is indeed the case. Wing digits can be inappropriately formed in the leg of a chick embryo when wing grafts expressing Tbx5 are inserted, and alternatively, wings can give rise to leg morphology when the Tbx4 gene is inserted (Figure 6) (Ohuchi et al, 1998; Gibson-Brown et al, 1998).

Figure 6. Tbx4 and Tbx5 During Chick Development.
Limb skeletons of 21 day old chick embryos (A) Normal chick wing skeleton morphology. (B) Graft of thigh region of chick embryo expressing Tbx4 inserted beneath the apical ridge of a chick wing – note the appearance of toe like digits. (C) Normal chick leg skeleton morphology. (D) Graft of shoulder inserted beneath the apical ridge of a chick leg – note the appearance of wing like digits (Isaac et al, 1998).
*Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20* also play a significant role in the development of the heart. Although developmental expression is sometimes overlapped, it has been shown that each gene plays an important role in cardiogenesis (Naiche *et al.*, 2005). *Tbx1* has been linked with cardiac FGF signaling and any defects in this pathway have been known to cause aortic arch artery remodeling defects (Vitelli *et al.*, 2002). In *Xenopus*, the African clawed toad, *Tbx5* expression was found to be located in the pre-cardiac mesoderm when the heart is established, as well as in the heart tube during morphogenesis (Horb and Thompsen, 1999). When the expression of *Tbx5* is repressed prior to gastrulation, the heart does not form properly (Horb and Thompsen, 1999).

Another T-box gene, *hrT*, is also involved in the growth of the heart. The *hrT* gene works to regulate the expression of the *Tbx5* gene, and in cell fate decisions within the heart, this gene is expressed in all developmental stages of the heart (Szeto *et al.*, 2002).

In the mouse, *Tbx20* is involved in the formation of the cardiogenic mesoderm at embryonic day 8.5, and the cardiac crescent and looping heart tube from embryonic day 9.5 (Kraus *et al.*, 2001). These aspects will soon form the four chambered heart (Kraus *et al.*, 2001). *Tbx20* is also involved in various aspects of the nervous system, and the formation of the chorion and the allantois, an expression that will persist in the umbilical cord (Kraus *et al.*, 2001).

Other T-box genes have also been found to be expressed in *Xenopus* embryogenesis. The T-box gene Xbra is involved in the formation of the ventral mesoderm (Cunliffe and Smith, 1994), as well as inducing endoderm formation (Horb and Thompsen, 1999), while *VegT* and *Eomesodermin* induce mesoderm markers (Horb and Thompsen, 1999).
1.2.3 T-BOX SIGNIFICANCE

Problems with the expression of T-box genes in humans can result in a number of congenital diseases. The Holt-Oram Syndrome (Figure 7B) is an autosomal dominant developmental disorder caused by a mutation in the \textit{Tbx5} gene, which occurs in approximately 1 in 100,000 people (Quan et al, 1997). This disease is characterized by cardiac problems, as well as skeletal disorders in the upper limbs – most notably on the left side (Quan et al, 1997). The severity of this disorder varies with each case, from small heart and limb defects, to crippling or life threatening problems (Quan et al, 1997).

![Figure 7. Tbx Disorders in Humans.](image)

The Ulnar-Mammary Syndrome (Figure 7A) affects limb, tooth, apocrine gland, and genital development (Bamshad et al, 1997). This syndrome is caused by a haplo-insufficiency in the \textit{Tbx3} gene (Bamshad et al, 1997). Both \textit{Tbx3} and \textit{Tbx5} genes are found on chromosome 12, hinting at a similar origin for both (Bamshad et al, 1997).
1.2.4 T-BOX GENE REGULATION

Although Tbx genes are implicated in a number of important embryogenesis events, the manner in which they function is still largely unknown. An analysis of these T-box sequences is shown in Figure 8, with conserved amino acids for all genes designated in bold – these amino acids constitute the T-box consensus sequence (Bollag et al., 1994).

Figure 8. Alignment of Various T-Box Amino Acid Sequences. Amino acids conserved throughout the T-box family are displayed in bold (Bollag et al., 1994).

