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TBX5, a Gene Mutated in Holt–Oram Syndrome, Is Regulated Through a GC Box and T-Box Binding Elements (TBEs)

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Abstract *TBX5* is a member of the T-box gene family and encodes a transcription factor that regulates the expression of other gene(s) in the developing heart and limbs. Mutations of TBX5 cause Holt–Oram syndrome (HOS), an autosomal dominant condition characterized by congenital heart defects and limb anomalies. How *TBX5* gene expression is regulated is still largely unknown. In order to identify transcription factors regulating TBX5 expression, we examined the 5′-flanking region of the human *TBX5* gene. We determined that up to 300 bp of the 5′-flanking region of the *TBX5* gene was necessary for promoter activity in mouse cardiomyocyte ECL2 cells. One GC box, three potential T-box-like binding elements (TBE-A, -B, and -C), and one NKX2.5 binding site were identified. Site-directed mutagenesis of the potential binding sites revealed that the GC box, TBE-B, TBE-C, and NKX2.5 are functionally positive for the expression of TBX5. DNA footprint analysis showed that these binding regions are resistant to DNaseI digestion. Electrophoretic mobility shift assays (EMSAs) further demonstrated the protein–DNA interactions at the GC box and the potential TBE-B, TBE-C, and NKX2.5 sites in a sequence-specific manner. The ability of TBX5 to regulate its own promoter was demonstrated by the ability of ectopically expressed human TBX5 to increase reporter expression. We conclude that the GC box, T-box-like binding elements, and NKX2.5 binding site play important roles in the regulation of TBX5 expression, and that TBX5 is likely to be autoregulated as part of the mechanism of its transcription. J. Cell. Biochem. 92: 189–199, 2004.

Key words: TBX5; Holt–Oram syndrome; promoter; regulation

TBX5 is a member of the T-box family of transcription factors. Mutations of TBX5 cause Holt–Oram syndrome (HOS), an autosomal dominant condition characterized by cardiac malformations and upper limb anomalies [Holt and Oram, 1960; Basson et al., 1994, 1997, 1999; Bonnet et al., 1994; Terrett et al., 1994; Newbury-Ecob et al., 1996; Sletten and Pierpont, 1996; Li et al., 1997; Yang et al., 2000; Cross et al., 2001; Garrity et al., 2002]. TBX5 is expressed in the developing heart, limb bud, lung, trachea, and retina and affects cardiac development and limb formation at an early stage [Li et al., 1997; Gibson-Brown et al., 1998; Basson et al., 1999; Bruneau et al., 1999; Yang et al., 2000; Cross et al., 2001; Huang, 2002; Brassington et al., 2003; Fan et al., 2003; Huang et al., 2003].

TBX5 expression is precisely controlled in the developing heart and limbs in humans and mice. Haploinsufficiency of TBX5 causes HOS. Overexpression of TBX5 in the heart showed loss of ventricular-specific gene expression and retardation of ventricular chamber morphogenesis in the embryos [Liberatore et al., 2000]. The hypothesis that TBX5 expression is precisely controlled in the developing heart and limbs is further supported by observation of such functions as the inhibition of cell proliferation

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[Hatcher et al., 2001; He et al., 2002], which requires precise levels of TBX5. Autoregulation is a common mechanism for control of transcription factors [Laffitte et al., 2001; Niehof et al., 2001]. Isolation of the functional promoter and identification of its transcriptional factors not only could lead to identification of the gene(s) regulating TBX5 expression and elucidation of the upstream molecular cascades of TBX5, thereby providing a unique window onto the causes of more common congenital heart diseases (CHDs) and limb anomalies, but also may allow us to examine the possibility of autoregulation.

TBX5 has three isoforms that differ in exon 1 (exon 1A, 1B, and 1C) [Basson et al., 1999], and all of them were alternatively spliced to exon 2. Among the three isoforms, 1C [Li et al., 1997; Basson et al., 1999] is the most abundant TBX5 isoform in heart (data not showed). Therefore, in this study, we focused on molecular dissection of the promoter of TBX5-1C. We found that up to 300 bp of the 5′-flanking region of the *TBX5* gene were necessary for promoter activity in mouse cardiomyocyte ECL2 cells. One GC box, three potential T-box binding sites (TBE-A, -B, and -C), and one NKX2.5 binding site were identified. Site-directed mutagenesis of the potential binding sites revealed that the GC box, TBE-B, TBE-C, and NKX2.5 sites are functionally important for the expression of TBX5. DNA footprint and electrophoretic mobility shift assays (EMSAs) further demonstrated the protein–DNA interactions at those sites in a sequence-specific manner. The ability of TBX5 to regulate the TBX5 promoter was demonstrated by co-transfecting the human TBX5 expression construct and TBX5 promoter luciferase reporter construct. Our results indicate that the GC box, TBEs, and NKX2.5 binding site play important roles in the regulation of TBX5 expression and that TBX5 is autoregulated as part of the mechanism of its transcription.

MATERIALS AND METHODS

Cell Lines

ECL2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% chicken embryo extract. MCF7 cells were grown in the same medium without chicken embryo extract. The cells were maintained in a 5% CO_2 incubator at 37°C.

