Novel Exons in the Tbx5 Gene Locus Generate Protein Isoforms with Distinct Expression Domains and Function*

Abir Yamak1, Romain O. Georges5,1, Massomeh Sheikh-Hassani1, Martin Morin5, Hiba Komati6, and Mona Nemer5,2

From the ‡Laboratory of Molecular Genetics and Cardiac Regeneration, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1N 6N5 and the §Graduate Program in Molecular Biology, Institut de Recherches Cliniques de Montréal (IRCM), Université de Montréal, Montréal, Québec H2W 1R7, Canada

Background: Tbx5 is the causative gene for Holt-Oram syndrome (HOS), characterized by forelimb abnormalities and heart problems; however, 30% of patients have no mutation in known exons.

**Results:** Tbx5 isoforms derived from novel exons have distinct expression domains and function.

**Conclusion:** Alternative splicing regulates Tbx5 function in heart and limb.

**Significance:** This is relevant for human mutational screening and for understanding Tbx5 function.

Tbx5 is the gene mutated in Holt-Oram syndrome, an autosomal dominant disorder with complex heart and limb deformities. Its protein product is a member of the T-box family of transcription factors and an evolutionarily conserved dosage-sensitive regulator of heart and limb development. Understanding Tbx5 regulation is therefore of paramount importance. Here we uncover the existence of novel exons and provide evidence that Tbx5 activity may be extensively regulated through alternative splicing to produce protein isoforms with differing N- and C-terminal domains. These isoforms are also present in human heart, indicative of an evolutionarily conserved regulatory mechanism. The newly identified isoforms have different transcriptional properties and can antagonize Tbx5a target gene activation. Droplet Digital PCR as well as immunohistochemistry with isoform-specific antibodies reveal differential as well as overlapping expression domains. In particular, we find that the predominant isoform in skeletal myoblasts is Tbx5c, and we show that it is dramatically up-regulated in differentiating myotubes and is essential for myotube formation. Mechanistically, Tbx5c antagonizes Tbx5a activation of pro-proliferative signals such as IGF-1, FGF-10, and BMP4. The results provide new insight into Tbx5 regulation and function that will further our understanding of its role in health and disease. The finding of new exons in the Tbx5 locus may also be relevant to mutational screening especially in the 30% of Holt-Oram syndrome patients with no mutations in the known Tbx5a exons.

Holt-Oram syndrome (HOS)3 is an autosomal dominant disorder characterized by upper limb and cardiac defects (1, 2). The most common structural heart abnormalities include atrial septal defects and ventricular septal defects. Conduction defects have also been commonly seen, and they mostly involve atrioventricular blocks. Hypoplastic left ventricles, mitral valve problems, and endocardial cushion defects have also been reported in HOS patients (1, 2). Genetic linkage analyses have mapped the disease to the chromosomal locus where Tbx5 is located, and mutations in Tbx5 have been found in patients with HOS. Moreover, Tbx5 expression pattern in the upper limb, atria, and left ventricle along with mouse genetics studies have strengthened the causative link between Tbx5 and HOS (3). Over 70 mutations in the Tbx5 locus have been identified so far in HOS patients (4). Many result in no protein production or in truncated proteins. Other more subtle mutations generate functionally impaired proteins with altered subcellular localization, DNA binding, transcriptional activity, and/or interaction with cofactors (5–7). These findings led to the suggestion that haploinsufficiency may be the mechanism of pathogenesis, but this remains uncertain in many cases. Interestingly, in about 30–35% of HOS patients, no mutations in Tbx5 coding sequences or intron-exon junctions are detected (8), which has raised the controversial suggestion of the existence of another as yet unidentified HOS-causing locus. An alternative explanation could be that unscreened mutations within presumed untranscribed regions of Tbx5 account for this low detection rate. Consistent with this, we recently reported the existence of a new exon downstream of the T-box whose alternative splicing results in a Tbx5 isoform lacking the entire C terminus, which contains several functional domains (9).

Tbx5 is a member of the large family of T-box transcription factors critical for early cellular commitment, differentiation, and organ development (10). T-box (or Tbx) proteins bind specific DNA motifs, called TBEs (T-box binding elements), to activate or repress target promoters. Tbx5 appears to act essentially as a transcriptional activator and cooperates with other transcription factors such as GATA4 and NKX2.5 to synergistically regulate downstream targets (3, 6, 11). As such, Tbx5 activity can be modulated at the DNA binding level and through protein-protein interactions. In addition to transcriptional regulators, Tbx5 was shown to interact with the cytoskeleton-associated LIM protein LMP4, which represses its transcriptional activity, possibly by stimulating its cytoplasmic...
Novel Tbx5 Exons Generate Distinct Protein Isoforms

