Differential Expression and Function of Tbx5 and Tbx20 in Cardiac Development*

The T-box transcription factors play critical roles in embryonic development including cell type specification, tissue patterning, and morphogenesis. Several T-box genes are expressed in the heart and are regulators of cardiac development. At the earliest stages of heart development, two of these genes, Tbx5 and Tbx20, are co-expressed in the heart-forming region but then become differentially expressed as heart morphogenesis progresses. Although Tbx5 and Tbx20 belong to the same gene family and share a highly conserved DNA-binding domain, their transcriptional activities are distinct. The C-terminal region of the Tbx5 protein is a transcriptional activator, while the C terminus of Tbx20 can repress transcription. Tbx5, but not Tbx20, activates a cardiac-specific promoter (atrial natriuretic factor (ANF)) and synergistically with other transcription factors. In contrast, Tbx20 represses ANF promoter activity and also inhibits the activation mediated by Tbx5. Of the two T-box binding consensus sequences in the promoter of ANF, only T-box binding element 1 (TBE1) is required for the synergistic activation of ANF by Tbx5 and GATA4, but TBE2 is required for repression by Tbx20. To elucidate upstream signaling pathways that regulate Tbx5 and Tbx20 expression, recombinant bone morphogenetic protein-2 was added to cardiogenic explants from chick embryos. Using real time reverse transcription-PCR, it was demonstrated that Tbx5 and ANF, is induced by bone morphogenetic protein-2. Collectively these data demonstrate clear differences in both the expression and function of two related transcription factors and suggest that the modulation of cardiac gene expression can occur as a result of combinatorial regulatory interactions of T-box proteins.

Members of the T-box family of transcription factors are expressed in a variety of embryonic structures and their functions include regulation of cell type specification, tissue patterning, and morphogenesis (1, 2). In the human population, mutations of T-box genes are associated with several developmental disorders. The congenital heart defects of Holt-Oram syndrome and DiGeorge syndrome are associated with genetic aberrations in Tbx5 (3, 4) and TBX1 (5), respectively. The role of T-box genes in heart development is supported by the cardiac expression of several T-box genes during cardiogenesis including Tbx5 and Tbx20 as well as Tbx1, Tbx2, and Tbx18 (6, 7). The overlapping but distinct expression patterns of many of these T-box genes suggest discrete transcriptional functions. Tbx5 and Tbx20 are co-expressed in the cardiac primordia; however, during chamber formation their expression patterns diverge (8–12). Although Tbx5 and Tbx20 are differentially expressed, it has yet to be determined that they differ in their regulatory functions in the development of the heart.

Many recent studies have focused on the function of Tbx5 because of its association with Holt-Oram syndrome (3, 4). Tbx5 is required for the normal development of the heart as homozygous null txb5 mice have hypoplastic atria and consequently do not survive past E10.5 (13). Mice heterozygous for the null txb5 allele phenocopy some cardiac abnormalities of Holt-Oram syndrome in humans, including atrial septal defects as well as first and second degree atrioventricular block (13). The importance of txb5 for heart development is supported by studies in zebrafish embryos where reduced txb20 expression results in abnormal heart morphogenesis (14). Despite the requirement of Tbx5 and Tbx20 for normal heart development, limited information is available regarding their specific transcriptional functions during cardiogenesis. Initial evidence for Tbx5 transcriptional regulatory function demonstrated that the promoters of atrial natriuretic factor (ANF) and connexin40 are direct downstream targets of Tbx5 and are cooperatively regulated with Nkx2.5 (13, 15–17). Tbx5 and GATA4 also activate the ANF promoter (11, 18); however, the cis-elements required for the cooperative interaction have not been identified. Tbx20 contains multiple transcriptional regulatory domains (19), but its role as an activator or repressor of cardiac gene expression has not been clearly defined. To better understand the transcriptional regulatory functions of Tbx5 and Tbx20, their expression and function were evaluated simultaneously.

Expression of Tbx5 and Tbx20 was examined in chick embryos to define their respective expression domains during cardiac development. The differential expression of Tbx5 and Tbx20 in the heart suggests that they have distinct regulatory roles in chamber formation. Reporter gene analysis performed using sequence from the ANF promoter demonstrated that Tbx5 and Tbx20 exhibit differential transcriptional regulatory functions. Additionally it was shown that the C termini of Tbx5...
and Tbx20 have functionally distinct transcriptional regulatory domains. Relatively little is known about the pathways responsible for regulating expression of Tbx5 and Tbx20 during initial stages of cardiogenesis. In explanted cardiogenic regions from chicken embryos, Tbx20 but not Tbx5 expression was induced by bone morphogenetic protein-2 (BMP2) treatment. Collectively these studies define distinct expression, transcriptional function, and regulation of the related transcription factors Tbx5 and Tbx20 during heart development.

