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GATA-4 and MEF2C transcription factors control the tissue-specific expression of the αT-catenin gene *CTNNA3*

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ABSTRACT

αT-catenin is a recently identified member of the α-catenin family of cell–cell adhesion molecules. Its expression is restricted mainly to cardiomyocytes, although it is also expressed in skeletal muscle, testis and brain. Like other α-catenins, αT-catenin provides an indispensable link between a cadherin-based adhesion complex and the actin cytoskeleton, resulting in strong cell–cell adhesion. We show here that the tissue-specificity of αT-catenin expression is controlled by its promoter region. By *in silico* analysis, we found that the αT-catenin promoter contains several binding sites for cardiac and muscle-specific transcription factors. By co-transfection studies in P19 embryonal carcinoma cells, we demonstrated that MEF2C and GATA-4 each have an activating effect on the αT-catenin promoter. Transfections with wild-type and mutant promoter constructs in cardiac HL-1 cells indicated that one GATA box is absolutely required for high αT-catenin promoter activity in these cells. Furthermore, we showed that the GATA-4 transcription factor specifically binds and activates the αT-catenin promoter *in vivo* in cardiac HL-1 cells. *In vivo* promoter analysis in transgenic mice revealed that the isolated αT-catenin promoter region could direct the tissue-specific expression of a *LacZ* reporter gene in concordance with endogenous αT-catenin expression.

INTRODUCTION

α-Catenins are key molecules of the E-cadherin-mediated cell–cell adhesion complex, because they make the indispensable link to the actin cytoskeleton. The importance of this link to confer strong and functional cell–cell adhesion is illustrated by tumor cells that have lost a functional αE-catenin protein, a change that is associated with the loss of cell–cell aggregation and the gain of invasive capacity (1–6). Re-introduction of exogenous functional α-catenin results in the restoration of cell–cell adhesion and the inhibition of the invasive capacity *in vitro* and *in vivo* (3,6–9). Besides its role as an invasion-suppressor molecule, α-catenin has a tumor-growth suppressive capacity. This was recently demonstrated by a conditional knock-out of αE-catenin in the epidermis (10). Ablation of αE-catenin expression in the skin results in a sustained activation of the Ras-MAPK pathway leading to hyperproliferation of the epidermal cells (10).

Recently, a new member of the α-catenin family was identified and termed αT-catenin. This novel α-catenin shows very high sequence homology to both αE- and αN-catenins (11). αT-catenin expression is restricted to certain tissues. It was first discovered in testis, but also found in cardiac and skeletal muscle and in the brain (11). The distinct expression pattern of αT-catenin contrasts with the ubiquitous expression of the closely related αE-catenin. In some cell types αE- and αT-catenin are co-expressed, as is found at the intercalated discs of cardiac muscle cells, whereas in other tissues, for instance in human testis, they are differentially localized (11).

Not much is known so far about the function of this recently identified αT-catenin. It has been shown that αT-catenin can bind strongly to β-catenin in heart and testis tissues, and *in vitro* data show that αT-catenin can function as a genuine α-catenin by providing a link between a cadherin-mediated cell–cell adhesion complex and the actin cytoskeleton (11). N-cadherin-mediated adhesion is critical for proper myofibril organization in cardiomyocytes (12), and altered cadherin expression in the myocardium leads to dilated cardiomyopathy (DCM) (13). DCM is a 'cytoskeletalopathy', as most of the DCM genes identified so far have an influence on actin cytoskeleton organization (14). Interestingly, the gene encoding human αT-catenin, *CTNNA3*, was mapped to chromosome 10q21 (15), a region that has been linked to certain cases of dominantly inherited autosomally DCM (16). In view of its high expression in the heart and its presumed role in anchoring the actin cytoskeleton to the cadherin-mediated cell–cell adhesion complex, αT-catenin is considered as a good

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candidate gene for inherited or sporadic DCM. However, so far no final evidence for such causative link has been found (15). Besides their structural role in cell–cell adhesion, α-catenins have also been implicated in signaling events. Similar to other α-catenins, αT-catenin is also able to inhibit Wnt signaling (17). Based on this finding and on the chromosomal position of αT-catenin, a link between αT-catenin gene variation and late-onset of Alzheimer disease has been proposed (17,18).