A phylogenetic analysis of T-box genes (Figure 9) shows the relatedness of the Tbx genes (Naiche et al., 2005). This tree demonstrates the existence of similar Tbx ancestors in both vertebrates and invertebrates. Through the evidence gathered for the homologous DNA binding domain sequences of Tbx proteins, a common ancestral background can easily be inferred.
Another important aspect in the elucidation of the function of the Tbx genes is the identification of the genes they target. In *Xenopus*, expression the Tbx gene *Xbra* has been found to activate the embryonic fibroblast growth factor (eFGF) (Isaacs et al, 1995). Some scientists have found that *Xbra* activates eFGF which then maintains the expression of *Xbra* (Isaacs et al, 1994; Schulte-Merker and Smith, 1995). It has been determined that Tbx genes encode transcription factors that regulate most of the necessary elements of embryogenesis.
BRACHYURY

The founding member of the T-box family of transcription factors is a gene called Brachyury (T) that is involved in the formation of the posterior mesoderm. The T locus is situated on murine chromosome 17, and was first cloned in 1990 by Herrmann et al; marking the first of a series of genes known as the T-box genes (Herrmann, 1990). The phenotypes for null and heterozygous T alleles have been the primary sources for knowledge of the function of the Brachyury gene. Loss of function of the T gene results in devastating effects. Mice that are homozygous for a deletion of the T gene produce insufficient mesoderm from the primitive streak, as well as poor production of the notochord and a premature cessation of the production of somites (Clements et al, 1996). These embryos ultimately die at approximately day 10.5 as a result of an improperly formed allantois that is not able to fuse to the placenta (Clements et al, 1996). Mice that are heterozygous for the deletion are able to survive; however, these mice show phonotypical abnormalities as a result. Although able to form the posterior somites necessary for development, the axis is not completely extended, and therefore these animals have a short tail or are tailless (Figure 10 – panel A) (Clements et al, 1996).
Through *in situ* hybridization techniques completed in 1991 by Bernhard Herrmann, the expression of the *T* gene was able to be tracked throughout mouse embryogenesis. The *T* gene is expressed early in the primitive streak, around 6.5 days post coitum (dpc) and first becomes heavily expressed at day 7.3 dpc. in the head process which is connected to the precursor of the notochord (see Figure 11A) (Herrmann, 1991). At 8 dpc., the *Brachyury*, or *T* gene is highly expressed in the primitive streak, as well as cells forming the head process and the notochord precursor, while it is down regulated in the mesoderm that is migrating away from the primitive streak (see Figure 11B) (Herrmann, 1991). The expression of *Brachyury* is evident in notochord cells until approximately day 17.5.
Homozygous mutants in *Brachyury* result from *T* gene deletion. This allows an analysis of loss of function for this gene (Wilson et al, 1993). Since *Brachyury* is only expressed in the primitive streak and the notochord, the phenotypical abnormalities that are evident in the allantois, neural tube, surface ectoderm, and somites are most likely a result of downstream effects (Wilson et al, 1993). These effects could occur from an interaction with abnormal T/T mutant cells, or from an ancestral cell that failed to express *T* (Wilson et al, 1993). Heterozygous T/+ mice exhibit a tailless phenotype. The tail initially grows in a normal fashion, but around 9.75 dpc, the tail begins a process of degeneration characterized by cell death (Wilson et al, 1993). At embryonic day 14 the tail is lost (Wilson et al, 1993).
2- PROJECT PURPOSE

2.1 THE RIVERA LAB

The aim of the Rivera lab in the Department of Cell Biology, University of Massachusetts (Worcester) is to elucidate the molecular and morphogenic mechanisms of mammalian development using mice as a model system. Currently, projects within the lab explore various hypotheses surrounding gastrulation and the formation of the primitive streak. Some of these projects include exploring the movement of the anterior visceral endoderm, the role of Wnt3 in axial development, as well as the movement of anterior visceral endoderm cells in the absence of the Cripto gene.

2.2 SPECIFIC PROJECT AIM

Although the Tbx genes have been proven to be involved in crucial aspects of mammalian development, their role in early post-implantation embryos is still not fully understood. The first step in addressing this question is to determine which Tbx genes are expressed at these stages. To achieve this goal, we focused on the expression of various T-box genes that included: Tbx1, Tbx3, Tbx4, Tbx5, Tbx19, and Tbx20. These genes were chosen due to their lethality early in embryogenesis, a fact suggesting their importance at critical stages of early development.
3- METHODS

3.1 MOUSE STRAINS AND DISSECTIONS

Wild-type embryos were collected from CD-1 mice obtained from Charles River Laboratories. Dissections were performed in phosphate buffer saline and M10 media as described by Rivera-Perez et al., 2003. Dissections were performed under a Leica 1500 LCD microscope, and pictures were obtained at either 11.5X or 10.5X magnification. Abnormal embryos were discarded. Embryos were staged according to morphological landmarks described by Rivera-Perez et al., (2003) and Downs and Davis (1993). The number of embryos used for RNA extraction were as follows: Fifty-two 4.5 dpc embryos, thirty 5.5 dpc embryos, thirty 5.75 dpc embryos, thirty-seven 6.5 embryos, and seventeen 6.75 dpc embryos.