Plasmid Constructs

Human TBX5-1C promoter-luciferase plasmids were constructed as follows: the fragments between −1347, −500, −400, −300, −250, −200, −150, −100, −45, and +75 bp of the 5′-flanking sequences of the human *TBX5* gene 1C promoter were PCR-amplified with pfuturbo or pfu ultra (Stratagene, La Jolla, CA) by using the corresponding primer pairs (Table I). All forward primers and reverse primers bear *Hind*III and *Kpn*I restriction enzyme sites, respectively. The PCR products were digested with *Hind*III and *Kpn*I and ligated into the *Hind*III and *Kpn*I sites of the luciferase reporter vector pGL3 Promega, Madison, WI). The potential transcription factor binding sites were mutated using a quick-change site-directed mutagenesis kit (Stratagene). The mutated sequences were the same as the mutated probes for EMSAs, as shown in Table I. All the constructs were verified by DNA sequence analysis.

Transfection and Luciferase Assay

Transfection was performed using the lipofectamide method (Invitrogen, Carlsbad, CA) in 24-well plates following the manufacturer's protocol. The day before transfection, 8×10^4 of ECL2 cells or MCF7 cells were plated in each well. For each well, 1 μl of lipofectamine reagents was mixed with 300 ng of various reporter plasmids, and 100 ng of the pRL-TK plasmid (Promega) in serum-free Opti-MEM and were then added to the respective wells. The cells were harvested after 48 h, and firefly luciferase and *Renilla* luciferase assays were performed using the Promega Dual-Luciferase Reporter Assay System. The activities of the luciferase constructs were normalized to the *Renilla* luciferase activity of the pRL-TK construct. All transfections were done in triplicate and repeated at least three times.

DNase I Footprinting Assay

The TBX5-1C promoter region −100 to +75 was PCR-amplified with forward primer 1C − 100 and reverse primer 1C + 75 (Table I). The region −300 to +50 bp was PCR-amplified with 1C − 300 and 1C + 75. The PCR products were purified using a Qiagen PCR purification kit and then end-labeled with ^{32}P-γ-ATP and T4 polynucleotide kinase. After the 3′ end was trimmed with *Hind*III digestion, the 5′ end-labeled fragment was gel-purified. The labeled

probe was incubated with or without ECL2 cell nuclear extract prepared as described [Muller et al., 1989]. This mixture was then treated with DNaseI for 2 min at room temperature, followed by phenol/chloroform extraction, and ethanol precipitation. The pellet was dissolved, boiled, and analyzed on a 10% denaturing gel. Maxam and Gilbert [1977] sequencing reactions with the labeled probe were used as ladders.

EMSAs

The nucleotide sequences of oligonucleotides used in EMSAs are shown in Table I. Complementary single-strand oligonucleotides were annealed, end-labeled with ^{32}P-γ-ATP, and T4 polynucleotide kinase, purified by G-25 sephadex columns (Roche, Indianapolis, IN), and used as probes. Unlabeled oligonucleotides were used as competitors. In each experiment, 4 μg of ECL2 cell or MCF7 nuclear extracts were incubated for 30 min at room temperature with 15 fmol labeled probe in binding buffer consisting of 200 ng of poly(dI-dC), 20 mM Tris-HCl, 2 mM $MgCl_2$, 80 mM NaCl, and 5% glycerol. For competition analysis, the 200-fold of unlabeled competitor DNAs was added to the binding mixture. After completion of the binding reaction, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel (37.5:1, acrylamide:bis-acrylamide) in 45 mM Tris-borate, 1 mM EDTA (pH 8.0) at 150 V for 2 h. The gels were dried and autoradiographed.

TBX5 Autoregulation Assay

To produce the replication-defective retrovirus expressing TBX5, a full-length human TBX5 cDNA was subcloned into *BamH*I and *Not*I sites of pFB-Neo (Stratagene). The constructs were transfected into a PT67 packaging cell (gift of Xing Dai Lab at UCI). The transfection was performed using an Invitrogen Transfection Kit as described above. The cells were grown in DMEM with 10% FBS and selected with 500 μg/ml G418 for 2 weeks starting 2 days after transfection. Forty-eight hours before harvesting the viruses, the medium was changed back to G418-free medium. The supernatants were filtered, and the titers were determined in NIH3T3 cells by following the manufacturer instructions (Stratagene). MCF7 cells were infected using the TBX5 expression construct pFB-TBX5, and vector pFB-Neo was used as a control. Luciferase assay was performed by transfecting cells with pGL3-1C-500 after 2 weeks of selection using G418 (500 μg/ml). The luciferase assays were performed as above. The nuclear extract from MCF7 cells infected with replication defective retrovirus expressing TBX5 was separated on 4–10% PAGEr Precast Gel (CAMBREX, Rockland, ME), and Western blot assay was performed to examined TBX5 overexpression. In the Western blot, rabbit anti-human TBX5 antibody was used as primary antibody and developed with donkey anti-rabbit Ig-horseradish peroxidase antibody (Amersham