The close relation of α-catenin genes and their partly overlapping expression patterns makes understanding the transcriptional regulation of these genes of considerable interest. Moreover, due to the particular expression pattern of αT-catenin being restricted to a very limited number of tissues, its promoter must be very tightly regulated and is thus an interesting object to study.

Extensive study of the cardiac-specific gene regulatory network has shown that MEF2C, Nkx2.5 and GATA proteins are key transcriptional regulators in the heart (19). GATA factors are Cys_4 zinc finger transcription factors with GATA-4, -5 and -6 playing roles in the development and proper function of the heart [for review see (20)]. Members of the MEF2 family of MADS-box transcription factors are expressed at high levels in all muscle cells, and MEF2C is essential for proper heart development [for review see (21)]. Overexpressed MEF2C can initiate cardiomyogenesis in embryonic carcinoma cells (22). In cardiomyocytes, MEF2C acts together with GATA factors to induce gene transcription (23). Apart from their role in heart-specific gene regulation, certain GATA factors and MEF2C are also expressed in other tissues exhibiting αT-catenin expression. MEF2C is found in skeletal muscle cells, where it strongly potentiates the transcriptional activity of MyoD, and in post-mitotic neurons in the brain, whereas GATA-4 is involved in gene regulation in the testis (24).

In the present study, we analyze the human αT-catenin promoter region both *in vitro* and *in vivo* and we determine the role of MEF2C and GATA-factors in the tissue-restricted expression of the αT-catenin gene.

MATERIALS AND METHODS

Cloning and sequencing of the human αT-catenin promoter

A human bacterial artificial chromosome (BAC) library (Genome Systems Inc.) was screened by a PCR specific for the first exon of the *CTNNA3* gene (5′-TGTCATCTGCCTCTCAATTTG-3′, 5′-ATGCTGCCTTTCTGTTTCTTC-3′). One positive BAC clone was obtained. Fragments containing exon 1 were identified by Southern hybridization with an exon-1-specific primer and subcloned into the pGEM11 vector to generate pGEM11-hαTctnprom. Clones of interest were identified by colony hybridization and sequenced. A sequence of 3412 bp of the human αT-catenin promoter was deposited in the GenBank database (GenBank accession no. AF361938).

Luciferase reporter plasmids and site-directed mutagenesis

A 3266 bp SacI–SpeI fragment from the pGEM11-hαTctnprom construct was blunt-ended and cloned into the blunt-ended HindIII site of the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI) to generate plasmid αTctnprom-luc1.

Site-directed mutations of two potential GATA-binding sites and one putative MEF2C-binding site were introduced into the αTctnprom-luc1 plasmid using the QuickChange site-directed mutagenesis kit (Stratagene). The following primers were used to generate the mutated constructs (mutations are in boldface and are underlined): 5′-TAACCTCCCCTTTCTTTCTTA**GG**CTGGGTGAACAACGCT-3′ and 5′-GAGCGTTGTTCACCCAG**CC**TAAGAAAGAAAGGGGAGGTTA-3′ for GATA box1. 5′-ACTAGCGGTTCAGGATTA**CC**TACCACCCACCCTGGCTTG-3′ and 5′-CAAGCCACCGTGGGTGGTA**GG**TAATCCTGAACCGCTAGT-3′ for GATA box2. 5′-GTCTGGCCTTTTTCAACGAAG**C**A**CGC**TCAGTAACAAGTTGTCAG-3′ and 5′-CTGACAACTTGTTACTGA**GCGTG**CTTCGTTGAAAAAGGCCAGAC-3′ for the MEF2C-binding site.

Eukaryotic expression vectors

A GATA-4 expression vector (pcDNA1/GATA-4) and an Nkx2.5 expression vector (pGK-Nkx2.5) were obtained from Dr J.Molkentin (25) and Dr I.Skerjanc (22), respectively. The MEF2C coding sequence was PCR amplified from human heart cDNA (BD Biosciences Clontech, Palo Alto, CA) using primers 5′-GGGACTATGGGGAGAAAAAAGA-3′ and 5′-TGTTGCCCATCCTTCAGAAAGT-3′, and cloned into the pEF6Ahismyc expression vector (Invitrogen, San Diego, CA).

