Distinct Expression and Function of Alternatively Spliced Tbx5 Isoforms in Cell Growth and Differentiation

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Mutations in the T-box transcription factor Tbx5 cause Holt-Oram syndrome, an autosomal dominant disease characterized by a wide spectrum of cardiac and upper limb defects with variable expressivity. Tbx5 haploinsufficiency has been suggested to be the underlying mechanism, and experimental models are consistent with a dosage-sensitive requirement for Tbx5 in heart development. Here, we report that Tbx5 levels are regulated through alternative splicing that generates, in addition to the known 518-amino-acid protein, a C-terminal truncated isoform. This shorter isoform retains the capacity to bind DNA, but its interaction with Tbx5 collaborators such as GATA-4 is altered. In vivo, the two spliced isoforms are oppositely regulated in a temporal and growth factor-dependent manner and are present in distinct DNA-binding complexes. The expression of the long isoform correlates with growth stimulation, and its reexpression in postnatal transgenic mouse hearts promotes hypertrophy. Conversely, the upregulation of the short but not the long isoform in C2C12 myoblasts leads to growth arrest and cell death. The results provide novel insight into posttranscriptional Tbx5 regulation and point to an important role not only in cell differentiation but also in cell proliferation and organ growth. The data may help analyze genotype-phenotype relations in patients with Holt-Oram syndrome.

The T-box transcription factor Tbx5 is a dosage-sensitive regulator of heart and limb development (28). In humans, mutations in the Tbx5 gene cause Holt-Oram syndrome, an autosomal dominant disorder characterized by a wide spectrum of malformations of the upper limbs and the heart (4, 23). Cardiac defects range from asymptomatic alterations in the conduction system to septal or more complex structural defects. This variable expressivity is observed even within families and suggests the existence of genetic and/or environmental modifiers. To date, over 50 different mutations in the Tbx5 locus have been identified in patients with Holt-Oram syndrome (17). Because many mutations lie within the coding region and result in a truncated or no protein, it has been proposed that Tbx5 haploinsufficiency may be the mechanism underlying Holt-Oram pathogenesis. Consistent with this hypothesis, mice heterozygous for a deleted Tbx5 allele display a state of haploinsufficiency. Intriguingly, these mutations that associate with cardiac versus limb defects, and the correlation between genotype and phenotype remains unclear. A better understanding of Tbx5 regulation and mechanisms of action is required to enhance our insight into the mechanism(s) of pathogenesis.

In addition to mutations in the coding sequences, mutations in intragenic regions have been identified in patients with Holt-
Oram syndrome (10, 17). Furthermore, it has been suggested that unscreened mutations within introns or regulatory sequences of the Tbx5 locus may account for the relatively low (35%) detection rate in familial as well as sporadic cases of Holt-Oram syndrome (5). Such mutations could affect RNA splicing, resulting in the production of less protein or altered splice isoforms. Examination of the hTbx5 locus indeed suggests the possible existence of splice variants resulting, in at least one case, in the production of a C-terminal truncated protein at amino acid (aa) 350 (GenBank accession number NM_080718.1). The existence of a shorter hTbx5 isoform was also reported previously by Basson et al. (4).

Alternative splicing resulting in distinct protein isoforms has been reported for other T-box proteins, notably the other cardiac family member Tbx20 (37) and Tbx3 (3), the gene mutated in ulnar-mammary syndrome. Analysis of the Tbx3 isoforms revealed that alternative splicing may be tissue specific and may account for distinctive organ effects (11). Alternative splicing at the Tbx20 locus can result in several C-terminal truncated or modified isoforms. However, the temporal and spatial expression of these isoforms as well as their function remain to be elucidated.

In this paper, we report the isolation and biochemical characterization of a novel Tbx5 isoform resulting from the usage of an alternate exon 8 that produces a C-terminal frameshift leading to the production of a shorter protein. We provide evidence that the two Tbx5 isoforms have distinct biochemical properties and are produced in a growth factor-regulated manner. Thus, Tbx5 dosage may be developmentally regulated through the generation of functionally distinct isoforms. The existence of alternatively spliced Tbx5 isoforms provides new insights for understanding the pathogenesis of Holt-Oram syndrome.

MATERIALS AND METHODS

Plasmids. Both isoforms of mouse Tbx5 were amplified by reverse transcription (RT)-PCR using the same oligonucleotides harboring the start codon (forward) and stop codon (reverse). They were subcloned into XbaI/BamHI digestion vector pCGN in phase with the hemagglutinin (HA) epitope. N- and C-terminal deletions of Tbx5 were generated in the same vector using PCR-mediated mutagenesis. The same Tbx5 inserts were also cloned into vector pcDNA to produce in vitro-transcribed/translated proteins. Maltose binding protein (MBP)-Tbx5 constructs were prepared as described previously (22). The ANF-luciferase constructs as well as the GATA-4 expression vectors were previously described (6, 9, 22). All constructs were confirmed by sequencing.

RT-PCR. The oligonucleotides used for the amplification of both Tbx5 isoforms were 5'-CAAACCTCAGAACACCCACC-3' (forward) and 5'-GCAGACA GACACCATCTCACC-3' (reverse). RT-PCR was carried out as previously described (31).