Table1. Oligonucleotides Used in Electrophoretic Mobility Shift Assays

	Sequences
500F1C	5' GCAGGTACCCCAGGGGCGTCTACGCGGTACGTT 3'
400F1C	5' GCAGGTACCATTGTGCCCATTGATCAGACTAA 3'
300F1C	5' GCAGGTACCGAAACCACCTCTCTCCACTCCCAC 3'
250F1C	5' GCAGGTACCCGCCCCTGCCGGGCCACTCTGGGA 3'
200F1C	5' GCAGGTACCCTCCCTCTGTCTCGTGTCCCCTCC 3'
150F1C	5' GCAGGTACCCTATGCAAGTCTGCAAGAGGGCAC 3'
100F1C	5' GCAGGTACCGCCCAGAGCTCCTCCGCGGGGGA 3'
75R1C	5' GCGAAGCTTGGGAACTCTTCACGAAGGGAGGTG 3'
Sp *	5' CTCGCACCCCGCCCCTGCCG 3'
Sp-mut*	5' CTCGCAGCTCACGCCTGCCG 3'
Sp1-Promega *	5' GCTCGCCCCGCCCCGATCGAAT 3'
Sp1-Non-specific *	5' GATCGAACTGACCGCCCGCGGCCCGT 3'
TBE-A, B, C *	5' GAGGTCTGAGGTGGACTCCCACCTC 3'
TBE-A-mut *	5' GACCTCTCAGGTGGACTCCCACCTC 3'
TBE-B-mut *	5' GAGGTCTGCGTTCGACTCCCACCTC 3'
TBE-C-mut *	5' GAGGTCTGAGGTGGACTGCCAGGTC 3'
NKX2.5 *	5' CCACCTCCCTTCGTGAAGAG3'
NKX2.5-mut *	5' CCACCTCCCTTCGTAACGAG 3'

* Reverse complimentary oligonucleotides are not shown

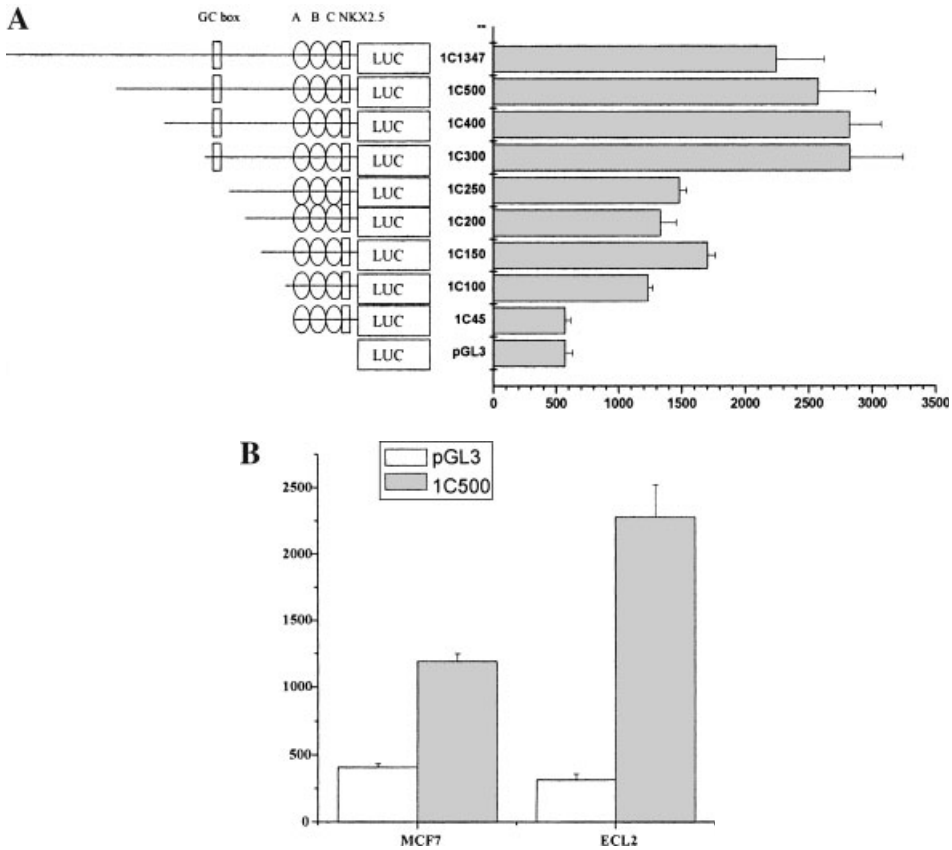

Fig. 1. Luciferase activity assays in ECL2 cells transfected with different lengths of TBX5-1C promoter constructs. **A**: The fragments between −1347, −500, −400, −300, −250, −200, 150, −100, −45, and +75 bp of the 1C 5′-flanking sequences of the human *TBX5* gene were PCR-amplified and subcloned into the *Kpn*I and *Hind*III sites of the vector pGL-3. The resultant plasmids were then transfected into ECL2 cells, and luciferase activity was examined. Transfection was performed using the lipofectamide method (Invitrogen). After an additional 48 h of incubation, the cells were used to measure firefly luciferase activity, and the results were normalized according to the *Renilla* luciferase activity. All transfections were repeated at least three times. The mean luciferase activity of transient transformants is presented as a filled bar, in relative light units (RLU), and the standard deviation is indicated by the thin line. **B**: The 1C-500 construct was transfected into MCF7 cells, a human breast cancer cell line and ECL2 cells and the luciferase activities were analyzed. The promoter activity of 1C-500 is significantly higher in ELC2 cells than that in MCF7 cells.

Pharmacia Biotech, UK Limited, Bucks, UK) as second antibody.

RESULTS

Proximal −300 to +75 bp of the 5′-Flanking Region of the TBX5-1C Promoter Are Important for the Promoter Activity in ECL2 Cells

Previously, we identified three isoforms of TBX5 that differed in exon 1 (exon 1A, 1B, and 1C) [Basson et al., 1999]. All of them were alternatively spliced to exon 2. This result suggests that TBX5 may have three transcriptional start sites. Among the three isoforms, 1C is consistent with the data published by others [Li et al., 1997; Brassington et al., 2003]. The transcription start sites in the developing human heart were confirmed by a primer extension and RACE RT/PCR (data not shown). To elucidate the regulatory region of the human *TBX5* gene, a P1 clone that included exon 1C and its previously isolated 5′-flanking region was used as a template, and then we subcloned a series of fragments comprising nucleotides −1347 to +75 relative to the transcription starting site.