Cloning of Novel Tbx5 Isoforms—Tbx5 transcripts with variable 3’ ends (Tbx5c and Tbx5d) were obtained using nested cDNA amplification of mRNA from embryonic hearts and limbs. cDNAs were amplified by RT-PCR using a 5’ oligonucleotide spanning the first codons of the T-box and at the 3’ end, an oligo(dT). The resulting products were cloned into the Bluescript plasmid, and individual clones were sequenced. Full-length clones containing the N-terminal sequences were then amplified using specific primers and subcloned in the pcDNA3 expression vector in phase with Kozak triple FLAG epitope. To isolate isoforms with variable 5’ end (Tbx5e), primer extension amplification was used with the primer sequence overlapping the 5’ end of the T-box sequences. Oligonucleotides used were: 5’-GGAGGTACCCGCGTGATAGGAGGCTTTG-3’ (forward for Tbx5a, Tbx5c, Tbx5d, and Tbx5b); 5’-GCAAGGT-ACGGAAGGATACAGCTTTTCTATCTG-3’ (forward for Tbx5e); 5’-CCCGAATTCCTAGATTCTTCCTGCA-CTCTG-3’ (reverse for Tbx5a and Tbx5e); 5’-GCACCT-GAGCTAATGAAAGGATGGTGAGAGAG-3’ (forward for Tbx5c); 5’-CCGGAAATTCATTTTTCTTCTATTTCTGCA-CTCTG-3’ (reverse for Tbx5d); and 5’-CCCTCGAGCTAAAGAGAGCAGGGGCTTTGCATCCGAG-3’ (reverse for Tbx5b). GATA4 and NKX2.5 expression vectors as well as the luciferase reporters were previously described (9, 17–19).

Cell Culture and Transfections—Cell lines were maintained in culture and transfected as described previously (9, 20) C2C12 were maintained in 10% FBS for 24 h, and the medium was switched to 1% BSA or 2% horse serum for differentiation when indicated. For co-transfection assays, the total amount of DNA was maintained constant by adding the appropriate amount of empty DNA vector. Fgf10-luc reporter construct was a kind gift from Dr. Benoit Bruneau from the Gladstone Institute of Cardiovascular Disease and was previously described (21). Nppa-luc, Bclx-luc, Cnd1-luc, and Bmp4-luc reporters were previously described (19, 20, 22). GAL4 fusion proteins were designed as described previously (23).

EMSA—Nuclear extracts were prepared from 293T cells. Binding reactions were done at room temperature using 1 µg of poly(dI-dC). The TBE probe used was from the Nppa promoter and previously described (9).

In Vitro Pulldown Assay—Production of the GST-TBX5a and MBP-NKX2.5 constructs (where MBP is maltose-binding protein) and the pulldown assays were done as described previously (24).

Protein Analysis—Western blots were done on nuclear extracts from 293T cells overexpressing the relevant Tbx5 constructs and FLAG or Tbx5 antibody as described previously (9). Endogenous Tbx5 proteins were analyzed in two relevant mouse lines, the TC13 cardiogenic cell line and in C2C12 myoblasts, as well as in 30-day-old mouse hearts. Protein extraction and analysis were as reported in Georges et al. (9). The N-terminal Tbx5a antibody was previously described. The C-terminal Tbx5a antibody as well as Tbx5c and Tbx5d antibodies were similarly raised in rabbits against the unique C-terminal domains: AA 331–425 for Tbx5a, AA 327–404 for Tbx5c, and AA 327–376 for Tbx5d.

Immunocytochemistry was done on 293T cells overexpressing the relevant FLAG-Tbx5 constructs as described previously (9). Mouse anti-FLAG M2 was purchased from Sigma (F1804). Immunofluorescence was performed on C2C12 cells overexpressing the indicated HA-TBX5 constructs. Myosin heavy chain and myogenin antibodies were both obtained from mouse hybridoma cells (MF20 and F5D, respectively). HA antibody was purchased from Santa Cruz Biotechnology (SC-805). Immunohistochemistry was done on mouse tissues at different embryonic stages as described previously (25).

RNA Analysis—Dropet Digital PCR was carried out on the QX200 Bio-Rad Droplet Digital PCR system using cDNA from embryonic and postnatal tissues as well as cardiac and muscle cells using sequence-specific primers as indicated and according to the manufacturer’s protocol. Primer sequences are available upon request. Quantitative PCR analysis was performed on C2C12 cells overexpressing the indicated Tbx5 constructs as described previously (25). siRNAs were obtained from Sigma and transfected in C2C12 cells using the HiPerFect transfection reagent from Qiagen (catalog number 301705). siRNA sequences are available upon request.
RESULTS

Identification of Novel Tbx5 Exons—To analyze endogenous TBX5 protein, we developed a specific antibody against the N-terminal TBX5 region; in Western blots, this antibody detected, in addition to the expected TBX5a band, immunoreactive bands that co-migrated with a TBX5 protein truncated of the last 118 amino acids (9). To determine whether these bands represent novel TBX5 isoforms, we used a PCR amplification strategy to isolate Tbx5 cDNAs from mouse heart and limb RNA. A 5'/H11032 oligonucleotide primer spanning the first codons of the T-box and a 3'/H11032 oligo(dT) primer allowed amplification of Tbx5 transcripts containing the T-domain together with any variable 3' sequences. Isolation and sequencing of several independent cDNA clones revealed the presence of four distinct transcripts that may result in four different TBX5 proteins; in addition to sequences corresponding to Tbx5a and the previously described short isoform (9), two new sequences were identified that would encode novel TBX5 isoforms of 404 and 376 AA, termed Tbx5c and Tbx5d, respectively (Fig. 1A). These two isoforms result from the use of two alternative exons (exon 9a or exon 10), which are mutually exclusive with exon 11. Tbx5c is generated from the splicing of exon 9 to exon 10 instead of exon 11, whereas Tbx5d originates from the usage of an alternative exon 9, named exon 9a, which emanates from the retention of additional intronic sequences 3' of exon 9 (Fig. 1C).