Cell culture

P19 embryonal carcinoma cells were cultured in α-minimal essential medium (Invitrogen) containing 5% newborn calf serum, 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HL-1 cardiac cells were cultured as described previously (26).

Transfections and reporter gene assays

Co-transfections in P19 cells were performed using FuGENE 6 Reagent (Invitrogen). These co-transfections included 0.5 μg of the wild-type or mutant αT-catenin promoter luciferase reporter constructs combined with 0.5 μg of the pcDNA1/GATA-4, pGK-Nkx2.5 or pEF6Ahismyc-MEF2C expression vectors, in various combinations. Total DNA for each transfection was corrected by the addition of empty expression vector. In each transfection, 0.5 μg of a CMV driven LacZ expression vector (pUT651; Eurogentec) was added as a control for the transfection efficiency. HL-1 cells were transfected using calcium phosphate precipitation in the presence of serum. The calcium phosphate precipitate contained 2 μg of the luciferase reporter plasmid and 1 μg of the pUT651 LacZ expression vector as a control for transfection efficiency. Fresh medium was added to the cells 12 h after transfection.

Forty-eight hours after transfection, the cells were lysed and luciferase and β-galactosidase activities were measured by adding a luciferase substrate buffer [40 mM Tricine, 2.14 mM $(MgCO_3)_4{\cdot}Mg(OH)_2{\cdot}5H_2O$, 5.34 mM $MgSO_4{\cdot}7H_2O$, 66.6 mM DTT, 0.2 mM EDTA, 521.2 μM Coenzyme A, 734 μM ATP and 940 μM Luciferin] and a β-galactosidase substrate (Galactostar kit, Tropix Inc., Bedford, MA), respectively.

Nuclear extract preparation and EMSA analysis

Nuclear extracts of HL-1 cells were prepared by controlled NP-40 lysis. Briefly, confluent cell monolayers in 75 mm^2

Table 1. Wild-type and mutant oligonucleotides for EMSA analysis and DNA precipitations

GATA box2 WT oligonucleotide	5′-GCGGTTCAGGATTAGATACCACCCA-CCC-3′
GATA box2 mutant oligonucleotide[a]	5′-GCGGTTCAGGATTA**CC**TACCACCCA-CCC-3′
MEF2C WT oligonucleotide	5′-TTTCAACGAAGTATTTTCAGTAACA-AGTTGTCAG-3′
MEF2C mutant oligonucleotide[a]	5′-TTTCAACGAAG**C**A**CGC**TCAGTAACA-AGTTGTCAG-3′

[a]Mutant nucleotides are in boldface and are underlined.

dishes were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by scraping in PBS, and centrifuged at 1500 *g* for 5 min. The pellets were washed once with PBS and pelleted again as before. The cell pellets were suspended in buffer 1 [10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5% glycerol, 2 mM Pefabloc (Pentapharm Ltd, Basel, Switzerland), 0.5 mM DTT and 0.15 IU/ml aprotinin] and the cells were allowed to swell on ice for 15 min. The cells were then lysed by adding 0.6% NP-40 in buffer 1 and vortex-mixed briefly. Lysates were centrifuged at full speed for 15 min and the cytoplasmic fraction was discarded. Nuclei pellets were resuspended in buffer 2 (20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 400 mM NaCl, 10 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM Pefabloc, 0.5 mM DTT and 0.15 IU/ml aprotinin) containing 1% NP-40, vortex-mixed well and centrifuged for 15 min at full speed. The supernatant (nuclear extract) was recovered and dialyzed overnight against 1.6× EMSA binding buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 2 mM DTT, 1 mM EDTA and 20% glycerol) with one buffer change.