Generation of Tbx5 antibody. Recombinant MBP-Tbx5 was generated by cloning the region encoding the first 60 aa of murine Tbx5 into the MBP vector as previously described (9). The recombinant protein was injected into New Zealand rabbits, and the antibody produced was purified as previously described (32). In addition to Tbx5, the following primary antibodies were used in the study: anti-HA (catalog number sc-805; Santa Cruz), anti-HA (clone 12CA5; Roche), anti-MyoD (catalog number 554130; BD Pharmingen), anti-MF-2 (Developmental Studies Hybridoma Bank), anti-edsin (catalog number BYA60851; Accurate Chemical & Scientific Corp.), anti-Flag-M2 and anti-Flag-M5 (catalog numbers F3165 and F4042, respectively; Sigma), anti-α-tubulin (catalog number sc-32293; Santa Cruz), and anti-Tb2 (catalog number 07-318; Upstate). The secondary antibodies used were biotinylated anti-goat, anti-rabbit, or anti-mouse antibody (catalog numbers BA-5000, BA-1000, and BA-2000, respectively; Vector Laboratories) and S-1122S, A-11029, and T-3605 from Molecular Probes.

Cell culture and transfections. Primary cardiomyocytes and NIH 3T3 and C2C12 cells were maintained in culture and transfected as described previously (2, 32). For immunocytochemistry, 30,000 cells per well were plated into 12-well dishes and transfected with 1 μg of expression vector. Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline (PBS) and then fixed at room temperature for 15 min with a 4% paraformaldehyde solution or 100% methanol. Lipofectamine (Gibco) was used for C2C12 cell transfection as recommended by the manufacturer. For cotransfection assays, the amount of the luciferase reporters was kept constant in all studies by the addition of the appropriate empty DNA vector. The experiments were repeated at least three times in duplicate and with different DNA preparations.

Immunofluorescence. Cells were incubated overnight with the primary antibodies in PBS–0.2% Triton X-100/NM_080718.1. The existence of a shorter hTbx5 isoform was also reported previously by Basson et al. (4).

Tbx5 isoform protein production and in vitro protein-protein interactions. In vitro protein-protein interactions were carried out using MBP fusion proteins for the long and short isoforms and in vitro-translated 35S-labeled proteins in rabbit reticulocyte lysates using the TNT system (Promega Corp., Madison, WI). Binding reactions and visualization were carried out as described previously by Durocher et al. (9). Western blots were carried out according to our previously published procedures using nuclear or whole-cell extracts (2).

Electrophoretic mobility shift assays. Nuclear or whole-cell extracts from cultured cardiomyocytes or 293T cells overexpressing Tbx5a or Tbx5b were used. Binding reactions were carried out at room temperature in the presence of 1 μg of poly(dI-dC). The brachyury (Br) and ANF and TBE2 probes were used for binding assays described previously (6).

Transgenic mice. To overexpress the long Tbx5 isoform specifically in the heart, mouse Tbx5a cDNA was subcloned in the CAG-CAT expression vector (a kind gift of M. Yanagisawa, Howard Hughes Medical Institute, Dallas, TX) in which the expression of Tbx5a was activated only after Cre-mediated recombination (20). Transgenic mice expressing CAG-CAT-Tbx5a were generated, genotyped, and bred according to standard procedures. These mice were crossed with transgenic mice expressing a tamoxifen-inducible Cre recombinase in the myocardin (33). Adult double-transgenic mice were treated with tamoxifen as described previously by Petrich et al. (33). Genotyping was carried out using PCR and standard protocols. For immunohistochemistry, hearts were processed as previously described (2).

RESULTS

Growth factor-regulated generation of alternatively spliced Tbx5 isoforms. We used RT-PCR to isolate cDNA clones containing the entire Tbx5 open reading frame from adult mouse hearts. Amplified DNA was subcloned, and 20 independent clones were fully sequenced. Of those clones, five corresponded to the expected sequence that encodes the 518-aa protein referred to as Tbx5a (Fig. 1A). The other 15 clones contained a 40-bp insertion at the 3′ border of the T-box coding region; this resulted in the addition of four new residues after aa 251 and a stop codon, thus generating a C-terminal truncated Tbx5 protein, Tbx5b (Fig. 1A and B). Sequence analysis of the genomic Tbx5 locus revealed that the insertion likely corresponds to an alternatively spliced exon 8. The fact that 75% of the isolated cDNA clones correspond to this splice isoform suggests that it may be the prevalent Tbx5 protein present in the adult heart. The presence of this novel transcript in Tbx5-expressing cells was confirmed using sequence-specific
RT-PCR-mediated amplification. As shown in Fig. 1C, this alternate splice isoform was detected in RNA from limb, P19 cells, cardiac cells (progenitor TC13 cells and atrial HL1 cells), and myoblast C2C12 cell lines.