MATERIALS AND METHODS

In Situ Hybridization—Fertilized White Leghorn chicken eggs (Safas Inc., Roanoke, IL) were incubated at 38 °C under high humidity, and embryos were collected at 1, 2, 5, and 10 days. Whole embryos or dissected hearts were fixed overnight in 4% paraformaldehyde, phosphate-buffered saline. Fixed embryos and hearts were dehydrated in a graded methanol, phosphate-buffered saline, 0.1% Tween 20 series and stored at −20 °C in 100% methanol. Whole mount in situ hybridizations were performed as described by Wilkinson (20) with reported modifications (21). Day 10 hearts were bisected with a razor blade prior to hybridization to visualize the developing valves and conduction system. Proteinase K digested were performed for 10–15 min, and color reactions were incubated for 1–5 h using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science). Digoxigenin-UTP-labeled antisense RNA probes were generated specifically for chicken Tbx5 and Tbx20. Generation of Tbx5 riboprobe has been described previously (9). The chicken Tbx20 sequence was amplified by RT-PCR from E3 heart RNA with degenerate primers 5'-CTGCTGAGTARTGRTG-3' and 5'-GTTGAYAAYAGAGAATA-3' (where R represents purine or pyrimidine). Amplification efficiency and sequence were confirmed by RT-PCR. Amplified cDNA sequence containing the 288 proximal base pairs of the ANF promoter was generated by performing PCR from E3 heart RNA with degenerate primers 5'-TCTGGGCTGAGCCATTCCACCGG-3' and 5'-AGGGATTTGTCGCAATTAG-3'. mRNA expression was analyzed by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. To compare the temporal and spatial regulation of Tbx5 and Tbx20, we have used luciferase reporter plasmids that contain Gal4 reporter cassette constructed by GATA4 and pEMSV-Nkx2.5 expression plasmids were provided by J. Molkenstuij.

RESULTS

Tbx5 and Tbx20 Are Differentially Expressed in the Developing Chicken Heart—To compare the temporal and spatial regulation of Tbx5 and Tbx20 mRNA expression in the heart, in situ hybridization was performed on chicken embryos and isolated hearts from 1–10 days of development (Fig. 1). Expression of both Tbx5 and Tbx20 is detected in the heart primordia of Hamburger-Hamilton stage 6 embryos (Fig. 1, A and B, black arrowheads). At this stage, Tbx5 is expressed at low levels in the anterior heart-forming region, whereas Tbx20 expression is apparent in the anterior heart-forming region and in the posterior lateral regions of the embryo (Fig. 1B, red arrowheads). By stage 8, Tbx5 and Tbx20 are co-expressed in the cardiac primordia immediately prior to cardiomyocyte differentiation.
and heart tube formation (Fig. 1, C and D, black arrowheads). Concurrently, the posterior lateral expression of Tbx20 is reduced, and expression becomes restricted to the cardiac primordia. At later stages, Tbx5 and Tbx20 are differentially expressed in the primitive heart tube and during heart chamber morphogenesis. In stage 12 embryos, Tbx5 expression becomes restricted to the posterior, atrial, and left ventricular regions of the heart tube (Fig. 1E, red arrowhead), while Tbx20 is expressed throughout the entire heart tube including the anterior outflow tract (Fig. 1F, red arrowhead).

Although Tbx5 and Tbx20 are co-expressed in the atria at E5 (Fig. 1, G and H, black arrowheads), their expression in the atrioventricular canal (AVC), ventricles, and outflow tract are distinct. Tbx5 is present in the atria and left ventricle but is not expressed in the right ventricle and outflow tract. Tbx20 expression, however, is enriched in the AVC, the outflow tract (Fig. 1H, red arrowheads), right ventricle (Fig. 1I, red arrowhead), and left ventricle but is reduced in the left ventricle. Interestingly the expression of Tbx5 and Tbx20 in the ventricles are complementary with sharp boundaries of expression where the ventricular septum will form (Fig. 1, G and H, blue arrowheads). After 10 days of development, Tbx5 is strongly expressed in the atria (Fig. 1J, black arrowhead) and in the developing conduction system (Fig. 1J, red arrowhead). Tbx20 expression is present in the atria (Fig. 1J, black arrowhead), the AVC, and specifically in the tricuspid and mitral valves (Fig. 1J, black arrow). These data show that Tbx5 and Tbx20 share a similar expression pattern in the early heart primordia and developing atria. However, Tbx5 and Tbx20 are differentially expressed in the atrioventricular valves and specialized myocardial lineages.