Oligonucleotide probes used for EMSA analysis are listed in Table 1. Each reaction mixture (20 µl) contained 12 mM HEPES, pH 7.9, 60 mM KCl, 1.2 mM DTT, 0.6 mM EDTA, 12% glycerol, 0.1% NP-40, 0.05% BSA, 1 µg poly(dI–dC), 2–10 µg nuclear extract and 50 fmol ^{32}P end-labeled probe (10 000 c.p.m.). The mixture was incubated at 30°C for 30 min and loaded directly onto a 6% non-denaturing polyacrylamide gel in 0.5× Tris–borate-EDTA buffer. For competition experiments, cold competitors were added to the reaction mixture simultaneously with the labeled probe. For supershift assays, a GATA-4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the DNA/nuclear extract mix, and the reactions were allowed to proceed for 20 min before loading the samples.

Biotinylated oligonucleotide precipitation assay

DNA precipitations were carried out as described previously (27). The biotinylated double-stranded wild-type and mutant oligonucleotides were the same as those used for the EMSA assays (Table 1). DNA bound proteins were collected with streptavidin–agarose beads (Pierce Biotechnology, Rockford, IL) and analyzed by western blotting using antibodies specific for GATA-4 and MEF2C (Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP)

We used the ChIP assay kit from Active Motif (Carlsbad, CA). Briefly, sample cell lysates were sonicated with a Sonics Vibra Cell (Newtown, CT), equipped with four 3 mm stepped microtips at 25% power, in this way simultaneously shearing four samples of 350 µl in 1.5 ml Eppendorf tubes. Samples were sonicated 15 times for 15 s with intermittent cooling on ice water for 30 s. The chromatin–antibody protein complex was eluted from the protein-G–Sepharose beads with freshly prepared elution buffer (0.2 M $NaHCO_3$ and 1% SDS). NaCl was added to each ChIP eluate to a final concentration of 200 mM before heating the mixture at 65°C for 4 h to reverse the cross link. The samples were digested with proteinase K for 2 h at 42°C. The DNA from the samples was purified using DNA minicolumns and eluted in water. The GATA-3, GATA-4 and GATA-6 antibodies used were from Santa Cruz Biotechnology.

Quantitative real-time PCR

Primers for PCR analysis for ChIP of the mouse αT-catenin promoter were designed using primer Express software (Perkin-Elmer Applied Biosystems). Sequences of primers for the murine proximal αT-catenin promoter sequence (−146 to −15) were 5′-TTATTTAGAGCTTCCGAAGA-3′ (forward), 5′-GGTGGGTGGTATCTAATCC-3′ (reverse); primers for a distal region of the αT-catenin promoter (−3373 to −3324), which we designated the distal promoter, were 5′-AATCCTGGGCCATACATGTT-3′ (forward) and 5′-TGTGCTGGCATCCATAAGAC-3′ (reverse). To detect the amplified promoter regions the SyBr green I reagent mix (Eurogentec, Belgium) was used. The average threshold cycle (Ct) values of triplicate measurements were used for all subsequent calculations on the basis of the delta Ct method.

RT–PCR analysis

RNA from cell lines and tissues was prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using pSuperscript Reverse Transcriptase (Invitrogen) and the residual RNA template was removed by RNAse H treatment. Primer sets were developed to specifically amplify αT-catenin (human αT-catenin: 5′-CTGCCTCTCAATTTGGTACT-3′ and 5′-AGGGGTCATCTGTAAATCTC-3′; murine αT-catenin: 5′-CCCCTTTCTCTCTTATCCTGAG-3′ and 5′-GCTGCCA-GCTCTTCCTTTAAA-3′), the different mouse GATA factors (GATA-1: 5′-GCCCAAGAAGCGAATGATTGT-3′ and 5′-GCCAGATGCCTTGCGGTTCCT-3′; GATA-2: 5′-TGA-AGACATGGAGGCGTTTG- 3′ and 5′-TCTAGGGCTGCG-TCAGGG-3′; GATA-3: 5′-GGGACATCCTGCGCGAACTG--3′ and 5′-CTCCAGCGCGTCATGCACC-3′) and the GAPDH housekeeping gene (human GAPDH: 5′-GAGGTGAAGGT-CGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′; murine GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′). Primers for GATA-4, GATA-5 and GATA-6 were used as described previously (28–30).