To assess the distribution of the two Tbx5 transcripts, we designed a pair of oligonucleotides corresponding to sequences in exons 7 and 9, which are common to both isoforms. The primers allow the simultaneous detection of Tbx5a (423-bp) and Tbx5b (463-bp) transcripts. As shown in Fig. 1D, both transcripts were detected in total RNA from embryonic hearts and limbs as well as in the adult heart. Interestingly, the ratio of the two transcripts was temporally regulated as Tbx5a abundance decreased while Tbx5b increased with development. Consistent with the cloning results, Tbx5b was the dominant isoform in the adult heart. We also analyzed Tbx5 expression in proliferating (myoblasts) and differentiated (myotubes) C2C12 cells (Fig. 1D, right). Similar to what was found in the developing heart, Tbx5b transcripts were more abundant in differentiated myotubes. These results confirm the presence of alternatively spliced Tbx5 transcripts in heart and limbs and suggest a positive correlation between Tbx5b and terminal differentiation on the one hand and Tbx5a and cell proliferation on the other.

Next, we analyzed the Tbx5 proteins produced from the isolated Tbx5 cDNAs using Western blots. For this, Tbx5a and Tbx5b sequences were subcloned into expression vectors to generate HA-tagged proteins. We also generated and characterized an anti-Tbx5 antibody that targets the first 60 aa and allows the detection of endogenous Tbx5 proteins. As shown in Fig. 2A, a full-length Tbx5 protein of ~64 kDa was found in cells expressing HA-Tbx5a, while a smaller, ~35-kDa protein was present in cells transfected with HA-Tbx5b expression vectors. Both proteins were detected using the HA antibody (Fig. 2A, bottom) as well as the Tbx5-specific antibody (top). The anti-Tbx5 antibody was specific and did not recognize other Tbx proteins such as Tbx2, Tbx4, and Tbx20. We used this antibody to assess the subcellular localizations of the two Tbx5 isoforms using immunocytochemistry. While the full-length Tbx5 isoform was localized exclusively to the nucleus, the shorter isoform was found in both the nucleus and the
cytoplasm (Fig. 2B). This result was consistently observed in several different cell types including cardiac tissue (TC13 cells and cardiomyocytes) (Fig. 2B and 3D), skeletal muscle (C2C12 cells) (see Fig. 9), and NIH 3T3 fibroblasts (Fig. 4B). The broader subcellular distribution of Tbx5b is consistent with the previously reported presence of a nuclear retention signal between aa 325 and 340, which is missing in Tbx5b (7). The anti-Tbx5 antibody was also used to detect endogenous Tbx5 using immunohistochemistry on sectioned mouse embryos. At embryonic day 13.75, immunoreactive Tbx5 was detected ex-
clusively in the heart and forelimbs (Fig. 2C and data not shown), consistent with the distribution pattern of Tbx5 transcripts (6); within the heart, Tbx5-positive cells were found mostly in the atria and the trabeculae of the left ventricles, a distribution that perfectly matches that of Tbx5 transcripts.

Having confirmed the specificity of the anti-Tbx5 antibody, we next used it to analyze the profile of Tbx5 proteins in cardiac cells. We previously showed that the cardiogenic progenitor TC13 cell line expresses Tbx5 as well as GATA-4 and other cardiac markers (31). We used this cell line to obtain native nuclear extracts and analyze Tbx5 proteins therein. To enrich for DNA-binding proteins, nuclear extracts were purified using ion-exchange chromatography on a phosphocellulose column. Three fractions were obtained using a step gradient of 100, 300, or 700 mM KCl containing non-chromatin-binding proteins (100 mM) or proteins moderately (300 mM) or tightly (700 mM) bound to chromatin. Proteins in the 300 and 700 mM KCl fractions were then size fractionated under nondenaturing conditions using a gel filtration Superose 6 column. Western blot analysis using the anti-Tbx5 antibody revealed the presence of both Tbx5a (~80 kDa) and Tbx5b (~35 kDa) in the 300 mM KCl fraction (Fig. 3A, top). The 700 mM fraction contained several Tbx5a-immunoreactive bands between 64 and 80 kDa (Fig. 3A, bottom). Our preliminary analysis suggests that these bands represent posttranslationally modified Tbx5a (M. Morin and M. Nemer, unpublished results). Bioinformatic analysis of Tbx5 indicated numerous potential phosphorylations as well as two potential sumoylations. Both Tbx5a and Tbx5b were also detected in nuclear extracts from other Tbx5-expressing cells including C2C12 cells, neonate cardiomyocytes cultures, and postnatal hearts (Fig. 3B; also see Fig. 7). Total nuclear extracts from atrial as well as ventricular cardiomyocytes contained an additional band at around 50 kDa, which comigrated with Tbx51-350 and may represent a cleavage product of Tbx5 or possibly another Tbx5 isoform. Interestingly, the Tbx5a protein was markedly up-regulated in atrial cardiomyocytes treated with 15% fetal calf serum (Fig. 3B, bottom). Gel shift analysis using whole-cell cardiomyocyte extracts and a probe corresponding to the high-affinity TBE of the ANF promoter was consistent with the prevalence of Tbx5a in atria versus ventricles and its inducibility by serum (Fig. 3C). As previously reported (6), this 18-bp probe specifically interacts with Tbx5 (see Fig. 5). Figure 3C shows that the DNA-binding complexes corresponding to Tbx5a binding are increased in cardiomyocytes treated with serum, while Tbx5b-containing complexes are decreased.