Once the additional exons were sequenced, we cloned the entire cDNA coding sequence of the novel isoforms from mouse embryonic atria (Tbx5d) and forelimb (Tbx5c) using primers spanning the first codon (ATG) and the stop codon of the different isoforms. Sequence analysis of the genomic TBX5 locus in existing databases (NCBI, Ensembl) revealed the possibility of a fifth TBX5 isoform, TBX5e (Fig. 1A). This isoform would result from the splicing of an alternative exon 1 in mice (termed exon 1b) with exon 3; thus, skipping exon 2 leading to

FIGURE 1. A, schematic representation of Mus musculus Tbx5 isoforms. At least five different isoforms are translated from the different splice variants. The red box is the DNA binding T-box domain of the protein. The purple and blue boxes are the regions that are divergent in the Tbx5c and Tbx5d, respectively. B, multiple sequence alignment of Tbx5a, Tbx5c, and Tbx5d showing the divergence in the C-terminal region of the three M. musculus Tbx5 isoforms. C, schematic representation of the genomic sequences showing the splice junctions. D, schematic representation of the exons alternatively spliced to produce the different protein isoforms in mice.
an N-terminal truncation of 50 AA (Fig. 1, C and D). Exon 1b is highly homologous to the human exon 1. To confirm the in vivo existence of such *Tbx5e* transcripts, a primer overlapping exon 1b and 3 was used in RT-PCR amplification together with a reverse primer spanning the stop codon of exon 11; this resulted in the isolation of *Tbx5e* cDNA clones from mouse heart RNA. Thus a N-terminally truncated TBX5 isoform is present in both humans and rodents.

Next, we analyzed the tissue distribution of *Tbx5c* and *Tbx5d*, which contain novel exons, using Droplet Digital PCR. In the case of *Tbx5a* and *Tbx5d*, a forward primer corresponding to sequences in the common exon 7 was used along with a reverse primer specific to exon 11 (*Tbx5a*) or exon 9a (*Tbx5d*). Forward and reverse primers were chosen in exon 10 in the case of *Tbx5c*. Using this strategy, we detected all isoforms in E11.5 atria and forelimbs. E11.5 ventricles expressed mostly *Tbx5a* and lower levels of the other isoforms. *Tbx5a* and *Tbx5c* transcripts were also detected in the hindlimbs, albeit at much lower levels (Fig. 2A). A similar expression pattern was seen in the postnatal heart where *Tbx5a* was always the predominant isoform (Fig. 2B). We also verified the presence of these isoforms in cardiogenic cell lines, notably the atrium-like HL-1 cells and the endocardial progenitors TC13 cells, as well as in skeletal muscle progenitors such as C2C12 myoblasts. The *Tbx5* expression pattern in HL1 resembled that of the E11.5 heart with the predominance of *Tbx5a* (Fig. 2C). *Tbx5a* was the only isoform present in TC13 cells but not in C2C12 myoblasts where *Tbx5c* was the predominant isoform and was dramatically up-regulated after differentiation.

We then used the N-terminal TBX5 antibody (9) to analyze the profile of TBX5 proteins in cardiac cells. Western blot analysis of nuclear extracts from TC13 cells revealed the existence of multiple immunoreactive bands (Fig. 2D) co-migrating with *Tbx5a*, *Tbx5b*, *Tbx5c*, and *Tbx5d*; *Tbx5a* and *Tbx5c* were also detected in C2C12 cells (Fig. 2D) although the *Tbx5a* band was much weaker. To verify the presence of *Tbx5e*, a similar blot was incubated with an antibody raised against a C-terminal epitope encoded by exon 11 (AA 331–425); this antibody can detect *Tbx5a* and *Tbx5e* but not *Tbx5b*, *Tbx5c*, and *Tbx5d*. Consistent with the Droplet Digital PCR results, a strong band co-migrating with *Tbx5a* was detected in TC13 nuclear extracts as well as a weaker band co-migrating with *Tbx5e*. A 50-kDa band that may be *Tbx5e* was also present in C2C12 cells (Fig. 2E). Next, we analyzed TBX5 complexes in postnatal hearts. Nuclear extracts were obtained using 300 mM NaCl extraction and dialyzed to 100 mM salt, and chromatin-binding proteins were enriched on a phosphocellulose column; chromatin-bound proteins were eluted at 300 mM salt and size-fractionated under non-denaturing conditions using a gel filtration Superose 6 column. Western blots on the resulting fractions were carried out using the N-terminal TBX5 antibody. Bands corresponding to *Tbx5a* (~64–80 kDa) were evident in several high molecular mass fractions. An immunoreactive band around 48 kDa was also present and would correspond to TBX5d (Fig. 2F). These results confirm the presence in the heart of TBX5a and TBX5d and suggest the existence of different protein complexes containing distinct TBX5 isoforms in Tbx5-expressing organs.