To define the minimal promoter region of TBX5, we performed luciferase reporter assays in ECL2 cells transfected with different lengths of promoter constructs. ECL2 cells are mouse cardiomyocyte cell line and were generously provided by Dr. Brunskill and Dr. Potter at the Cincinnati Children's Hospital [Brunskill et al., 2001]. The gene-expression profiles showed the transcription of *tbx5*, *Nkx2.5*, *GATA4*, *dHAND*, cardiac troponin C, and other cardiac-specific genes [Brunskill et al., 2001]. As shown in

Figure 1A, 300 bp of 5′ upstream region of 1C are able to drive luciferase reporter expression. There is no significant difference in luciferase activity among −1347, −500, −400, and −300 bp. After deletion of the −300 to −250 bp, the luciferase activity decreased to about 50% as compared with the 1C − 300 constructs, suggesting that a strong positive regulator element is located in this region. When the promoter region was deleted to −45 bp, the luciferase activity decreased to the control level. To search for the potential transcription factor binding sites, we analyzed the sequences by using the tool at (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). A GC-box located at −265 to −275 bp was found, which harbored a transcription factor Sp binding site. Since many transcription factors have autoregulatory mechanisms by which they are able to control their own expression levels, and the consensus TBX5 binding sequence (A/G)GGTGT(C/G/T)(A/G), which is part of the Brachyury binding site [Kispert and Herrmann, 1993; Muller and Herrmann, 1997], has been defined using in vitro random oligonucleotide selection [Ghosh et al., 2001], we analyzed the TBX5 promoter region and found three T-box-like binding sites, as shown in Figure 2 (TBE-A, -B, and -C). The first two sites are in tandem, while the third is in inverted orientation. Each of the potential binding sites has only one nucleotide different from those of the TBX5 binding site published [Ghosh et al., 2001]. We also found a potential transcription factor NKX2.5 binding site near the T-box-like binding sites.

The construct 1C-500 was transfected into MCF7 cells, a human breast cancer cell line without endogenous TBX5 expression, and ECL2 cardiomyocytes luciferase activity was analyzed (Fig. 1B). The luciferase activity in MCF7 is significant lower in MCF7 than that in ELC2 cells. This result suggest 1C-500 has a much weaker promoter activity in MCF7 cell line and some transcription factors specific in ECL2 cardiomycytes are required for 1C-500 promoter activity. Another interpretation would be a negative regulator in MCF7 cell line which inhibits the promoter activity.

Mutation Analysis of the Potential cis-Acting Elements

To understand the contribution of the GC box, the potential T-box-like binding elements and NKX2.5 binding elements to TBX5-1C promoter activities, we performed mutagenesis on those potential transcription factor binding sites and tested the effects of mutations on reporter gene expression. As shown in Table I, for each potential transcription factor binding site we introduced 2–4 bp changes to the 1C-500 constructs. Figure 3 showed that mutation at the potential TBE-A binding site did not change luciferase activity significantly, while the mutant TBE-B and -C sites significantly decreased luciferase activity ($P < 0.001$ and 0.05, respectively), suggesting that TBE-B and -C are functionally important for TBX5-1C promoter activities. Mutations at the GC box and NKX2.5 sites decreased luciferase activity 84 and 63%, respectively. These studies demonstrate that the GC box and the potential TBE-B, -C, and NKX2.5 sites are functionally important for TBX5 expression.

Physical Interaction of Protein–DNA at the GC Box and at the Potential TBE-B, -C, and NKX2.5 Sites

To test whether any transcription factors bind those sites, we performed a DNaseI footprinting assay. As shown in Figure 4A, the GC box region was protected from DNAseI digestion. Figure 4B shows that two regions were protected from DNAseI digestion: one spanned the potential TBEs and may extend to the NKX2.5 site, and the other is between −46 and −22, which contains a potential homeobox protein HOX-1.3 (HOXA5) binding site. This result further supports our hypothesis that the potential the GC box, TBE-A, -B, and -C and NKX2.5 binding sites interact with transcription factors.

To further confirm the specificity of the protein–DNA interaction, we performed EMSAs using nuclear extracts of ECL2 cells. The oligonucleotides that correspond to those transcription factor binding sites were end-labeled and incubated with ECL2 nuclear extracts in the presence or absence of a 200-fold molar excess of competitor oligonecleotides (Fig. 5). After completion of the binding reaction, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel (37.5:1, acrylamide:bis-acrylamide) in TBE buffer, and the gel was dried and exposed to X-ray film. Figure 5 confirmed the protein–DNA interaction at those sites. As shown in Figure 5A, lane 2, two major DNA–protein binding complexes were detected in the