Generation of transgenic mice

A construct containing 2917 bp of the human αT-catenin promoter, the αT-catenin exon 1 sequence and 153 bp of the intron 1 sequence was designed to direct expression of the *LacZ* reporter gene. To obtain this, the luciferase coding sequence from the pGL3Basic vector was exchanged with the β-galactosidase coding sequence. The plasmid was digested

with SalI to reduce the amount of vector sequences, leaving intact a 6681 bp fragment containing the above-mentioned αT-catenin gene fragment and the β-galactosidase coding sequence. This fragment was purified by agarose gel electrophoresis followed by two consecutive ethanol precipitations, and injected into the pronuclei of fertilized eggs from FVB mice. The manipulated oocytes were transferred into the oviducts of pseudopregnant foster mothers. The transgenic mice were generated in collaboration with Thromb-X nv (Leuven, Belgium).

Transgenics in the progeny were identified by PCR analysis of genomic DNA isolated from tail tissue. The primer set used was specific for the transgene, with one primer located in the αT-catenin promoter region (5′-TGTAACTCTGTGACCAC-CAAGAAG-3′) and the other in the LacZ coding sequence (5′-TTGAGGGGACGACGACAGTA-3′). Southern-blot analysis with a β-galactosidase-specific probe was performed to verify transgene integration and the absence of rearrangements of the transgene.

Analysis of β-galactosidase expression

The expression of the transgene was analyzed in the F1 progeny, derived from backcrosses with non-transgenic FVB mice. Tissues (and organs) were dissected and fixed for 2 h at 4°C in 0.1 M sodium phosphate (pH 7.3) containing 2% paraformaldehyde, 0.2% glutaraldehyde, 0.01% sodium deoxycholate and 0.02% NP-40. After three 30 min washes in a 0.1 M sodium phosphate buffer (pH 7.3) containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40, tissues were stained overnight at 30°C in X-gal staining solution [0.1 M sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ and 1 mg/ml X-gal]. To examine the distribution of transgene expression histologically, organs were dissected, fixed, transferred to 15 and 30% sucrose in the PBS for 4 and 16 h, respectively, and embedded in OCT freezing medium. Sections of 10 μm were cut on a cryostat, washed in 0.1 M sodium phosphate and incubated in X-gal staining solution.

β-Galactosidase enzyme activity was measured using the Galacto-star kit (Tropix Inc.). The tissues were homogenized in lysis buffer (0.1 M potassium phosphate, pH 7.8, containing 0.2% Triton X-100, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin). After two freeze–thaw cycles, tissue debris was removed by brief centrifugation. Protein concentrations were measured by the Bio-Rad assay, and equal amounts of tissues were used for the determination of enzymatic activity.

Immunohistochemical analysis

Cryosections of 10 μm were prepared from paraformaldehyde–glutaraldehyde-fixed tissues. The sections were washed in PBS and pre-incubated with 10% goat serum for 10 min. They were then incubated with primary antibody for 45 min at room temperature. The primary antibody used was a polyclonal αT-catenin specific antibody (11), diluted in PBS with 1% BSA. The secondary antibody used was a biotin-labeled goat-anti-mouse Ig (Dako, Carpinteria, CA), which was linked to a streptavidin–biotin complex coupled to horseradish peroxidase. Detection was carried out by incubation with the chromogenic peroxidase substrate diaminobenzidine for 5 min (Biogenex, San Remon, CA).

RESULTS

The αT-catenin promoter contains putative binding sites for several cardiac- and muscle-specific transcription factors

Sequence analysis of the human αT-catenin promoter region and a search in the transcription factor binding site database (Transfac database; www.genomatix.de; www.biobase.de) (31) revealed several putative binding sites for cardiac- and muscle-specific transcription factors. A MEF2C consensus site, several GATA-binding sites, an Nkx2.5 site and a binding site for helix–loop–helix transcription factors eHAND and dHAND were identified. All these regulatory elements were within 300 bp upstream of the transcription initiation site. Parts of the mouse and rat promoter regions were identified by BLAST analysis using the mouse ortholog of the human αT-catenin. Sequence alignments showed high sequence conservation only in the proximal αT-catenin promoter region. Interestingly, two GATA boxes, the MEF2C site and the HAND binding site, are conserved in the three different species (Figure 1).