Next, we wanted to verify whether similar splice isoforms exist in humans. *In silico* analysis of the mouse and human TBX5 genomic sequences revealed the presence of similar alternate splice site junctions for *Tbx5c* and TBX5d. For TBX5d, this predicted the existence of alternate splicing within exon 9 to produce the splice variant shown in Fig. 3A, which turned out to be identical to the reported transcribed sequence MN_181486, which would produce a 350-AA isoform whose last 22 AA are divergent from TBX5a (Fig. 3B). The presence of this cDNA sequence was also reported by Basson et al. (1) in the initial identification of TBX5 as the HOS gene. Interestingly, only the first three additional AA are conserved between mouse and humans, suggesting that protein conformation, and not primary sequence, may need to be conserved. Another transcript that would encode the human equivalent of TBX5e can also be found in the Ensembl database (NM080717). Based on mouse–human sequence alignment, we were able to predict the human sequence of *TBX5c*, which seems to be more conserved among mouse and humans than the TBX5d-specific region (Fig. 3C). We designed primers specific for human *Tbx5a*, *Tbx5c*, and *Tbx5d* by targeting the unique exons of each isoform. RT-PCR analysis revealed the presence in human heart tissues of the expected size transcripts (Fig. 3D), indicating that similar isoforms are present in human and rodent hearts.

**Differential Expression of Distinct TBX5 Isoforms**—To study the expression pattern of the different isoforms at different mouse embryonic stages, we generated two new antibodies raised against the C-terminal epitopes of *Tbx5c* (AA 327–404) and *Tbx5d* (AA 331–417), respectively. The antibody specificity was confirmed by immunofluorescence done on C2C12 cells transfected with HA-TBX5a, HA-TBX5c, or HA-TBX5d using HA antibody co-stained with C-terminal TBX5a, TBX5c, or TBX5d antibodies (data not shown). Immunohistochemistry was then performed using the C-terminal antibodies specific to TBX5a, TBX5c, and TBX5d. The N-terminal directed antibody that detects all TBX5 isoforms containing this domain was also used on mouse embryos (E11.5, E15.5) and neonate (P1.5) tissue sections (Fig. 4, A and B). All isoforms were detected in the myocardium as well as the endocardium of E11.5 mouse hearts. At E15.5, in addition to their expression in the atria, the isoforms were also detected in the atrioventricular valves where TBX5c and TBX5d were more predominant. TBX5a and TBX5c but not TBX5d were also detected in the forelimbs at E15.5. Expression of the isoforms in the atrioventricular valves persisted in the postnatal mouse heart (P1.5) where TBX5c and TBX5d continued to be the more abundant isoforms in the mitral and tricuspid valves. In contrast, TBX5a was the most abundant isoform in the atrioventricular bundle. The differential expression of TBX5 isoforms in the limbs and in different heart cells raises intriguing questions regarding their specific functions in organ development.

**TBX5 Isoforms Possess Distinct Biochemical Properties**—We investigated the biochemical properties of the newly identified TBX5 isoforms. Western blot analysis revealed that all N-terminally FLAG-tagged isoforms were well expressed in transfected 293T cells (Fig. 5A). Immunocytochemistry revealed distinct subcellular localization with TBX5c and TBX5d detected in both the nucleus and the cytoplasm, indicating that TBX5
sequences between AA 327 and 518 promote nuclear localization. This is in line with the reported presence of a nuclear localization signal between AA 325 and 340 (9, 26). EMSA revealed that all isoforms were able to bind the cognate TBE site of the \textit{Nppa} promoter with good affinity (Fig. 5C). Because the various newly identified transcripts appear to be co-expressed with the long \textit{Tbx5a}, we tested the consequences of their presence on TBX5a binding to its cognate site (Fig. 5D). When present in equimolar ratio with HA-TBX5a, all isoforms tested (FLAG-TBX5c, FLAG-TBX5d, and FLAG-TBX5e) appeared to
form heterodimers (the lower asterisk corresponding to the isoform homodimer and the upper asterisk corresponding to the heterodimer) that were supershifted with both the FLAG and HA antibodies (last two right lanes of each panel). This result is in line with the fact that homo- and heterodimerization of TBX proteins is mediated by the T-box, which is identical for all TBX5 isoforms tested.

We also tested the ability of the various isoforms to form a ternary complex with NKX2.5 on the composite TBE-NKE (NKX2.5 binding element) site. As shown in Fig. 5E, when co-expressed with NKX2.5, TBX5a, TBX5c, TBX5d, and TBX5e efficiently formed a slower migrating ternary complex (indicated by asterisk). The protein composition of the complex was confirmed by the addition of TBX5 (N-terminal) and NKX2.5 antibodies, which both resulted in supershifting the ternary complex.

The TBX5 heterodimers as well as the TBX5-NKX2.5 complexes were also confirmed by in vitro pulldown assay as shown in Fig. 5F. Interestingly, TBX5c and TBX5d as well as TBX5e bound NKX2.5 and TBX5a with a higher affinity than TBX5a; this raises the possibility that they can compete for TBX5a binding to NKX2.5 and confirms their ability to form DNA binding heterodimers with TBX5a.