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-500  GTTTTGCAACTCAACCTAGTGGGTTTCCAGCGGTGCGCAAAAGTTTGCGA
-450  GCATCCACCACTGCGCTGCTTAGAAATTGTGCCCATTGATCAGACTAAAA
-400  ATAATAGTCGTCGTGATTACAAAACAAAATAGAGTGCCTCGTGCCTCGGC
-350  GGGCCCTGGTACAATAATTATTCTCTACAGAAACCACCTCTCTCCACTCC
-300  CACCCCTACTCCACCACCCACTCGCACCCCGCCCCTGCCGGGCCACTCTG
                          GC box
-250  GGACGAATTGCATTCTTGGACCTTTCTCTCCGCAAGGCACATTACGGAGA
-200  ACTCCCTCTGTCTCGTGTCCCCTCCACGACAACCCAGTAATTATTTCTAT
-150  GCAAGTCTGCAAGAGGGCACTGAGTTATCGGACATCCCAAGCCTAACCAG
-100  CTAGAGCGGCGCCTCGGTATTCATTTGCCCAGAGCTCCTCCGCGGGGGAT
 -50  TTAAAAATAATAATAATAATAATAAGGATCCCATGCCTTATGCAAGAGAC
   0  CTCAGTCCCCCGGAACAACTCGATTTCCTTCCAATAGAGGTCTGAGGTGG
                                        TBE-A    TBE-B
 +50  ACTCCCACCTCCCTTCGTGAAGAGTT
       TBE-C          NKX2.5
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Fig. 2. The TBX5-1C 5′-flanking sequence and potential transcription factor binding sites. Several potential transcription factor sites were identified by submitting the genomic sequence in the 5′-flanking region of TBX5 to the Transcription Factors Search (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). The region spanning −265 to −275 contains the GC box. The 500 bp 5′-flanking region of the TBX5 promoter was analyzed for potential TBX5 binding sites (A/G)GGTGT(C/G/T)(A/G). Three potential T-box binding sites (TBE-A, -B, and -C) were present. The first two sites are in tandem, while the third one is in inverted orientation. Each of the potential binding sites has only one nucleotide different from the TBX5 binding site. Sites B and C have perfect palindrome sequences. One potential NKX2.5 site is only a few base pairs away.

GC box. These two complexes were competed away by the unlabeled the GC box sequences (lane 3), but not by the oligonucleotide-carrying mutations at the GC box (lane 4). The unlabeled Sp1-specific oligonucleotide from Promega (lane 5), but not the nonspecific Sp1 competitor (lane 6), was also able to compete away the binding. These results suggest that bands I and II are the DNA–protein complexes of the Sp transcription factor and the GC box.

To confirm the protein–DNA complexes at the potential TBE-A, -B, and -C sites, the oligonucleotide corresponding +37 to +61 was labeled and used as a probe for EMSA. As shown in Figure 5B, three DNA–protein complexes (I, II, and III) were detected in this region (lane 2). Unlabeled wild-type oligonucleotides with all three potential T-box binding sites were able to compete away the signals (lane 3), and like the wild-type oligonucleotides, oligonucleotides mutated at the TBE-A site (lane 4) could also compete away all the three interaction bands, suggesting that the potential TBE-A site is not part of the complex. However, 200-fold molar excess oligonucleotides with either a mutant TBE-B site (lane 5) or the mutant TBE-C site (lane 6) were not able to compete away complexes II and I or III and I, respectively. These results suggest that TBE-B and -C sites, but not the TBE-A site, are involved in DNA–protein complex formation.

Since transcription factors interacting with TBE sites could be cell- and tissue-specific. We performed nuclear extracts from ECL2 cells and MCF7 cells to test if DNA–protein complexes at TBEs are tissue-specific. As shown

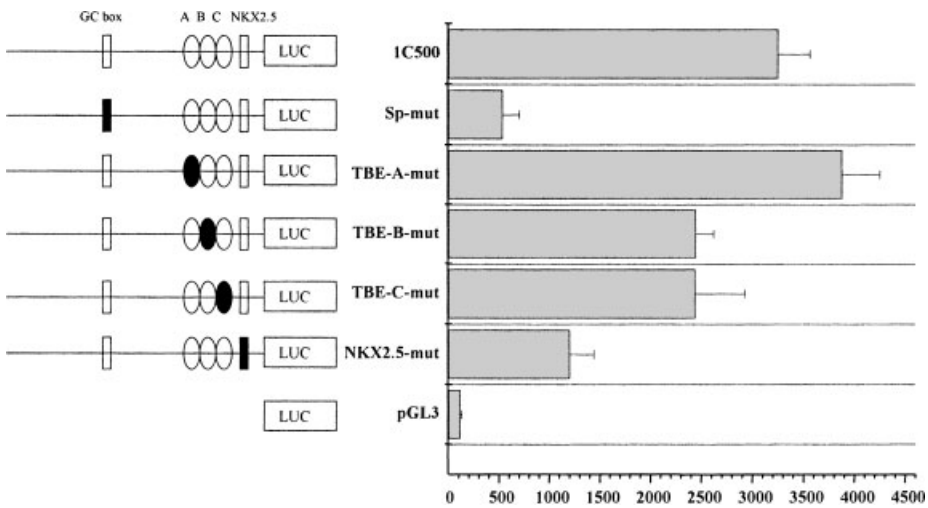

Fig. 3. Effects of mutations at the transcription factor binding sites on reporter expression. Using a site-specific mutagenesis kit (Strategene), site-specific mutations were performed by altering three or four nucleotides at the GC box, TBE-A, -B, and -C, and NKX2.5 sites of 1C-500 (Table I). Mutant constructs were analyzed for luciferase expression in ECL2 cells, as described in ''Materials and Methods.'' The mean luciferase activity of transient transformants is presented as a filled bar, in RLU, and the standard deviation is indicated by the thin line.