The subcellular localization of Tbx5 in cardiomyocytes treated or not treated with serum was analyzed (Fig. 3D). First, HA-tagged Tbx5a and Tbx5b were transfected in cardiomyocytes, and their cellular localizations were determined using anti-HA antibody. Consistent with the localization of the two isoforms in all cell types analyzed (Fig. 2B and 4B), we found that Tbx5a is exclusively nuclear even in the absence of serum, while Tbx5b is both nuclear and cytoplasmic even in presence of serum (Fig. 3D, bottom). In contrast, in the absence of serum, endogenous Tbx5 immunoreactivity was found in both the nucleus and the cytoplasm of desmin-positive cells, while in the presence of serum, Tbx5 immunoreactivity was exclusively nuclear. These results likely reflect changes in Tbx5a/Tbx5b ratios following serum treatment (as suggested by data shown in Fig. 3B and C). Together, the findings support the likelihood that Tbx5 splicing is regulated in a growth-dependent manner.

Alternatively spliced Tbx5 isoforms have distinct transcriptional activities. In order to assess the biochemical properties of the Tbx5 isoforms described above, we carried out a structure-function analysis of Tbx5. Mutant proteins that lacked the N-terminal 52 aa or that harbored various C-terminal deletions were produced. It was noted that except for the isoforms lacking the first 52 aa, which were systematically expressed at lower levels, all other mutant proteins were expressed at similar levels (Fig. 4A). Deletion of the N-terminal domain did not alter nuclear localization, but deletion of aa 350 to 300 resulted in both nuclear and cytoplasmic localization (Fig. 4B). The finding that Tbx5 mutants lacking aa 300 to 350 are not exclusively nuclear is consistent with the previously reported presence of a nuclear localization signal between aa 325 and 340 of hTbx5 (7). The transcriptional activities of these constructs were assessed using the Tbx5 target promoter ANF. Figure 4C shows that the deletion of the last 118 aa (Tbx51-332) led to a significant drop in promoter activation, indicating the loss of a transactivation domain (TAD). A further decrease in transcriptional activity was observed when aa 300 to 255 were removed. Deletion of the N-terminal domain did not significantly decrease promoter activation but caused a right shift in the dose-response curve, likely reflecting the lower level of protein accumulation. Together, these results suggest the presence of three important functional domains in Tbx5, in addition to the T box, one in the N terminus that is likely to be involved in protein stabilization and two TADs within the C-terminal domain between aa 400 and 518 and between aa 250 and 300, with both C-terminal TADs being absent in the shorter Tbx5b splice isoform.

The inability of Tbx5b to activate the ANF promoter extended to cardiomyocytes (Fig. 5A). However, increased Tbx5b levels did not inhibit ANF promoter activity in cardiomyocytes, indicating that this isoform is not acting in a dominant-negative manner on the ANF promoter, as may have been expected. We directly tested whether Tbx5b antagonizes Tbx5a transcriptional activity. When increasing doses of Tbx5b were cotransfected with Tbx5a, no inhibition of promoter activation was observed in any cell type (Fig. 5B and data not shown). Instead, a small but consistent enhancement of Tbx5a was observed with increasing amounts of Tbx5b, reflecting either the titration of an inhibitor or the formation of more active Tbx5a/Tbx5b complexes. Gel shift analyses were carried out to assess the ability of Tbx5b to bind DNA in the presence or absence of Tbx5a using the ANF TBE as well as the palindromic brachyury site as a probe. Tbx5b retained the ability to bind both sequences albeit at a lower affinity than Tbx5a and, as a result, did not interfere with Tbx5a binding (Fig. 5C). Moreover, while Tbx5a binds DNA as a dimer, no Tbx5a/Tbx5b dimers were evident; rather, the addition of Tbx5a decreased Tbx5b binding. Together, the results suggest either that Tbx5b has a reduced affinity for DNA or that Tbx5a-DNA complexes are more stable. Either way, the C-terminal domain of Tbx5 appears to contribute to DNA binding.