The C Termini of Tbx5 and Tbx20 Have Distinct Transcriptional Regulatory Functions—Differential expression of Tbx5 and Tbx20 may be related to diverse functions in the development of the heart. Although homologous in the T-box DNA binding region (63.0% identity), Tbx5 and Tbx20 share no obvious homology outside of this domain (15.1% identity in the N terminus and 10.1% in the C terminus). To determine the transcriptional regulatory functions of their divergent domains, fusion proteins were generated containing the C terminus of either Tbx5 or Tbx20 and the Gal4 DNA-binding domain. The amino acids used in the fusion proteins relative to the T-box region are shown in Fig. 2A. The Gal4-Tbx5 fusion protein contains amino acids 266–518 of Tbx5, and the Gal4-Tbx20 fusion protein contains amino acids 294–445 of Tbx20. These fusion constructs were co-transfected into NIH 3T3 cells with the G5E1b-luciferase reporter gene that contains five sequential Gal4 binding sites linked to the E1b promoter (29).

Co-transfection of the G5E1b-luciferase reporter with Gal4-Tbx5-(266–518) led to a more than 250-fold increase in expression relative to the reporter co-transfected with the Gal4 DNA-binding domain alone (Fig. 2B). In contrast, co-transfection with Gal4-Tbx20-(294–445) resulted in a significant decrease in the level of reporter activity relative to that observed with the Gal4 DNA-binding domain alone (Fig. 2B). Additional evidence for the repressor activity of Tbx20 was provided by co-transfection of Gal4-Tbx20-(294–445) with a reporter gene containing five sequential Gal4 and two LexA binding sites (5xGal4–2xLexA-E1B-luc) (35). This reporter allows repressor activity of Gal4-Tbx20-(294–445) to be assessed by the ability to reduce the activation mediated by a strong activator. The 5xGal4–2xLexA-E1B-luc reporter was co-transfected with the Gal4-Tbx20-(294–445) and a fusion construct containing the LexA DNA-binding domain fused to VP16. Co-transfection of Gal4-Tbx20-(294–445) significantly decreased the high reporter activity mediated by the LexA-VP16 construct (Fig. 2C). These data provide additional evidence that the C terminus of the Tbx20 protein contains a transcriptional repressor domain. Taken together, these experiments demonstrate that the C terminus of Tbx5 acts as an activator, while the C terminus of Tbx20 acts as a repressor.

Tbx20 Antagonizes Tbx5 Activation of ANF Reporter Gene Expression—The relative abilities of Tbx5 and Tbx20 to activate cardiac gene expression was assessed using a reporter
gene consisting of the proximal 3003 base pairs of the rat ANF promoter linked to the luciferase gene (−3003)ANF-luciferase (25, 26). The ANF promoter contains the consensus binding sequences of several cardiac transcription factors and has been extensively used to examine cardiac gene-regulatory mechanisms (13, 15, 17, 36–40). NIH 3T3 cells were co-transfected with the (−3003)ANF-luciferase reporter and with pAC-CMV-Tbx5 or pAC-CMV-Tbx20 expression plasmids, and ANF transcriptional activation was assessed 48 h later. (−3003)ANF-luciferase expression significantly increased −2.3-fold compared with the empty vector control when co-transfected with Tbx5. ANF reporter activity, however, was significantly decreased (−30%) when co-transfected with Tbx20 compared with the empty vector control (Fig. 3A). To determine whether Tbx20 can antagonize Tbx5 function, (−3003)ANF-luciferase was co-transfected with increasing amounts of pAC-CMV-Tbx20 (0.1–1.5 μg) and a constant amount of pAC-CMV-Tbx5 (0.5 μg). Tbx5 activation of (−3003)ANF-luciferase expression decreased as the quantity of Tbx20 expression plasmid transfected increased and was completely abrogated by the maximal transfected ratio (1.5:0.5 μg) of Tbx20:Tbx5 (Fig. 3A). Together these results indicate that Tbx20 alone can repress ANF promoter activity and also is able to inhibit the ability of Tbx5 to activate ANF gene expression.