4.5 dpc embryos were extracted via uterine flushing with M10 media and a 26g needle. 5.5-6.75 dpc embryos were removed manually from the uterus. The ectoplacental cone and parietal yolk sac were removed to prevent contamination of maternal RNA.

3.2 RNA EXTRACTION AND ISOLATION

All embryo samples were placed in 1 ml of Trizol Reagent (Invitrogen) and allowed to lyse. 0.2 mL of chloroform was added to every 1 mL of Trizol solution. The tubes were shaken vigorously by hand for 15 seconds, allowed to incubate at room temperature, then centrifuged at 12,000 g for 15 minutes at 4°C in order to allow for a phase separation. The aqueous phase was then precipitated with isopropyl alcohol (0.5 mL per 1 mL of Trizol used). The samples were allowed to incubate at room temperature
for 3 minutes, and then centrifuged at 12,000 g for 15 minutes at 4°C. After this step, the RNA precipitate forms a pellet on the side of the tube.

The supernatant was then removed and the pellet was washed with 75% ethanol (1 mL per every 1 mL of Trizol used). The samples were then centrifuged at 7,500 g for 5 minutes at 4°C. The RNA pellets were then dried and dissolved in RNase free water.

### 3.3 cDNA SYNTHESIS

cDNAs were synthesized using an Invitrogen kit (Cat No. 12371-019). The reaction mixture consisted of 10 µL of RNA sample, 1 µL of 50 µM Oligo (dT), and 1.5 µL of 10 mM dNTP mix. The mixture was heated to 65°C for 5 minutes then incubated at 4°C for 1 minute. The first strand synthesis media consisted of 2 µL of 10x RT buffer, 4 µL of 25 mM MgCl₂, 2 µL of 0.1 M DTT, 1 µL of RNaseOut (40 U/µL), and 1 µL of 200U/µL SuperScript III Reverse Transcriptase. The SuperScriptase III RT was omitted in the negative control.

10 µL of cDNA synthesis media was mixed with each RNA/Primer mixture. The samples were then incubated at 50°C for 1 hour. The reaction was inactivated by heating the samples to 85°C for 5 minutes, and then chilled at 4°C. RNase H (2U) was then added to each sample, and the samples were incubated at 37°C for 20 minutes. The cDNA samples were then stored at -20°C.

### 3.4 RT-PCR ANALYSIS

Reaction mixtures for RT- PCR reactions included 2 µL of cDNA sample, 4 µL of 5 x PCR buffer, 1.6 µL of 2.5 pM dNTPs, 2 µL of each 10 pM primer pair, 0.8 µL of MgCl₂, and 0.1 µL of Taq DNA Polymerase. The PCR parameters were as follows:
denaturing at 95°C for 40 seconds, annealing for 40 seconds, and elongation at 72°C for 40 seconds. The annealing temperature for β-Tubulin, Tbx3, Tbx5, and Tbx19 was 55°C, and the annealing temperature for Brachyury, Tbx1, Tbx4, and Tbx20 was 60°C. The PCR was run for 41 cycles. The sequences of primers were the following: Brachyury, forward, 5′-AGTATGAACCTCGGATTCACATCG-3′, and reverse, 5′-GCAGATGAATTGTCCGCATAGG-3′; β-tubulin, forward, 5′-TCACT-GTGcCTGAACTTACC-3′, and reverse, 5′-GGAACATAGCCGTAACACTGC-3′ (Baharvand et al., 2007); Tbx1 forward, 5′-CAAGGCAGGC-AGACGAAT-3′, and reverse, 5′-GGCTTCTGCAGCGTCTTTG-3′; Tbx3 forward, 5′-GGATAAAACACGGATTTACTTTGG-3′, and reverse, 5′-CAGTGTGAGCTGCTTTTCT-3′; Tbx4 forward, 5′-CGGTTGCAAGCAGAGAG-3′, and reverse, 5′-GATGCCAAGGAGAGAAACC-3′, and reverse, 5′-GGTTCTGCAGCGTCTTTG-3′; Tbx5 forward, 5′-GCTGATAACAAATGGTCCGTA-3′, and reverse, 5′-AGCTTTCTATCAAATTC-3′; Tbx19 forward, 5′-GCTGCCAGCCAGCCAGGCTCTTA-3′, and reverse, 5′-GATGCCAAGGAGAGAAACC-3′, and reverse, 5′-GCTTCTATCAAATTC-3′; Tbx20 forward, 5′-GCTGCCAAGAGCCAGGCTCTTA-3′, and reverse, 5′-GAACAAGACCTCATTCCCTTC-3′.
4- RESULTS