In the heart, Tbx5a can modulate target gene transcription in a DNA-binding-independent manner via associations with other DNA-binding transcription factors such as Nkx2.5 and GATA-4 (6, 12, 18). The observation that increased levels of
FIG. 3. Endogenous Tbx5 expression in cardiac cells. (A and B) Western blot analysis of Tbx5 in fractionated nuclear extracts from TC13 cardiac progenitors (A), in total nuclear extracts from atrial and ventricular cardiomyocyte cultures (B), and from atrial cardiomyocytes treated or not treated with fetal bovine serum (FBS) (B, bottom). The positions of the long and short isoforms are indicated by closed and open arrows, respectively. (C) DNA-binding profile of endogenous Tbx5 in total atrial and ventricular cardiomyocyte extracts. The binding of Oct1 is used as a loading control (Ctl). (D) Confocal microscopy analysis of Tbx5 subcellular localization in cardiomyocytes treated or not treated with fetal bovine serum. To-Pro3 stains the nuclei, and desmin antibody stains the cardiomyocytes. Note the nuclear redistribution of endogenous Tbx5 in cells treated with serum. (Bottom) Localization of transfected HA-tagged Tbx5a/Tbx5b in cardiomyocytes.
Tbx5b did not affect ANF promoter activity in cardiomyocytes raised the possibility that Tbx5b may be compromised in its ability to physically or functionally interact with coactivators. To address this hypothesis, we directly tested the ability of Tbx5b to physically and functionally interact with GATA-4, an essential regulator of ANF and a known Tbx5 collaborator. Although Tbx5 and GATA-4 were shown to physically associate and cooperate in ANF promoter activation (12), little is known regarding the domains of GATA-4 or Tbx5 required for the interaction. First, we tested whether GATA-4 TADs were required for synergy with Tbx5. GATA-4 contains two TADs in its N- and C-terminal regions (26). The removal of the first 96 aa of GATA-4 dramatically decreased its transcriptional activation of the ANF promoter but did not interfere with its ability to synergize with Tbx5 (Fig. 6A). Similarly, the removal of the entire C-terminal domain, which severely impairs GATA-4 transcriptional activity, did not affect synergy, nor did the deletion of the entire N terminus and the first zinc finger. Point mutations within the first zinc finger that abolish (V217G) (8) or do not abolish (E215D) (30) the GATA-4 interaction with FOG2 had no effect on the GATA-4 association with Tbx5 (data not shown). However, the removal of both activation domains as well as a mutation within the basic domain that abolishes DNA binding abrogated synergy (Fig. 6A).
We conclude that one GATA-4 TAD is sufficient to support synergy and that residues in the basic region C terminal to the second zinc finger are essential for functional Tbx5 interactions. GATA-4 mutants were then tested for their abilities to physically associate with Tbx5 (Fig. 6B). Pull-down assays revealed that C273G, which disrupts the second zinc finger and DNA binding, as well as K299A/H301L greatly reduce Tbx5 interactions (Fig. 6B, compare the intensity of lane 2 to those of lanes 7 and 8). The removal of either or both activation domains of GATA-4 did not affect physical associations with Tbx5 (Fig. 6B, lanes 3, 4, and 5). A GATA-4 fragment containing the second but not the first zinc finger retained the ability to interact with Tbx5 (Fig. 6B, lane 9). We conclude that GATA-4 contacts Tbx5 mainly through its second zinc finger and residues just downstream within its basic region.

Next, we tested the domains of Tbx5 required for the GATA-4 enhancement of ANF promoter activation. As shown in Fig. 6C, the removal of the N terminus of Tbx5 decreased synergy with GATA-4, but the removal of the last 218 aa, which removes the major TAD (Fig. 4), had little effect on GATA-4 synergy. However, neither Tbx5b nor Tbx51-255 was able to cooperate with GATA-4 in ANF promoter activation, suggesting that aa 255 to 300, which contain the second TAD, are required for GATA-4 cooperativity (Fig. 6C) on the ANF promoter. Surprisingly, Tbx5b retained the ability to enhance GATA-4 activation of a GATA-dependent promoter, suggesting that Tbx5b can act as a transcriptional activator and a collaborator of GATA-4 on a subset of Tbx5 targets (Fig. 6C). Pull-down studies confirmed that Tbx5b retains a weak but consistent ability to physically contact GATA-4 but that the optimal physical interaction with GATA-4 requires sequences outside the T box that are absent in Tbx5b, resulting in reduced GATA-4 affinity (Fig. 6E).
FIG. 6. Mapping of the physical and functional interaction domains of GATA-4 and Tbx5. (A) Transfections of various GATA-4 mutants with Tbx5 in NIH 3T3 cells. MutZn2 is a point mutation in the second zinc finger of GATA-4 (C273G) that abolishes DNA binding; K299A/H301L also abolishes DNA binding. (B) GATA-4 contacts Tbx5 through its second zinc finger domain. Pull-down assays were performed using MBP-Tbx5a and in vitro-produced 35S-labeled GATA-4 proteins (DM is the K299A/H301L mutant), luciferase (Luc), and GATA-6. (C) Functional interaction with GATA-4 requires N- and C-terminal domains of Tbx5 and is promoter context dependent. Transfections were carried out in NIH 3T3 cells using the indicated reporters. Note how Tbx5b is able to differentially activate the two promoters. The results show the change in the enhancement of GATA-4 transcriptional activity. (D) Cotransfections of the ANF-Luc reporter with Nkx2.5 and Tbx5a/Tbx5b. Note how Tbx5b is unable to support Nkx2.5 synergy. The results show the means ± standard deviations of data from four determinations. (E and F) Pull-down assays using different MBP fusion proteins and in vitro-translated 35S-labeled full-length GATA-4 (G4) (E) or wild-type (WT) and mutant Nkx2.5 proteins (F). Note how Tbx5b has a greatly reduced affinity for GATA-4, and note its inability to interact with Nkx2.5.
In contrast, no functional interaction could be observed between Tbx5b and Nkx2.5 over the ANF or connexin 40 promoters (Fig. 6D and data not shown). Pull-down studies indicated that, unlike Tbx5a, Tbx5b could not be retained on an MBP-Nkx2.5 resin and that no Nkx2.5 proteins could be retained on an MBP-Tbx5b column (Fig. 6F and data not shown).

The above-described biochemical analysis together with the finding that Tbx5a and Tbx5b are present in distinct DNA-binding protein complexes (Fig. 3A) suggest that the two isoforms may have differential downstream targets and regulate distinct cellular processes. The results also indicate that the ANF promoter is a Tbx5a but not a Tbx5b target.