Next, we tested their transcriptional effects on target promoters. As stated earlier and schematically shown in Fig. 6A, TBX5a harbors three transactivation domains, one in the N-terminus (TAD1) and two in the C terminus (TAD2 and TAD3). TAD1 is absent in TBX5e, whereas TBX5c/TBX5d lack TAD3 (Fig. 6A). Co-transfection experiments were carried out using three target promoters: Nppa (a differentiation marker (3)), BCLX (a survival marker (18)), and Ccnd1 (cyclin D1, a proliferation marker). The results revealed unexpected promoter-specific differences in the transcriptional activity of TBX5 isoforms. When compared with TBX5a, TBX5e and TBX5a1–327 were weaker activators of the Nppa promoter; modest, if any, activation was observed with TBX5c and TBX5d on this promoter. These results are consistent with our previous structure-function study (9) and suggest that maximal Nppa activation requires all three TADs; they also suggest that the additional C-terminal sequences do not contribute activation functions (Fig. 6B, left panel). When tested on the BCLX promoter, the activity of TBX5a and TBX5e was almost similar; TBX5c was able to activate transcription, albeit to a lower extent, whereas TBX5d was inactive. This result suggests a strong dependence on the C-terminal TADs and reveals distinct behaviors for TBX5c and TBX5e on this promoter. These results are consistent with our previous structure-function study (9) and suggest that maximal Nppa activation requires all three TADs; they also suggest that the additional C-terminal sequences do not contribute activation functions (Fig. 6B, left panel). When tested on the BCLX promoter, the activity of TBX5a and TBX5e was almost similar; TBX5c was able to activate transcription, albeit to a lower extent, whereas TBX5d was inactive. This result suggests a strong dependence on the C-terminal TADs and reveals distinct behaviors for TBX5c and TBX5d. (Fig. 6B, middle panel). In contrast, activation of the Ccnd1 promoter was independent of TAD3 as evidenced by the ability of TBX5a1–327 to maximally activate the promoter; however, the absence of the N-terminal domain reduced activation by half (Fig. 6B, right panel). Interestingly, as in the case of Nppa, TBX5c and TBX5d were less active than TBX5a1–327, suggesting that the additional C-terminal domain interferes with existing TADs.

Next we tested the consequences of co-expressing the new isoforms on TBX5a transcriptional activity. Co-transfections of the Nppa-luciferase reporter with TBX5a were carried out in
the presence of increasing concentrations of the TBX5c, TBX5d, or TBX5e; the concentrations used resulted in 1:1, 3:1, and 10:1 ratios of isoform:TBX5a. As shown in Fig. 6C, all isoforms resulted in a dose-dependent attenuation of TBX5a activation. These results, together with the gel shift data in Fig. 5, C and D, likely reflect the presence on the promoter of heterodimers with weaker activating properties than TBX5a homodimers. Next, we tested the transcriptional properties of the TBX5-NKX2.5 complex. Neither TBX5c nor TBX5d was able to synergize with NKX2.5 (Fig. 6D), whereas TBX5e was
able to cooperate with NKX2.5 to a nearly similar extent as TBX5a. Given that all isoforms formed stable ternary complexes with NKX2.5, and the nuclear and cytoplasmic distribution of TBX5c and TBX5d, C, DNA binding properties of TBX5 isoforms. Electrophoretic mobility shift assay was performed using nuclear extracts from 293T cells overexpressing the indicated TBX5 isoform. The exogenous TBX5 binding is indicated by an asterisk. Supershift/blocking was done with homemade anti-TBX5 antibody (N-terminal TBX5 in the case of TBX5a, TBX5c, TBX5d, and 1–327; C-terminal TBX5 in the case of TBX5e). SS, supershift. D, ability of novel TBX5 isoforms to form heterodimers with TBX5a. EMSA was performed as above: 1:1 ratios of HA-TBX5a and the indicated FLAG-TBX5 isoform were used as indicated. The asterisk marks the binding of the individual isoform or the complex when applicable. Note that all isoforms are capable of forming a heterodimer with TBX5a, and a supershifted complex with both FLAG and HA antibodies. E, formation of a TBX5-NKX2.5 complex. EMSA was carried out as above. The asterisk marks the binding of the complex. Supershift was done with anti-TBX5 or anti-NKX2.5 antibody. 100× self-cold probe was used as a competitor. F, in vitro translated radiolabeled TBX5 proteins (or luciferase protein as a negative control) were incubated with glutathione-Sepharose beads containing GST-TBX5a fusion protein (middle panel) or amylose beads containing MBP-NKX2.5 fusion protein (lower panel) (where MBP is maltose-binding protein). Note that the isoforms form heterodimers with TBX5a and ternary complex with NKX2.5. The autoradiograph shown is representative of two independent experiments. Quantitation of the NKX2.5-TBX5 binding relative to the NKX2.5-TBX5a binding is shown in the bottom panel. Values are corrected over the corresponding TBX5 inputs.

These results are consistent with our previous findings that the T-box is essential for GATA4-TBX5 interaction and that sequences in the N and C terminus contribute to the overall synergy (9). To test whether the TBX5c- and TBX5d-specific domains have a transcriptional activity on their own, reporter gene transactivation assays were performed with expression plasmids encoding fusion proteins of these domains with the DNA binding domain of Gal4 (Gal4-DBD). As shown in Fig. 6F,
TBX5a TAD3 is able to activate transcription on its own (10X). This activity is greatly enhanced in the presence of TAD2 (30X), although TAD2 has minimal activity on its own (2.2X). Neither TBX5c-specific nor TBX5d-specific domains seemed to possess autonomous transcriptional activity. Moreover, they had no effect (positive or negative) on TAD2 transcriptional activity (Fig. 6A). Together the results reveal that the different TBX5 isoforms have distinct transcriptional properties.