Members of the T-box family have been shown to be very important in different developmental stages of mammals, with respect to limb, heart, and brain formation. However, despite the importance of this gene family there is still fairly little known about their role in early embryogenesis. The first step forward in elucidating this role is determining if any of these genes are expressed at early post-implantation stages. In order to address this question, mouse embryos were dissected at 4.5, 5.5, 5.75, 6.5, and 6.75 days post-coitum, and the expression of Brachyury, Tbx1, Tbx3, Tbx4, Tbx5, Tbx19, and Tbx20 was analyzed through RT-PCR.

4.1 EMBRYO DISSECTION AND STAGING

In order to receive clear results of the expression of different T-box genes, it was important that the embryos be staged in the correct manner. The light cycle for the laboratory mice is from 7:00 AM to 7:00 PM. When a mating is set, it is assumed that the mating happens around midnight, and the next day is 0.5 days post-coitum (dpc). Although this staging system is somewhat accurate, there can be some discrepancies. Variables exist that can either advance or retard the developmental process; the mating could take place before or after midnight, the litter size could be very large or very small, or the female could be older or younger. At any rate, it is important to correctly stage the embryos based on morphology and not simply on time.

The first stage examined (Figure 12) was at 4.5 dpc. These embryos are recently implanted in the uterus, and it is still possible to detach them from the uterine wall.
Embryos at this stage can be recognized by the presence of primitive endoderm (shown in pink in Figure 12).

Distinguishing the 5.5 dpc embryos (Figure 13, Panel A) from the 5.75 dpc embryos (Figure 13, Panel B) was a bit more challenging. At 12:00 AM on the fifth day post – coitum, a litter could, and most likely does, consist of both 5.5 and 5.75 dpc embryos. In order to correctly characterize these embryos at the correct stage, they need to be staged according to morphology. According to Rivera-Perez et al, 2003, embryos at 5.5 dpc have a thickening of cells at the distal tip of the visceral endoderm (DVET), while those at 5.75 dpc have a similar thickening at the lateral side of the embryos (LVET). This method of staging was used to characterize these embryos.

![Figure 12. Mouse Embryo at 4.5 dpc. After implantation, these embryos consist of three cell types: inner cell mass, primitive endoderm and trophectoderm. Green denotes the inner cell mass, grey, the trophectoderm, pink, the primitive endoderm.](image)

![Figure 13. Mouse embryos dissected at 5.5 and 5.75 dpc. Yellow denotes the visceral endoderm, blue, the epiblast, light grey, the extraembryonic ectoderm, dark grey, the ectoplacental cone. (Panel A) Embryos at 5.5 dpc can be staged by the presence of morphologically distinct cells at the distal tip of the embryo; this is known as distal visceral endoderm thickening (DVET) (Rivera-Perez et al, 2003). (Panel B) Embryos at 5.75 dpc. Cells from the distal tip are now in the prospective anterior side of the embryo, known as the lateral visceral endoderm thickening (LVET) (Rivera-Perez et al, 2003).](image)
At 6.5 – 6.75 dpc (Figure 14) a similar embryo staging problem exists, however, at this stage it is much easier to morphologically differentiate between 6.5 and 6.75 dpc. At 6.5 dpc (Figure 14, Panel A) the embryo is at a pre-streak stage (Downs and Davies, 1993). At around 6.75 dpc (Figure 14, Panel B), the streak has formed (Downs and Davies, 1993). These embryos were from all stages of streak, including very early and late streak.