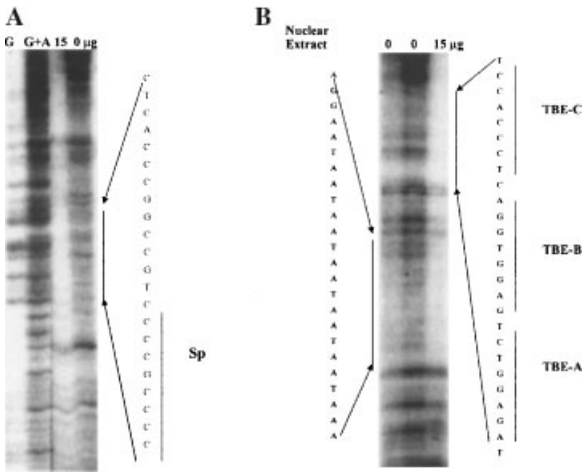

Fig. 4. DNaseI footprinting assay. The TBX5 promoter 5′-flanking fragments were prepared with PCR amplification using the primers bearing *Kpn*I and *Hind*III (Table I) and then labeled with [^{32}P] γ-ATP and T4 DNA polynucleotide kinase. The labeled end was trimmed with *Hind*III, and the resultant one-end-labeled probe was incubated with ECL2 cell nuclear extracts (15 μg). Probe 60,000 cpm per reaction was used. This mixture was then treated with DNaseI (0.03–0.003 U) at room temperature, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved, denatured, and analyzed on a 10% denaturing gel. Probes incubated with ECL2 cell nuclear extract were run in parallel with probes without nuclear extract. **A**: DNaseI footprinting for the GC box. **Lanes 1** and **2** show two Maxam and Gilbert sequencing reactions performed with the DNA fragment and run as ladders. **Lanes 3** and **4** indicate DNaseI-treated probe with or without ECL2 nuclear extract. The protected area is indicated with a line. **B**: DNaseI footprinting for TBE-A, -B, and -C sites. DNaseI-treated probe is shown with (lane 3) or without (lanes 1 and 2) ECL2 nuclear extract. The Maxam and Gilbert sequencing reactions also were performed and run as ladders (data not shown). The three potential T-box binding sites, as indicated by a line on the **right**, were in the protected area. Another protected area was also indicated on the **left**.

in Figure 5C, the protein–DNA complex are different from each other. Oligonucleotides with TBE sites form three complexes with ECL2 nuclear extract (DNA–protein complexes, I, II, and III). The different protein–DNA interaction in ECL2 cells and in MCF7 cells further suggests that transcription factor cell-specific in ECL2 cells acts on the TBX5 promoter.

The end-labeled oligonucleotide containing the potential NKX2.5 binding site also binds to protein in nuclear extract and unlabeled mutant NKX2.5 oligonucleotide has less ability to compete the binding compared with the unlabeled wild-type (data not shown).

Studies of Autoregulation of TBX5

To test whether TBX5 regulates its own promoter, we tested the effects of site-directed mutations in potential TBE-A, -B, and -C, respectively, on luciferase activities in ECL2 cells. As discussed above, mutations in the potential TBE-B and -C decreased luciferase activities.

These results were replicated in a cell line that ectopically expressed TBX5. Human TBX5 protein was ectopically expressed by infecting MCF7 cells with replication-defective retrovirus carrying a full-length TBX5 (pFB-TBX5-Neo). Western blot assay shows TBX5 expression in MCF7 cells infected with pFB-TBX5-Neo (Fig. 6A). Luciferase reporter driven by TBX5 promoter was tested in this ectopically expressed TBX5 cell line and the control cell line, which was infected with pFB-vector only. Three independent experiments were performed. As shown in Figure 6B, all three experiments showed that luciferase activity in TBX5 expressing MCF7 is significantly higher than in the control. These results suggest that TBX5 is a positive regulator for TBX5 promoter.

DISCUSSION

TBX5 is a transcription factor of the T-box gene family, and mutations in TBX5 are sufficient to cause HOS, which is characterized by congenital heart defects and limb anomalies. Although secundum-type atrial septal defect (ASD) and ventricular septal defect (VSD) are the most common heart defects, almost every type of cardiac anomaly has been reported in this condition. Normal heart development relies on the proper expression of hundreds of genes. Mutations in some of these could lead to cardiac malformations. Despite recent advances, molecular etiologies of the majority cardiac malformations are unknown. So far, only a few genes have been identified by molecular studies of rare genetic syndromes. The goal of this study is to gain a better understanding of the molecular events of TBX5 action that are involved in cardiogenesis and to identify genetic factors contributing to human CHD. Elucidation of the upstream transcription factors that control TBX5 transcription may open a window onto the causes of more common CHDs and limb anomalies.