Effect of TBX5 Isoforms on Skeletal Muscle Differentiation—As stated above, TBX5c is the predominant isoform in skeletal myoblasts, and its expression is greatly increased in differentiated myotubes. To determine the effect of TBX5c in these cells, we carried out gain and loss of function studies. First, we transfected myoblasts with TBX5a or TBX5c and monitored their fate in quiescent (1% bovine serum albumin) or differentiating (2% horse serum) medium. As shown in Fig. 7A, TBX5c but not TBX5a was able to induce differentiation in quiescent cells as evident by the myogenic markers myosin heavy chain (MF20) (left panel) and myogenin (right panel). TBX5c was able to accelerate myotube formation when cells were switched to the differentiation medium, in contrast to TBX5a, which inhibited differentiation.

We then tested the effect of down-regulation of Tbx5a or Tbx5c on myocyte differentiation. C2C12 myoblasts were transfected with a universal control siRNA and with two different Tbx5a-specific or Tbx5c-specific siRNAs. Cells were examined after 2 and 4 days in differentiation medium. Down-regulation of Tbx5a was able to remove the inhibitory effect of TBX5a on myotube formation at day 2 (48 h) and day 4 (96 h), whereas down-regulation of Tbx5c but not Tbx5a reduced myotube formation (Fig. 8, A and B), indicating that TBX5c plays an important role in skeletal muscle differentiation.

To get insight into the mechanisms underlying TBX5a and TBX5c effects on muscle progenitors, we monitored changes in

---

**FIGURE 6. Differential transcriptional activities of the Tbx5 isoforms.** A, schematic representation of the different Tbx5 isoforms with the various transactivation (TAD) domains indicated. Note that Tbx5c and Tbx5d lack TAD3, and Tbx5e lacks TAD1. B, transcriptional activity of Tbx5 isoforms was determined in NIH3T3 cells using three different Tbx5 target reporters and increasing doses of the indicated Tbx5 proteins. C, effect of co-expression of the novel isoforms on Tbx5a activity. The three bars in each case represent increasing ratios of the relevant isoform versus Tbx5a as follows: 1:1, 3:1, and 10:1. D and E, synergistic activity of the various Tbx5 isoforms with Nkx2.5 (D) or Gata4 (E) on the Nppa promoter. Note the weak/absent synergy of Tbx5c and Tbx5d with Nkx2.5 and Gata4. The results shown are from one representative experiment out of at least three carried out in duplicates. Error bars indicate S.E. F, the variant regions of Tbx5c and Tbx5d do not possess autonomous transcriptional activity. C2C12 cells were transfected with the indicated GAL4-DBD fusion protein together with (UAS)₅-Tk reporter plasmid. Note that Tbx5c and Tbx5d fusion proteins do not activate transcription (Tbx5c 327–404 and Tbx5d 327–376), nor do they repress it (Tbx5c 238–404 and Tbx5d 238–376 when compared with 238–327). The results are one representative experiment of three.
expression of several genes linked to myocyte proliferation or differentiation using quantitative PCR analysis. As shown in Fig. 9A, TBX5a induces Igf1 and Bmp4 expression, whereas TBX5c inhibits Igf1 and Fgf10 transcript levels. Igf1 is involved in C2C12 proliferation and differentiation (27). Bmp4 was previously shown to inhibit C2C12 myotube formation (28). TBX5a was previously shown to activate Fgf10 promoter (21), and its induction is important for myocyte proliferation (29, 30). As reported previously, TBX5a but not TBX5c activated transcription from the Fgf10 promoter (21). TBX5c inhibited TBX5a activation of both Fgf10 and Bmp4 promoters. (Fig. 9, B and C). These results support a role of Tbx5c in myocyte differentiation and indicate that Tbx5a and Tbx5c have opposing functions on skeletal muscle progenitor expansion and differentiation.

DISCUSSION

TBX5 is a dosage-sensitive regulator of heart and limb development (31) and is the causative gene of HOS. Little is known about the mechanisms regulating TBX5 levels and activity. In this study, we provide further evidence that Tbx5 is highly regulated through alternative splicing and identify the existence of novel exons including two that result in TBX5 proteins containing new C-terminal domains. All isoforms retain the ability to bind DNA as homodimers, as well as heterodimers with TBX5a or NKX2.5. Nonetheless, they display distinctive transcrip-
Novel Tbx5 Exons Generate Distinct Protein Isoforms

Using isoform-specific antibodies, we confirm the in vivo presence of Tbx5c and Tbx5d isoforms in developing mouse heart and forelimbs where they display both overlapping but also specific expression domains relative to Tbx5a. Tbx5c, which is the dominant isoform in skeletal muscle progenitors, enhances their differentiation, and its down-regulation blocks myotube formation. The data provide for the first time insight into Tbx5 protein distribution in Tbx5-expressing cells and uncover a critical role for a novel isoform in muscle differentiation. In addition to helping us understand the role of Tbx5 in health and disease, the data may be significant for elucidating genotype-phenotype correlations in patients with mutations in the Tbx5 gene.

Genotype-phenotype correlations are a major clinical challenge in congenital heart disease in general, and in HOS patients in particular, mainly due to the wide range of mutations and the variability in disease expressivity within family

FIGURE 8. Tbx5c down-regulation inhibits myotube formation. A and B, C2C12 myoblasts were transfected with a universal control (Ctrl) siRNA or siRNA specific to Tbx5a or Tbx5c and incubated in 2% horse serum differentiating medium for 48 h (A) or 96 h (B). Note that down-regulation of Tbx5a allows myotube formation, whereas down-regulation of Tbx5c greatly reduces myotube formation. The results are one representative experiment of two.