![Figure 14. Mouse Embryos at Pre-Streak and Streak Stages.](image)

**Figure 14. Mouse Embryos at Pre-Streak and Streak Stages.** Yellow denotes the visceral endoderm, blue, the epiblast, light grey, the extraembryonic ectoderm, dark grey, the ectoplacental cone. The black hatched area is the primitive streak. **(Panel A)** Embryo around 6.5 dpc the proamniotic cavity has extended to the extra embryonic ectoderm, and the beginnings of the primitive streak are now evident. **(Panel B)** Embryo around 6.75 dpc. At the posterior side of the embryo, opposite the LVET, large amounts of mesoderm cells are present and the primitive streak has formed.

### 4.2 RT-PCR ANALYSIS

After each stage of embryo was recovered, an RT-PCR analysis of these stages was conducted on total RNA extracted from the embryos. In order to ensure there was enough cDNA in each sample for a primer to detect, an RT-PCR analysis was run with primers for β-Tubulin, a protein expressed in every cell (Figure 15). As can be seen from this tubulin positive control figure, cDNA is present in every sample. Lane 7 does not
contain template. In all RT-PCR gels shown in this report, lane 1 contains the ladder, lane 2 contains stage 4.5 dpc embryos, lane 3 contains DVET stage embryos (~5.5 dpc), lane 4 contains LVET stage embryos (~5.75 dpc), lane 5 contains Pre-Streak stage embryos (~6.5 dpc), lane 6 contains Primitive Streak stage embryos (~6.75 dpc), lane 7 has no template, lane 8 contains sample of adult mouse liver, lane 9 contains sample of adult mouse heart, lane 10 contains sample of adult mouse brain.

Figure 15. RT-PCR Analysis of Various Mouse Embryonic Stages for β-Tubulin as a Control. All lanes show the expression of β-Tubulin, lane 7 contains no template. Lanes 2-10 (with the exception of lane 7) contain mouse embryos at the following dpc, respectively, 4.5, 5.5, 5.75, 6.5, and 6.75, adult mouse liver, adult mouse heart, adult mouse brain.

Figure 16 shows the RT-PCR of the gene Brachyury. Brachyury is known to be expressed from DVET to Primitive Streak stages (Rivera-Perez and Magnuson, 2005).

Figure 16. RT-PCR Analysis of the Expression of Brachyury in Mouse Embryos. Brachyury is expressed at all stages post 4.5 dpc, but is not expressed in the heart, liver or brain of the adult mouse.
This serves as a positive control. The expected size of the *Brachyury* amplicon is 433 bp. As can be seen, *Brachyury* is expressed at all embryonic stages past 4.5 dpc; however, *Brachyury* is not expressed in the liver, heart, or brain of the adult mouse.

*Tbx1* (Figure 17) can be seen expressed at the latest stage of development examined (lane 6), and not in any adult organs. However, this band should be viewed with some discretion. The expected size of the *Tbx1* amplicon is 625 bp, yet this band seems to fall short of half that size.

*Tbx3* (Figure 18) is shown to be expressed at all stages of development examined.
The expected size of \( Tbx3 \) is 316 bp. In the heart and brain, \( Tbx3 \) shows a band that is much larger than the expected size.

\( Tbx4 \) (Figure 19) is shown to be expressed at 5.75 dpc (LVET) and 6.5 dpc (Pre-Streak), but not in adult tissues. The expected size for \( Tbx4 \) is 260 bp.

\( Tbx5 \) (Figure 20) is not expressed at any embryonic stages examined. However, it is clear that the primers are working because \( Tbx5 \) is found to be expressed in the liver, heart, and brain of adult tissues. The expected size of \( Tbx5 \) is 424 bp.
Tbx19 (Figure 21) is also not expressed at any embryonic stage examined, yet the primers are working as Tbx19 is shown to be expressed heart and brain tissues. The Tbx19 amplicon is expected to 283 bp. Tbx20 (Figure 22) is shown to be expressed at 5.75 dpc (LVET), 6.5 dpc (pre-streak), and 6.75 dpc (streak) (lanes 4, 5, and 6, respectively), but was not expressed in the adult liver, heart, and brain tissues. The expected size of the Tbx20 amplicon is 374 bp.
5- DISCUSSION