MEF2C and GATA-4 transactivate the αT-catenin promoter synergistically

We used co-transfection studies to assess the contribution of the above-mentioned *cis*-acting elements and their respective transcription factors to the activation of the αT-catenin gene (Figure 2A). Co-transfection into P19 cells of a MEF2C expression plasmid with an αT-catenin promoter luciferase construct activated the promoter on average 12-fold (Figure 2B). Mutation of the MEF2C consensus site abolished this activating effect almost completely. Also GATA-4 was able to transactivate the αT-catenin promoter after co-transfection into P19 cells, although only about 3- to 4-fold (Figure 2C). Only mutagenesis of the GATA box2 element could destroy this activation effect. Remarkably, the mutation of this GATA box2 site also diminished the effect of MEF2C on the promoter (Figure 2B, 5). Indeed, mutation of both the MEF2C site and the GATA box2 element is needed to completely destroy the activating effect of MEF2C on the αT-catenin promoter (Figure 2B, 6).

Co-transfection of GATA-4 and low amounts of MEF2C with the promoter constructs led to the synergistic activation of the αT-catenin promoter, pointing to cooperation between MEF2C and GATA-4 in activating the αT-catenin promoter (Figure 3).

Heterodimers of eHAND or dHAND and E47 transcription factors could also transactivate the αT-catenin promoter in a co-transfection promoter reporter assay, while the Nkx2.5 transcription factor had no effect on the αT-catenin promoter (data not shown).

GATA box2 is required for αT-catenin promoter activity in HL-1 cardiac muscle cells

The αT-catenin promoter was tested for tissue-specific activity by the transfection of a reporter construct in cell lines of a different origin and its activity was compared with the SV40 promoter activity (Figure 4A). HL-1 is a mouse cardiac-muscle cell line that can be repeatedly passaged in culture without losing its cardiac-specific phenotype (26). Using RT–PCR and western-blot analysis with an αT-catenin-specific