FIGURE 9. A, quantitative PCR analysis on C2C12 myoblasts transfected with pCG, Tbx5a, or Tbx5c expression vectors. Note that Tbx5a increases Igf1 and Bmp4 transcript levels, whereas Tbx5c inhibits Igf1 and Fgf10 expression. *, p < 0.05 Tbx5a versus pCG; #, p < 0.05 Tbx5c versus pCG. B and C, NIH3T3 cells transfected with Tbx5a, Tbx5c, or Tbx5a with increasing doses of Tbx5c and Fgf10 (B) or Bmp4 (C) reporter constructs. Note that Tbx5c inhibits Tbx5a activation of both promoters. *, p < 0.0005 versus Tbx5a. Error bars indicate S.E.
members. As shown in Fig. 10, many of the known mutations in the N- and C-terminal region of TBX5 would affect TBX5a but not other isoforms; for example, mutations in exon 2 do not alter TBX5e, whereas mutations in exon 11 affect TBX5e but not TBX5c or TBX5d. This is noteworthy given the findings presented in this study that suggest that subgroups of TBX5 target genes will be differentially impacted by such mutations. Moreover, decreased TBX5a activity resulting from mutations in exon 11, which harbor TAD3 (and are specific to TBX5a), might not only decrease progenitor expansion but might also favor premature differentiation given the altered ratio of TBX5a and TBX5c activity in these cells. Coordinated cell proliferation, apoptosis, and differentiation are critical for heart and limb morphogenesis. Through alternative splicing that generates protein isoforms that can promote or antagonize each one of the processes, Tbx5 might play a distinctive role at various stages of heart and limb development. Such a paradigm may help explain the prevalence of certain phenotypes in patients harboring specific TBX5 mutations and deserves to be further explored. Lastly, for nearly 30% of HOS patients, no mutation has been found in the known TBX5 exons. Our results provide additional sequences within the TBX5 locus for mutational screening of patients with HOS and other heart and/or limb abnormalities. Moreover, intragenic mutations, such as those in introns 2, 7, 8, and 9, whose mechanism of pathogenesis is still uncertain, may be affecting expression of the isoforms reported in this study. Regulation of TBX5 alternative splicing would affect the level at which specific isoforms are produced and could contribute to the variable phenotype expressivity. Consequently, our findings provide novel insights that will help elucidate the mechanisms of action of TBX5 and shed more light on genotype-phenotype correlations in HOS and possibly other human congenital heart diseases involving TBX5.

**Acknowledgments**—We thank Drs. Benoit Bruneau and Jacques Drouin for gifts of plasmids, Chantal Lefebvre and Nathalie Bouchard for technical assistance and Hélène Touchette for secretarial help.

**REFERENCES**

1. Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Souths, J., Grayzel, D., Kroumpouzou, E., Traill, T. A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J. G., and Seidman, C. E. (1997) Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. Nat. Genet. 15, 30–35

2. Li, Q. Y., Newbury-Ecob, R. A., Terrett, J. A., Wilson, D. I., Curtis, A. R., Yi, C. H., Gebuhr, T., Bullen, P. J., Robson, S. C., Strachan, T., Bonnet, D., Lyonnet, S., Young, I. D., Raeburn, J. A., Buckler, A. J., Law, D. J., and Brook, J. D. (1997) Holt-Oram syndrome is caused by mutations in TBX5, a member of the Barchbury (7) gene family. Nat. Genet. 15, 21–29

3. Bruneau, B. G., Nemier, G., Schnitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessier, M., Nemier, M., Seidman, C. E., and Seidman, J. G. (2001) A murine model of Holt-Oram syndrome defines roles of the
Novel Tbx5 Exons Generate Distinct Protein Isoforms

T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106, 709–721

4. Heinritz, W., Shou, L., Moschik, A., and Froster, U. G. (2005) The human Tbx5 gene mutation database. Hum. Mutat. 26, 397

5. Fan, C., Liu, M., and Wang, Q. (2003) Functional analysis of Tbx5 mis-sense mutations associated with Holt-Oram syndrome. J. Biol. Chem. 278, 8780–8785

6. Garg, V., Kathiriya, I. S., Barnes, R., Schueterman, M. K., King, I. N., Butler, C. A., Rothrock, C. R., Eapen, R. S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J. C., and Srivastava, D. (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with Tbx5. Nature 424, 443–447

7. Ghiot, K., Packham, E. A., Bonser, A. J., Robinson, T. E., Cross, S. J., and Brook, J. D. (2001) Characterization of the Tbx5 binding site and analysis of mutations that cause Holt-Oram syndrome. Hum. Mol. Genet. 10, 1983–1994

8. Borozdin, W., Bravo Ferrer Acosta, A. M., Bamshad, M. J., Botzenhart, E. M., Froster, U. G., Lemke, J., Schinzl, A., Spranger, S., McGaughran, I., Wund, D., Chrzanowska, K. H., and Kohlhase, I. (2006) Expanding the spectrum of Tbx5 mutations in Holt-Oram syndrome: detection of two intragenic deletions by quantitative real time PCR, and report of eight novel point mutations. Hum. Mutat. 27, 975–976

9. Georges, R., Nemer, G., Morin, M., Lefebvre, C., and Nemer, M. (2008) Distinct expression and function of alternatively spliced Tbx5 isoforms in cell growth and differentiation. Mol. Cell. Biol. 28, 4052–4067