In addition, studies in human and animal models have shown that TBX5 expression may be precisely controlled in the normal heart. Overexpression or insufficicency of TBX5 causes cardiac malformation [Basson et al., 1999; Liberatore et al., 2000; Bruneau et al., 2001;

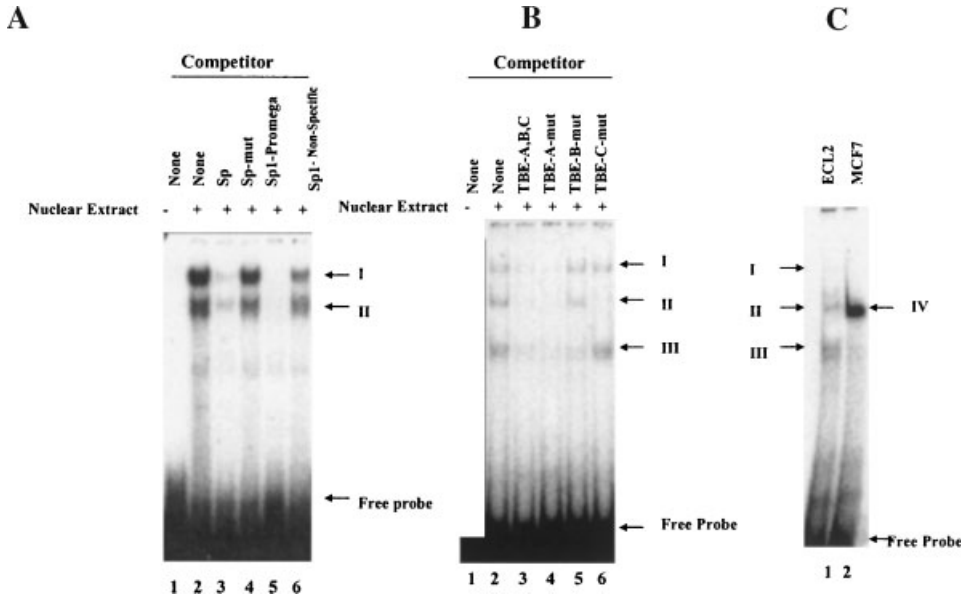

Fig. 5. EMSA of TBX5. **A**: The oligonucleotides containing the GC box were end-labeled and incubated with ECL2 nuclear extracts in the presence or absence of 200-fold unlabeled oligonucleotides. Two major DNA–protein complexes are indicated as I and II. **Lane 1** shows free probe without nuclear extract; **lane 2**, labeled probe incubated with ECL2 nuclear extracts without unlabeled competitor. In **lane 3**, 200-fold unlabeled oligonucleotides were added. **Lane 4** shows 200-fold unlabeled oligonucleotide with mutations in the core sequence of the GC box. **Lane 5** shows the 200-fold unlabeled Sp1-specific oligonucleotides from Promega. In **lane 6**, 200-fold Sp1 nonspecific oligonucleotides were added as competitor. **B**: Electrophoretic mobility shift assays (EMSAs) of the potential TBE-A, -B, and -C sites. Oligonucleotides including the three potential T-box binding sites were radiolabeled and used as probe. The probe was incubated without (**lane 1**) and with nuclear extract (**lane 2**). Three DNA–protein complexes were indicated as I, II, and III in lane 2. In lane 3, 200-fold of the unlabeled oligonucleotides were added to probe/nuclear extract reaction, and all the three major DNA–protein complexes were competed away. In **lanes 4–6**, unlabeled oligonucleotides with mutations at TBE-A site (lane 4), TBE-B-site (**lane 5**), or TBE-C site (lane 6) were added to the probe/protein mixture. Please note that the mutations at the TBE-B and -C sites were not able to compete away complexes II and I or III and I, respectively. **C**: EMSA was performed with TBEs probe and nuclear extract from ECL2 cells or from MCF7 cells As shown in Figure 5B, three DNA–protein complexes (complex I, II, and III) were formed when ECL2 cardiomyocyte nuclear extract was used. In contract, complex IV was visible when nuclear extract from MCF7 cells was used.

Huang, 2002]. However, it is unknown precisely how TBX5 expression is regulated. Studying the mechanisms by which TBX5 is regulated may allow us to examine the possibility of autoregulation.

This study revealed that up to 300 bp of the 5′-flanking region of the TBX5-1C promoter was necessary for promoter activity in cardiomyocyte cells. We then identified several regulatory elements, a GC box at nucleotide positions −265 to −275, three potential T-box binding sites (TBE-A, +38 to +45; TBE-B, +45 to +52; and TBE-C, +53 to +60), and one NKX2.5 binding site (+68 to +75). Previously, Ghosh et al. [2001] performed an in vitro random oligonucleotide selection using a TBX5 protein produced in *E. coli* and derived the consensus sequence (A/G)GGTGT(C/G/T)(A/G), which is part of the Brachyury binding site [Kispert and Herrmann, 1993]. Seventeen clones contained 2 such core sequences, in tandem or in an inverted orientation, separated by 1–14 nucleotides. The alignment of sequences from the 17 clones provided the consensus (A/G/T)GGTG(T/C)(T/G/C)(A/G/C). For the TBE-A, -B, and -C sites in the TBX5-1C promoter, there was 1 bp difference in each of the TBX5 binding sites from those of the consensus sequences identified by Ghosh et al. [2001]. This difference may reflect T-box binding site in mammalian cells.

To demonstrate the physiological importance of the potential transcription sites, we performed site-specific mutagenesis studies. Mutation at the potential TBE-B and -C sites, and at the GC box and NKX2.5 sites significantly decreased luciferase activity, suggesting that the GC box and the potential TBE-B, -C, and NKX2.5 sites are functionally important for the activation of the promoter function.

The protein–DNA interactions at those transcription factor binding sites were demonstrated by DNaseI footprinting and by EMSAs.