To verify the chromatographic profiles of Tbx5a and Tbx5b during postnatal heart development, nuclear extracts were prepared from neonate and adult hearts. The enriched DNA-binding fraction (elution at 300 or 700 mM KCl over the phosphocellulose ion-exchange column) was size separated using gel filtration. In neonate extracts, Tbx5a was the predominant isoform in the 300 mM fraction and the only detected isoform in the 700 mM fraction (Fig. 7A and data not shown). Interestingly, Tbx5a and GATA-4 coeluted in the same fractions (Fig. 7A). In adult hearts, Tbx5b was abundantly present in the 700 and the 300 mM fractions (Fig. 7B and data not shown) and was mostly present in very-high-molecular-weight complexes. Western blots also revealed a coelution of Nkx2.5 with the 70-kDa Tbx5a isoform and the presence of GATA-4 in fractions containing Tbx5a (70- and 80-kDa forms) as well as Tbx5b (Fig. 7B). This elution profile is consistent with the in vitro analysis detailed above and in line with the possibility that the two Tbx5 isoforms participate in distinct cellular pathways. Moreover, the higher abundance of Tbx5b than Tbx5a in the adult heart contrasted with the higher abundance of Tbx5a than Tbx5b in the neonate heart and in proliferating TC13 cardiogenic progenitors (Fig. 3A) and C2C12 myoblasts (data not shown) and is consistent with data in Fig. 1D and 3B, all of which show a positive correlation between Tbx5a and cell proliferation and Tbx5b and cell differentiation.

**Effect of altering Tbx5 ratios.** Given the distinct properties of Tbx5a and Tbx5b as well as their differential temporal expression levels during development and in response to growth factors, we tested the consequences of altering their distributions. Since Tbx5b is the predominant isoform in postnatal ventricles, we decided to assess the effect of the reexpression of Tbx5a. For this, we generated CAG-CAT-Tbx5a transgenic mice in which Tbx5a expression is activated only after Cre-mediated recombination. To induce Tbx5a expression in the adult heart, we crossed this line with a tamoxifen-inducible anti-myosin heavy chain-driven Cre transgenic line. Adult double-transgenic mice were treated with tamoxifen for 1 week and analyzed. As shown in Fig. 8, this treatment resulted in the expression of Tbx5a in the ventricles (Fig. 8, top) as well as the upregulation of ANF (Fig. 8, bottom). Interestingly, the reexpression of Tbx5a in adult ventricles led to cardiac hypertrophy, as evidenced in Fig. 9. Tbx5a-overexpressing hearts were visibly larger than controls (Fig. 9A). Histological analysis revealed that this is likely due to myocyte hypertrophy (Fig. 9B, left). Immunohistochemical analysis of proliferation markers including cyclin A and Ki67 found no detectable evidence of increased proliferation in the Tbx5a transgenic hearts (Fig. 9B, middle and right). Given that hypertrophy is the response of terminally differentiated cardiomyocytes, these data are consistent with a growth-promoting role of Tbx5a that is reminiscent of that of GATA-4 (24). Thus, in addition to its role in atrial specification, Tbx5a may regulate myocyte growth.

We also analyzed the effect of altering the Tbx5a/Tbx5b ratio in C2C12 myoblasts. As stated above, Tbx5a is the predominant isoform in proliferating myoblasts. Therefore, we tested the effect of overexpressing Tbx5b therein. As shown in Fig. 10A and B, the transient transfection of Tbx5b consistently resulted in marked cell death and very few Tbx5b-positive cells (Fig. 10A, top). Cell morphology was also consistently altered, with many cells appearing to be either elongated or condensed. This was not the case when either Tbx5a or GATA-4 was similarly transfected in C2C12 myoblasts (Fig. 10A, bottom, and data not shown). TUNEL assays were carried out to assess
whether Tbx5b altered cell survival. As shown in Fig. 10B and C, cells transiently expressing Tbx5b had a threefold increase in the rate of apoptosis, whereas cells expressing Tbx5a had a low level of apoptosis similar to those of cells that did not express the transgene.

These observations suggest that increased levels of Tbx5b may cause growth arrest, a result consistent with the prevalence of Tbx5b in terminally differentiated myocytes. Together, the data are consistent with the notion that Tbx5 isoforms have distinct biochemical and functional properties and that Tbx5 function may be regulated at the splicing level. The results also point to a role for Tbx5 in cell growth.

**DISCUSSION**

It is now well established that Tbx5, the gene mutated in Holt-Oram syndrome, is a dosage-sensitive regulator of heart and limb development. However, little is known regarding the modulation of Tbx5 levels and activity. In the present work, we provide evidence for Tbx5 regulation through alternative splicing that generates two isoforms with distinct biochemical properties in a temporally specific and growth factor-dependent manner. The regulated production of alternatively spliced Tbx5 isoforms provides a new paradigm that may help to elucidate the variable expressivity and genotype-phenotype relation in patients with Holt-Oram syndrome.

**Functional Tbx5 domains and regulation of Tbx5 levels and activity.** Tbx5 has been shown to bind to target promoters and activate transcription alone or in collaboration with other transcriptional regulators such as Nkx2.5 (6) or GATA-4 (12). In the present work, we found that in addition to an intact T box, sequences C terminal of the T box that are present in only one of the Tbx5 isoforms contribute to the physical association with GATA-4 and Nkx2.5. The decreased affinity of Tbx5b for GATA-4 affects its ability to potentiate the GATA-4 activation of the ANF promoter but not that of a GATA-dependent BNP promoter. These results raise the possibility that Tbx5b, which lacks the entire C-terminal domain, may retain the ability to activate the transcription of a subgroup of Tbx5 target genes.