The current project was designed to identify members of the Tbx family that might play a role in early post-implantation stages of development. To achieve this goal, Tbx1, Tbx3, Tbx4, Tbx5, Tbx19, and Tbx20 were analyzed for their expression in 4.5 dpc, DVET, LVET, Pre-Streak, and Streak stages of mouse embryo development. From the data gathered in this project, Tbx1, Tbx4, and Tbx20 were shown to be expressed from LVET to Streak stages - the later stages of development examined. Tbx1 was expressed at only Streak stage, Tbx4 was expressed at LVET and Pre-Streak stages, and Tbx20 was expressed at LVET, Pre-Streak and Streak stages. Tbx3 was expressed at all stages examined. Tbx5 and Tbx19 were not detected at any of the stages examined. In support of these results, each primer tested showed at least one positive band at the correct size, and the β-Tubulin control provided proof that cDNA was present in every sample. These controls substantiate the data acquired.

This work also reiterates previous reports in the literature. Tbx1 was shown to be expressed at embryonic day 6.75; this corroborates the evidence presented by Hu et al. (2004) that Tbx1 is first expressed in pharyngeal mesoderm – a tissue that can only be present in post gastrulation stages. Tbx3 was interestingly expressed at all stages examined – and can even be pinpointed to an even earlier expression at 4.0 dpc through RT-PCR analysis (Bollag et al., 1994). The earliest that Tbx5 has been shown to be expressed was at embryonic day 8.0 in the heart tube formation (Bruneau et al., 1999). Although the work of Bruneau and co-workers was done through in-situ hybridization – a far less sensitive method for determining expression levels, the RT-PCR analysis supports this data. Previous data has shown that Tbx20 is expressed at embryonic day 7.5
or later according to Kraus et al., 2001, yet the data shown from the RT-PCR analysis indicates that Tbx20 is expressed as early as 5.75 dpc. It is interesting to view the very different expression patterns for these four genes, because they have all been identified as having key roles in cardiac development (Naiche et al., 2005). Tbx3 and Tbx20 have been shown to be expressed well before gastrulation stages, while Tbx5 is shown to not be present at all in early development. These results could allude to a number of different explanations, including very early cell fate decisions on the part of Tbx3 and Tbx20, or a different function early in development that Tbx5 does not share.

Tbx19 was shown to not be expressed in any of the stages examined. This data is in agreement with the results from Lamolet et al. 2001, where Tbx19 was shown to not be present until embryonic day 12.5, and only in the pituitary gland cells, as was evident through in-situ hybridization. It should be noted that as expected, Tbx19 was present in adult brain tissue, suggesting that the lack of detection was not due to a technical problem. Through RT-PCR, the expression of Tbx4 has been shown at 5.75 and 6.5 dpc, yet the work of Naiche and Papaioannou (2003) refutes the expression of Tbx4 before 7.5 dpc in the developing allantois. This discrepancy could be based on different methods of measuring expression levels, as they did not mention the method they used.

In attempting to perform the RT-PCR analysis, it was first necessary to obtain a detectable amount of RNA from the embryo samples. This initially proved to be a problem, especially in the 4.5 dpc embryos, whose mass was considerably smaller than that of the later stages. After a trial and error period, the correct amounts were determined (approximately 50 4.5 dpc embryos) as can be seen in the β-Tubulin control (Figure 15). Another problem confronted was a differing of band size, as seen in the
results section. *Tbx1*, for instance, exhibited a band at around 300 bp, when the expected size was 625 bp. Since this is a specific band that is not seen in the negative control, it cannot be due to contamination. Most likely this band is due to alternative splicing. The *Tbx1* primers were designed to span several exons, since the exons were so small. All other T-box primers used were designed to span one or two exons. It is possible that some exons were spliced out, explaining the smaller band. The heart and brain bands of *Tbx3* are also different than expected. *Tbx3* is expressed at all stages examined, at the size expected (316 bp). This is proof that the primers are working correctly. A different size band in the adult tissues could be due to a different transcript of *Tbx3* produced by alternative splicing.

In the future, these RT-PCR approaches should be repeated to confirm the results obtained. After that, it will be necessary to understand the location of the expressions of the genes present at the stages examined. Since *Tbx20* and *Tbx3* are definitively expressed in early development, these two genes should be examined more closely. Whole-mount In-situ hybridization should be performed for these two genes at the same stages described here. This would show where *Tbx3* and *Tbx20* are being expressed – either ubiquitously or restricted to a specific region. In conjunction with the results obtained from these experiments, loss or gain of function experiments should then be performed in order to understand the function of these genes during early mammalian development.
6- BIBLIOGRAPHY