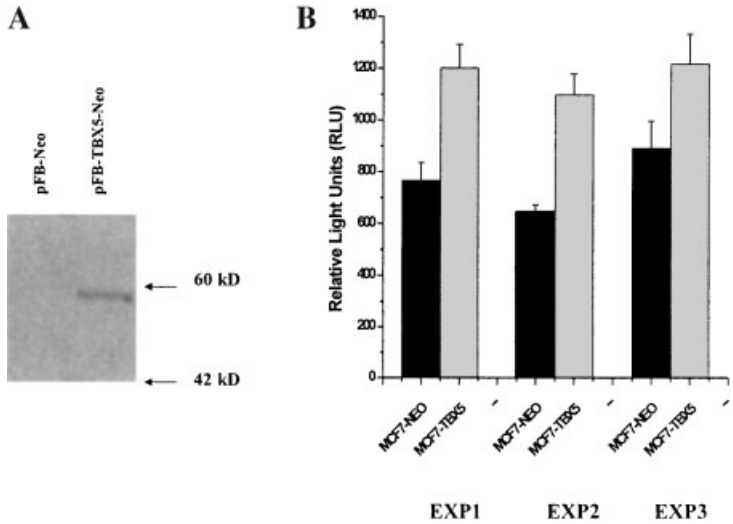

Fig. 6. Autoregulation of TBX5 in MCF7 cells. To further address whether TBX5 can regulate its expression via an autoregulation mechanism, a luciferase assay was performed in MCF7 cells infected by pFB-TBX5-Neo expression vector. An MCF7 cell line expressing TBX5 was established by infecting cells with replication-defective retrovirus pFB-TBX5-Neo and selected using 500 μg/ml G418 for 2 weeks. The cell line infected with replication-defective retrovirus pFB-Neo was used as control. **A**: Western blot assay to examined the expression of ectopical TBX5 in MCF7 cell infected with pFB-TBX5-neo. TBX5 protein was indicated with a arrow. **B**: 1C-500-luciferase reporter construct was transfected into the cell line expressing TBX5. Luciferase activity was analyzed as described above. Three independent experiments in triplicates all showed that luciferase activity significantly increased in the cells expressing TBX5, in contrast to the control cells. The mean luciferase activity of transient transformants is presented as a filled bar, in RLU, and the standard deviation is indicated by the thin line.

We found that all those transcription factor binding regions were resistant to DNaseI digestion. The specificity of the protein–DNA interaction was tested using competitive EMSAs. Our results confirmed that the protein–DNA interaction acts in a sequence-specific manner and protein–DNA interaction in ECL2 cardiomycyte could be tissue- or cell-specific.

Many transcription factors have autoregulatory mechanisms by which they are able to control their own expression levels. To investigate whether TBX5 can control its expression by interacting with its own promoter, the 500 bp 5′-flanking region of the TBX5 promoter was analyzed for potential TBX5 binding sites. We identified three potential T-box binding sites (TBE-A, -B, and -C) (Fig. 2). The TBE-A and -B sites appear in tandem, while the TBE-C site is in inverted orientation. Mutations at TBE-B and -C caused a significant decrease in luciferase activity.

To further study the possibility of TBX5 autoregulation, we also co-transfected the human TBX5 expression construct and luciferase reporter genes driven by the TBX5-1C promoter to test to the effects of ectopically expressed TBX5 on promoter activities. We found that luciferase activity was significantly increased (by an average of 1.6-fold) in the cells overexpressing human TBX5.

As TBX5 is expressed in many organs besides the cardiomyocytes, we speculate that both tissue-specific and ubiquitous transcription factors are involved in transcription of the *TBX5* gene. The transcription factor at the GC box is a Sp family with four members, Sp1, Sp2, Sp3, and Sp4. Since the function domain of Sp2 is more diverse, it was suggest that Sp2 should not be considered a member of the Sp family [Kolell and Crawford, 2002]. Sp4 is mainly expressed in central nervous system. Ubiquitously expressed Sp1 and Sp3 have been implicated in the control of a wide variety of genes, and [Hagen et al., 1992]. Although it is able to bind to GC box, Sp3 has both activation and inhibitory effects [Hagen et al., 1994]. Strong transcriptional activation effect of the GC box in our study and two strong DNA–protein complexes in EMSA (Fig. 5A) suggested the GC box might be a Sp1 and/or Sp3 binding site.

NKX2.5 is a transcription factor [Hiroi et al., 2001] that has been found to interact with the N-terminal half of TBX5. Mutations of NKX2.5 also cause familial ASD [Schott et al., 1998] and other cardiac malformations [Benson et al., 1999]. The results of the site-directed mutagen-

esis construct of NKX2.5 and of the competitive EMSAs and DNAaseI footprint suggest that the potential NKX2.5 binding site played significant roles in activating TBX5 transcription. Since the potential NKX2.5 binding site is so close to T-box binding sites, this result further supports previous observations that TBX5 and NKX2.5 form a complex, acting on the promoter and executing its functions. In addition, one of the regions that were protected in DNA footprint analysis contains a potential homeobox protein HOX1.3 (HOXA5) binding site, which is a transcription factor regulating gene expression, morphogenesis, and differentiation during embryonic development [Hershko et al., 2003]. Thus, it will be intriguing to further investigate the significance of this potential interaction. Using antibody against human TBX5 and NKX2.5 to perform the Supershift mobility assay to confirm TBX5– and NKX2.5–DNA interactions is in progress. We should point out that although we have identified many potential transcription factor binding sites which are important for reporter expression in ECL2 cells, many important elements may not be included in this region, particularly negative regulators.

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