Previous studies indicated that the C terminus of Tbx5 harbors a transcriptional activation domain(s). Using Gal chimeras, Plageman and Yutzey (34) previously reported that aa 266 to 515 of mouse Tbx5 contain a potent TAD. Others, using a similar Gal4-Tbx5 fusion in a modified yeast one-hybrid system, reported that an autonomous TAD resides between aa 339 and 379 (38). In the present work, we tested the abilities of
various Tbx5 C-terminal deletion mutants to activate a Tbx5 target promoter. Our results indicate that the C-terminal domain of Tbx5 is required for transcriptional activation within the context of the native protein. Additionally, we found two discrete domains within the C terminus that contribute to activation. The first is located between aa 400 and 518, and the second is located between aa 255 and 300, which was not previously described. Our results do not exclude the possibility of additional TADs, such as the one reported between aa 339 and 379, that may function cooperatively with downstream domains in the context of the native Tbx5 protein.

The C-terminal domain of Tbx5 was shown to interact with

FIG. 9. Reexpression of Tbx5a in adult hearts leads to cardiomyocyte hypertrophy. (A) Gross morphologies of freshly dissected hearts from control and Tbx5a-overexpressing hearts. Note how tamoxifen-induced expression of Tbx5a is associated with cardiac enlargement. The hearts shown are representative of two separate experiments, each carried out with four mice in each group. (B) Analysis of heart sections from vehicle (top)- and tamoxifen (bottom)-treated transgenic mice. (Left) Masson-trichrome staining. Note the enlarged size of the myocytes. (Middle and right) Immunohistochemical labeling with the proliferation markers Ki67 (middle) and cyclin A (right). No change was detected following the reexpression of Tbx5a in the adult heart. IVS, interventricular septum.
FIG. 10. Differential effects of transient transfection of Tbx5 isoforms in C2C12 myoblasts. (A) Transient expression of HA-tagged Tbx5 proteins in C2C12 myoblasts in the presence of serum. Tbx5-expressing nuclei are in red. The smaller amount of Tbx5b-positive cells was consistently obtained in over 10 different experiments carried out by three independent experimenters. Also note the altered morphology of Tbx5b- but not Tbx5a-transfected cells. MyoD staining is used as a control. (B) Tbx5b increases cell apoptosis. TUNEL assays were carried out on C2C12 cells transiently transfected with a Flag-tagged Tbx5a or Tbx5b vector. Cells expressing the transgenes are labeled in red (with anti-Tbx5) and yellow (with anti-Flag), and apoptotic nuclei are in green. (C) Quantitative analysis of apoptosis. The data shown are expressed as the ratio of TUNEL-positive cells in transfected cells to that in nontransfected cells in the same petri dish. The results are from two transfections and represent the means of data from at least 10 different fields.
transcriptional coactivators such as WW domain proteins, whose presence enhances Tbx5 activity (29). In this case, a functional interaction is abrogated when aa 317 to 318 are deleted. In the future, it will be interesting to test whether either TAD identified in the present work is involved in this interaction. The C-terminal domain of Tbx5 was also shown to associate with the Lim protein LMP-4, leading to decreased nuclear Tbx5 levels and relocalization to the cytoskeleton (21). While the exact Tbx5 domain involved in this interaction has not been mapped, it is noteworthy that in our study, a deletion of the last 178 aa did not alter the subcellular distribution. Whether this reflects an interaction of LMP-4 with aa 239 to 350 will be worth testing. In this respect, our results showed that the deletion of aa 300 to 350 causes a nuclear-cytoplasmic redistribution of Tbx5 consistent with the previously reported presence of a nuclear localization signal between aa 325 and 340 (7). Thus, nuclear Tbx5 levels and activity can be regulated posttranscriptionally through protein-protein interactions, many of which target the Tbx5 C-terminal domain.

The data presented provide, for the first time, information on endogenous Tbx5 proteins. Other than the expected 70- and 35-kDa bands comigrating with Tbx5a and Tbx5b, other immunoreactive bands were detected in Tbx5-expressing cells, suggesting extensive posttranslational modifications. In silico analyses of Tbx5 revealed the existence of numerous phosphorylation sites, mostly in the C terminus, as well as consensus motifs for other modifications (including sumoylation), which would produce higher-molecular-weight isoforms. Remarkably, the elution profile suggests that these modifications may alter protein-protein interactions, as evidenced by the fact that the different isoforms are present in distinct DNA-binding fractions.