Tbx5, but Not Tbx20, Cooperatively Acts with GATA4 and Nkx2.5 to Activate ANF Expression—The ability of Tbx5 or Tbx20 to cooperate with GATA4 and Nkx2.5 in activating (−3003)ANF-luciferase was determined in transfected NIH 3T3 cells. pAC-CMV-Tbx5 and pAC-CMV-Tbx20 were co-transfected with either pMT2-GATA4 or pEMSV-Nkx2.5 expression plasmids and the (−3003)ANF-luciferase reporter. Tbx5, Nkx2.5, and GATA4 each activated (−3003)ANF-luciferase expression (−1.9-, −7.4-, and −4.1-fold, respectively) (Fig. 3B). A synergistic activation of (−3003)ANF-luciferase was observed when Tbx5 was co-transfected with Nkx2.5 (−28.0-fold) (Fig. 3B). However, when Tbx20 was co-transfected with Nkx2.5, this synergistic activation was not observed, and the level of (−3003)ANF-luciferase activation was comparable to that of Nkx2.5 alone (−7.0-fold) (Fig. 3B). Synergistic activation of (−3003)ANF-luciferase was also observed when Tbx5 was co-transfected with GATA4 (−36.5-fold); however, this synergy was not achieved with Tbx20 and GATA4 together (−5.4-fold) (Fig. 3B). These data indicate that Tbx5 and Tbx20 differ in their abilities to cooperate with other cardiac transcription factors in the regulation of ANF promoter activity.

The TBE Sites of the ANF Promoter Mediate Transcriptional Regulation by Tbx5 and Tbx20—The cis-elements in the ANF promoter were examined to determine the sequences required for synergistic activation mediated by Tbx5 and GATA4. The proximal 288 base pairs of the ANF promoter contain two GATA4, two Nkx2.5, and two Tbx5 consensus binding sequences (Fig. 4A) (13, 17, 36, 38). Co-transfection of
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Mutations of the distal T-box binding element (TBE2) did not interfere with the synergistic activation mediated by Tbx5 and GATA4 (−28.7-fold) (Fig. 4B). However, when the more proximal element was mutated (TBE1), Tbx5 and GATA4 were unable to synergistically activate (−288)ANF-luciferase (−11.0-fold) (Fig. 4B). This result indicates that TBE1 but not TBE2 is required for synergistic activation of the ANF promoter by Tbx5 and GATA4. The TBE1 and TBE2 mutant reporters were also co-transfected with pAC-CMV-Tbx5 or pAC-CMV-Tbx20 alone to determine which sites are required for their transcriptional activity. Mutation of the proximal TBE1 site but not TBE2 prevented Tbx5 from significantly activating the ANF promoter (Fig. 4C). Interestingly, the observed repression of ANF mediated by Tbx20 was eliminated by mutation of the distal TBE2 site. These data suggest that TBE1 is required for Tbx5 transcriptional activation, while TBE2 mediates Tbx20 transcriptional repression.

Holt-Oram Syndrome Tbx5 Alleles Have Compromised Gene-regulatory Functions—Mutations in Tbx5 coding sequence are associated with HOS (3, 4). To determine whether the HOS alleles of Tbx5 have compromised function, corresponding mutations were introduced into the protein coding sequences of mouse Tbx5. A missense mutation at amino acid 237 (R237Q), which has previously been shown to result in deficient DNA binding (16, 17), was introduced in the highly conserved T-box. A nonsense mutation also was generated at amino acid 279 (R279ter), resulting in a truncated protein lacking the majority of the transactivation domain identified in Fig. 2B. The expression plasmids pAC-CMV-Tbx5(R237Q) and pAC-CMV-Tbx5(R279ter) were co-transfected with the (−3003)ANF-luciferase reporter into NIH 3T3 cells. Neither Tbx5(R237Q) (−1.3-fold) nor Tbx5(R279ter) (−1.1-fold) could activate the reporter to the same levels as Tbx5 (−2.2-fold) (Fig. 5). Additionally, neither Tbx5(R237Q) nor Tbx5(R279ter) synergized with Nkx2.5 or GATA4 to activate (−3003)ANF-luciferase (Fig. 5). These results demonstrate that mutations in Tbx5 associated with Holt-Oram syndrome affect its ability to activate transcription alone and in conjunction with Nkx2.5 or GATA4.