10. Hariri, F., Nemer, M., and Nemer, G. (2012) T-box factors: insights into the evolutionary emergence of the complex heart. Ann. Med. 44, 680–693

11. Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R., and Komuro, I. (2001) Tbx5 associates with Ncx2–5 and synergistically promotes cardiomyocyte differentiation. Nat. Genet. 28, 276–280

12. Camarata, T., Bimer, K., Kulisz, A., Chew, T. L., Yeung, J., and Simon, H. G. (2006) LMP4 regulates Tbx5 protein subcellular localization and activity. J. Cell Biol. 174, 339–348

13. Zaragoza, M. V., Lewis, L. E., Sun, G., Wang, E., Li, L., Said-Salman, J., Feucht, L., and Huang, T. (2004) Identification of the TBX5 transactivating domain and the nuclear localization signal. Gene 330, 9–18

14. Kulisz, A., and Simon, H. G. (2008) An evolutionarily conserved nuclear export signal facilitates cytoplasmic localization of the Tbx5 transcription factor. Mol. Cell. Biol. 28, 1553–1564

15. Stirmann, C. U., Petchkine, D., Grimm, C., and Müller, C. W. (2010) Structural basis of TBX5-DNA recognition: the T-box domain in its DNA-bound and -unbound form. J. Mol. Biol. 400, 71–81

16. Plageman, T. F., Jr., and Yutzey, K. E. (2004) Differential expression and function of Tbx5 and Tbx20 in cardiac development. J. Biol. Chem. 279, 19026–19034

17. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) The cardiac transcription factors Nkx2–5 and GATA-4 are mutual cofactors. EMBO J. 16, 5687–5696

18. Nadeau, M., Georges, R. O., Laforest, B., Yamak, A., Lefebvre, C., Beauregard, J., Paradis, P., Bruneau, B. G., Andelfinger, G., and Nemer, M. (2010) An endocardial pathway involving Tbx5, Gata4, and Nos3 required for atrial septum formation. Proc. Natl. Acad. Sci. U.S.A. 107, 19356–19361

19. Lavallée, G., Andelfinger, G., Nadeau, M., Lefebvre, C., Nemer, G., Horb, M. E., and Nemer, M. (2006) The Kruppel-like transcription factor KLF13 is a novel regulator of heart development. EMBO J. 25, 5201–5213

20. Aries, A., Paradis, P., Lefebvre, C., Schwartz, R. J., and Nemer, M. (2004) Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. Proc. Natl. Acad. Sci. U.S.A. 101, 6975–6980

21. Agarwal, P., Wylie, J. N., Galceran, J., Arkhitko, O., Li, C., Deng, C., Grosschedl, R., and Bruneau, B. G. (2003) Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. Development 130, 623–633

22. Nemer, G., and Nemer, M. (2003) Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. Dev. Biol. 254, 131–148

23. Charron, F., Tsimiklis, G., Arcand, M., Robitaille, L., Liang, Q., Molkentin, J. D., Meloche, S., and Nemer, M. (2001) Tissue-specific GATA-factor genes are transcriptional effectors of the small GTPase RhoA. Genes Dev. 15, 2702–2719

24. Yamak, A., Latinkic, B. V., Dali, R., Temsah, R., and Nemer, M. (2014) Cyclin D2 is a GATA4 cofactor in cardiogenesis. Proc. Natl. Acad. Sci. U.S.A. 111, 1415–1420

25. Yamak, A., Temsah, R., Maharsy, W., Caron, S., Paradis, P., Aries, A., and Nemer, M. (2012) Cyclin D2 rescues size and function of GATA4 haploinsufficient hearts. Am. J. Physiol. Heart Circ. Physiol. 303, H1057–H1066

26. Collavoli, A., Hatcher, C. J., Je, J., Okin, D., Deo, R., and Basson, C. T. (2003) Tbx5 nuclear localization is mediated by dual cooperative intra-molecular signals. J. Mol. Cell Cardiol. 35, 1191–1195

27. Sato, M., Ito, A., Kawabe, Y., Nagamori, E., and Kamihira, M. (2011) Enhanced contractile force generation by artificial skeletal muscle tissues using IGF-I gene-engineered myoblast cells. J. Biosci. Bioeng. 112, 273–278

28. Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibalée, C. F., and Lendahl, U. (2003) Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130, 6089–6099

29. Hosokawa, R., Oka, K., Yamaza, T., Iwata, J., Urata, M., Xu, X., Brinas, P., Jr., Nonaka, K., and Chai, Y. (2010) TGF-β mediated FGF10 signaling in cranial neural crest cells controls development of myogenic progenitor cells through tissue-tissue interactions during tongue morphogenesis. Dev. Biol. 341, 186–195

30. Rochais, F., Sturmi, R., Chao, C. M., Mesbah, K., Bennett, M., Mohun, T. J., Bellusc, S., and Kelly, R. G. (2014) FGF10 promotes regional foetal cardiac myocyte proliferation and adult cardiomyocyte cell-cycle re-entry. Cardiovasc. Res. 104, 432–442

31. Mori, A. D., Zhu, Y., Vahora, I., Nieman, B., Koshiha-Takeuchi, K., Davidson, L., Pizard, A., Seidman, J. G., Seidman, C. E., Chen, X. J., Henkelman, R. M., and Bruneau, B. G. (2006) Tbx5-dependent rheostatic control of cardiac gene expression and morphogenesis. Dev. Biol. 297, 566–586