Finally, our study reveals a new mechanism for regulating Tbx5 expression and activity that involves the alternative splicing of the Tbx5 gene to produce two isoforms with distinct temporal distributions and activities. The novel isoform, Tbx5b, lacks the entire C-terminal domain but retains the ability to bind DNA. The altered DNA-binding profile may provide a possible mechanism to explain why an isoform lacking TADs does not act as an inhibitor of the full-length Tbx5 protein. Indeed, we found that Tbx5b formed predominantly monomeric complexes with DNA that were displaced by Tbx5a, leading exclusively to Tbx5a homodimeric complexes. No Tbx5a/Tbx5b heterodimers could be detected by gel shift assays. In pull-down experiments, Tbx5a was efficiently retained on a glutathione S-transferase–Tbx5a column but not on a glutathione S-transferase–Tbx5b column (data not shown). These results suggest that sequences C terminal of the T box are involved in optimal Tbx5-DNA interactions, possibly through dimer formation. A previous report also suggested that residues outside the T box affect Tbx5 binding to an artificial palindromic site in a complex manner; for example, Tbx51-237 bound DNA but largely as a monomer, but the addition of 4 aa (Tbx51-237) abrogated DNA interactions (14). Consistent with this, the elution profile of endogenous Tbx5 proteins in cardiac cells showed that the interaction of high-affinity Tbx5b with chromatin occurs in the context of a large DNA-binding complex (Fig. 7). Together, these results indicate that the C-terminal domain of Tbx5 contributes to high-affinity DNA binding and influences complex formation.

Expression and effects of Tbx5 isoforms in cell growth and differentiation. In addition to their distinct biochemical properties, the expression pattern of Tbx5 isoforms suggested a differential role in cellular proliferation. Both in vivo and in cultured cells, the expression of Tbx5a correlated with proliferative growth, while Tbx5b was predominant in more differentiated, less proliferative cells. Thus, the ratio of Tbx5b/Tbx5a in the heart increased during embryonic development, and by embryonic day 13.5, Tbx5b was by far the predominant isoform. By this stage, heart morphogenesis is near completion and, importantly, myocyte proliferation is tempering down. In cultured postnatal myocytes, it is possible to reverse this ratio by growth factor stimulation (Fig. 3). Interestingly, Tbx5a was predominant in neonate hearts, but the Tbx5b/Tbx5a ratio increased in adult hearts, suggesting that Tbx5a may also be involved in postnatal hypertrophic growth. The reexpression of Tbx5a in adult hearts using spatial and temporal inducible transgenic lines led to cardiac hypertrophy (Fig. 9), consistent with a causative link between Tbx5a and cardiomyocyte growth. A similar growth factor-dependent distribution of the two isoforms was observed in C2C12 myoblasts, where Tbx5a is dominant in proliferating myoblasts, while Tbx5b is present at a higher level in differentiated myotubes (Fig. 1B). The misexpression of Tbx5b but not Tbx5a in proliferating C2C12 cells caused growth arrest and apoptosis (Fig. 10). Further analysis of the role of Tbx5b in cell survival and/or cell cycle arrest will require the generation of inducible Tbx5b-expressing transfectants.

In addition to its role in cellular differentiation, accumulating evidence supports a role for Tbx5 in cell growth regulation. For example, several studies suggested a role for Tbx5 in limb outgrowth, in part through Fgf10 expression, rather than a role in limb specification or identity (1, 27, 36). Tbx5 has also been implicated in forelimb regeneration (19). At the level of the heart, a gain of Tbx5a function in transgenic chick or mouse hearts was shown to result in growth retardation and hypotrauculation (16, 25). Although Hatcher et al. previously suggested that their results demonstrate that Tbx5a is a negative regulator of proliferation (16), the phenotype may well reflect a loss of ventricular specification, as shown previously by Liberatore et al. (25). The loss of Tbx5 function in knockout mice did not support a negative role for Tbx5a in growth regulation (6). More recently, it was shown that Tbx5 depletion in Xenopus laevis decreases cardiac cell numbers and interferes with cell cycle progression (15). Our results are consistent with a growth-promoting effect of Tbx5a in the heart as well as in C2C12 myoblasts, where a persistent expression of Tbx5a promoted proliferation even in the absence of serum (data not shown). Ongoing experiments are aimed at identifying Tbx5a targets in cell growth.

Regulation of Tbx5 splicing and implications for Holt-Oram syndrome. An understanding of genotype-phenotype relations remains a major clinical challenge for patients with Holt-Oram syndrome due in part to variable intrafamily expressivity and the large spectrum of mutations. Some of these mutations lie in the C-terminal coding region and would affect the Tbx5a isoform solely. Based on our results, it is intriguing to speculate that in such patients, only a subset of Tbx5 target genes and processes may be disrupted, resulting in milder phenotypes. The heartstrings mutation in zebrafish, which is due to a mu-
tation in Tbx5 that leads to the formation of a C-terminally truncated protein (Tbx5Δ・316), causes a less severe cardiac and limb phenotype than does the null Tbx5 mutation (13) and is consistent with our hypothesis. Moreover, in a recent study, a gain of Tbx5 function in cultured cardiogenic cells revealed that an equivalent human mutant (R297er) alters the ability of Tbx5a to induce some but not all target genes (35).

Finally, our results point to new sequences within the Tbx5 locus in which mutations may be associated with Holt-Oram syndrome or other congenital cardiac and/or limb defects. Regulated splicing at the Tbx5 gene would alter the level of Tbx5a and/or the ratio of the two isoforms, thus impacting total Tbx5 levels. Considering the exquisite Tbx5 dosage sensitivity, we propose that the regulation of Tbx5 splicing may provide a paradigm that helps explain the variable expressivity of a given Tbx5 mutation within families as well as the genetic back-ground-dependent severity of defects resulting from a Tbx5 haploinsufficiency.

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