Expression of Tbx20, but Not Tbx5, Is Induced by BMP2 in Cultured Cardiogenic Embryo Explants—The factors responsible for regulating Tbx5 and Tbx20 expression during initial stages of embryonic heart development were examined. In culture, lateral cardiac primordia explanted from stage 5 chicken embryos are capable of differentiating into beating cardiomyocytes, while explanted mesendodermal medial to the cardiac primordia cannot. In the presence of BMP2, however, the medial mesendodermal cells are competent to differentiate and can express cardiac-specific markers (41). To determine the inductive mechanisms that control Tbx5 and Tbx20 expression during the initial stages of heart formation, lateral and medial mesendodermal explants from Hamburger-Hamilton stage 5 chick embryos were treated with recombinant BMP2 (200 ng/ml). After 6 h in culture, RNA from lateral or medial explants was isolated and subjected to RT-PCR analysis. Standard RT-PCR revealed that expression of Tbx20 and Nkx2.5 is strongly induced in the medial cells when recombinant BMP2 is added to the explant culture (Fig. 6A). In contrast, Tbx5 expression was relatively unaffected by BMP2 treatment.

Quantification of the RNA levels was also carried out using real-time RT-PCR and normalized to the levels of GAPDH expression. Representative experimental results shown in Fig. 6, B–D, demonstrate the striking induction of Tbx20 and Nkx2.5, but not Tbx5, in the medial cells following BMP2 treatment, confirming the results visualized in Fig. 6A. In these representative experiments, expression of Nkx2.5 and
Tbx20 was induced 89.5- and 40.0-fold, respectively, while Tbx5 was induced 2.1-fold. The average medial to lateral expression ratios of Tbx20, Tbx5, and Nkx2.5 were calculated from seven experiments. The ratios of Tbx20 (-0.04) and Nkx2.5 (-0.13) expression significantly increased with the addition of BMP2 (Tbx20, -0.63; Nkx2.5, -1.4) (Fig. 6E). In some cases, the levels of Tbx5 were slightly induced by the addition of BMP2 (Fig. 6, A and B); however, this induction did not significantly change the medial to lateral expression ratio (untreated, -0.19; BMP2-treated, -0.24) (Fig. 6E). Induction of Tbx5 and Tbx20 expression also was analyzed subsequent to treatment with FG2, FG4, or FG8; however, no significant increase in expression was observed with any of these growth factors (data not shown). These data demonstrate that Tbx20 expression in the heart-forming region is responsive to BMP2 but that Tbx5 expression is not. Therefore, Tbx5 and Tbx20 expression likely is regulated by distinct inductive pathways during the earliest stages of heart development.

DISCUSSION

Differential expression and transcriptional function of Tbx5 and Tbx20 suggest that they have distinct roles during cardiac development. In chicken and mouse embryos, the expression of Tbx5 and Tbx20 overlap in the early heart-forming region, but as cardiac morphogenesis and chamber formation progresses, they are differentially localized. In the four-chambered heart, the atrial chambers express both Tbx5 and Tbx20. However, only Tbx5 is expressed in the left ventricle and developing conduction system, while Tbx20 is expressed in the right ventricle, outflow tract, AVC myocardium, and the atrioventricular cushions and valves. Co-expression of Tbx5 and Tbx20 in the early heart-forming region suggests they have related func-
described in Fig. 2.

Tbx5 and Tbx20 have been shown to disrupt the transcriptional function of the protein by interfering with DNA binding, interactions with other cardiac transcription factors, and/or transcriptional activation (15–18, 59). Supporting these studies, we have demonstrated that the R237Q and R279ter mutant Tbx5 proteins are compromised in

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their abilities to cooperatively activate the ANF promoter with GATA4 and Nkx2.5. These data suggest that the cardiac abnormalities of Holt-Oram syndrome are likely caused by the inability of Tbx5 mutant proteins to activate gene expression from target promoters. Similarly, the transcriptional functions of GATA4 mutant proteins associated with human congenital heart disease are also compromised (18). Mutations in human GATA4 and NKX2.5 genes are associated with congenital cardiac abnormalities similar to Holt-Oram syndrome including conduction system and atrioventricular septal defects (18, 60, 61). Because Tbx5, GATA4, and Nkx2.5 can cooperatively regulate cardiac gene expression, it is possible that mutations in any of these genes disrupt the transcriptional activation complex. In addition to ANF, other cardiac genes such as connexin40 and cardiac α-actin are cooperatively regulated by Tbx5/Nkx2.5 and Nkx2.5/GATA4, respectively (13, 62, 63). Therefore, altered functions of T-box, GATA, or Nkx proteins could lead to misregulation of several shared downstream target genes. The cooperative nature of these regulatory interactions likely contributes to the common cardiac congenital defects observed with mutations of diverse transcription factors expressed in the developing heart.

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