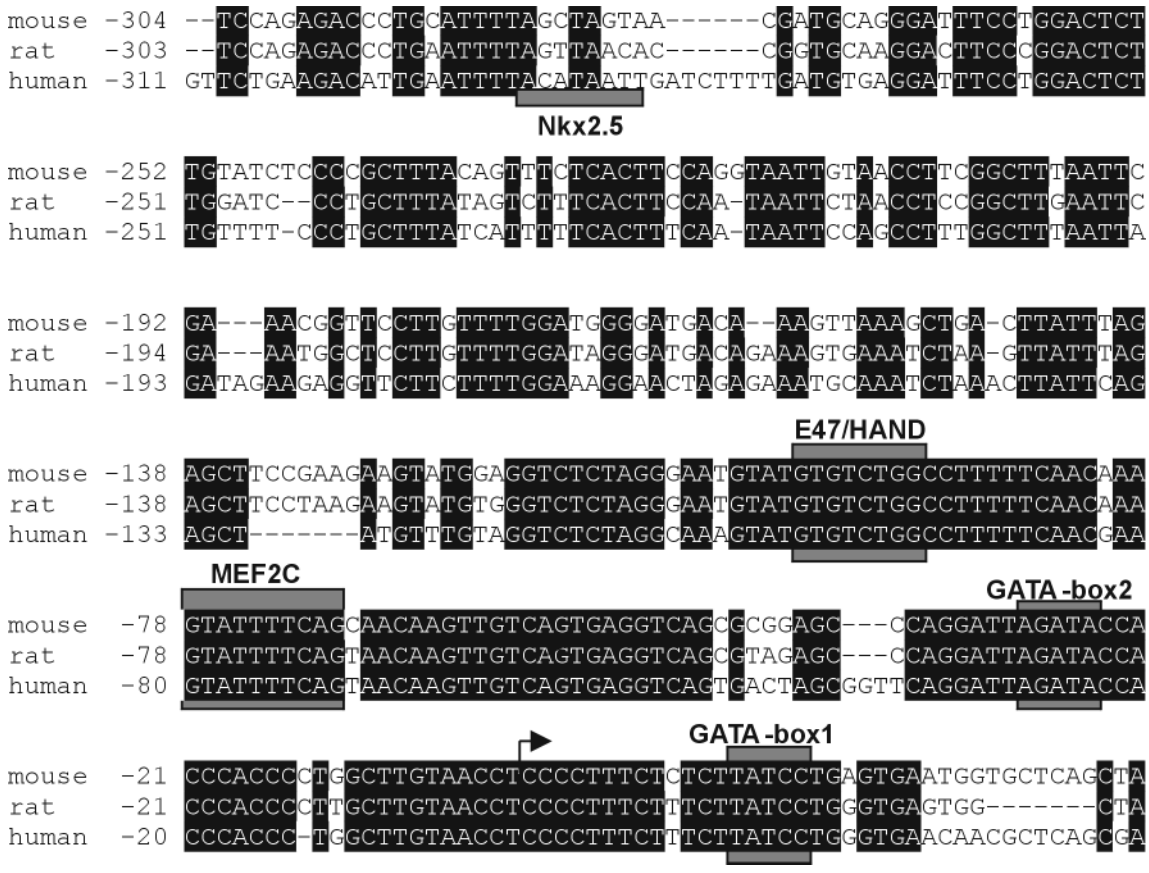

Figure 1. Alignment of the 5′ flanking sequences of the human, mouse and rat αT-catenin genes. The numbering is relative to the transcription initiation site (indicated by arrow). The putative regulatory regions as predicted by the Matinspector Software are indicated by gray boxes.

polyclonal antibody (11), we found that HL-1 cells express αT-catenin (Figure 4B), while all other cell lines tested did not express αT-catenin. Hence, HL-1 cells should have all the necessary transcription factors to confer expression of the αT-catenin gene. Indeed, the activity of the αT-catenin promoter in these cells was demonstrated by transient transfection with αT-catenin promoter luciferase constructs (Figure 4A). In contrast, the αT-catenin promoter showed no activity after transfection in the mouse embryonal carcinoma cell line P19, in the PC3 prostate carcinoma cell line or in the MCF7/AZ epithelial cell line, pointing to a requirement for tissue-specific regulatory factors. To determine whether the MEF2C site and the GATA boxes are actually required for αT-catenin promoter activity in HL-1 cells, we mutated the respective core binding sequences (Figure 4C). Mutation of the GATA box2 element reduced the αT-catenin promoter activity in HL-1 cells to background levels. Mutation of the MEF2C site, however, had no effect on the αT-catenin promoter activity, although HL-1 cells do express the MEF2C transcription factor (data not shown). These results show that the GATA box2 element is absolutely required for the expression of αT-catenin in cultured cardiac muscle cells.

The GATA-4 and GATA-6 transcription factors specifically bind the GATA box2 in HL-1 cells

We examined whether the GATA box2 sequence is indeed bound by GATA proteins in cardiac muscle cells. Semiquantitative RT–PCR expression analysis of HL-1 cells for all the six known GATA factors revealed that GATA factor 4 is most prominently expressed, whereas GATA-3, GATA-5 and GATA-6 are weakly to moderately expressed (data not shown). No expression of GATA-1 or GATA-2 was detected. In an EMSA using wild-type and mutant probes encompassing the GATA box2 element (Table 1), the labeled GATA box2 probe formed a sequence-specific DNA–protein complex when incubated with nuclear extracts from HL-1 cells (Figure 5A). The formation of this complex was prevented by addition of a 100-fold excess of the unlabeled probe whereas the competition was far less extensive when using excess cold mutated GATA-box2 oligonucleotide or excess of a probe containing the MEF2C site. To determine if GATA-4 was involved in the shifted complex, we performed a supershift analysis with a GATA-4-specific antibody. As can be seen in Figure 5B, addition of this antibody to the binding reaction resulted in a weak supershifted band. To further confirm binding of GATA-4 to the GATA box2 site, a DNA precipitation assay was performed. A biotinylated wild-type GATA box2 oligonucleotide was able to extract GATA-4 from HL-1 nuclear extracts, which was not possible with the mutated oligonucleotide (Figure 5C). Moreover, ChIP analysis revealed that a proximal αT-catenin promoter region containing the GATA-box2 is bound *in vivo* by transcription factors GATA-4 and GATA-6 but not by GATA-3 (Figure 5D). No

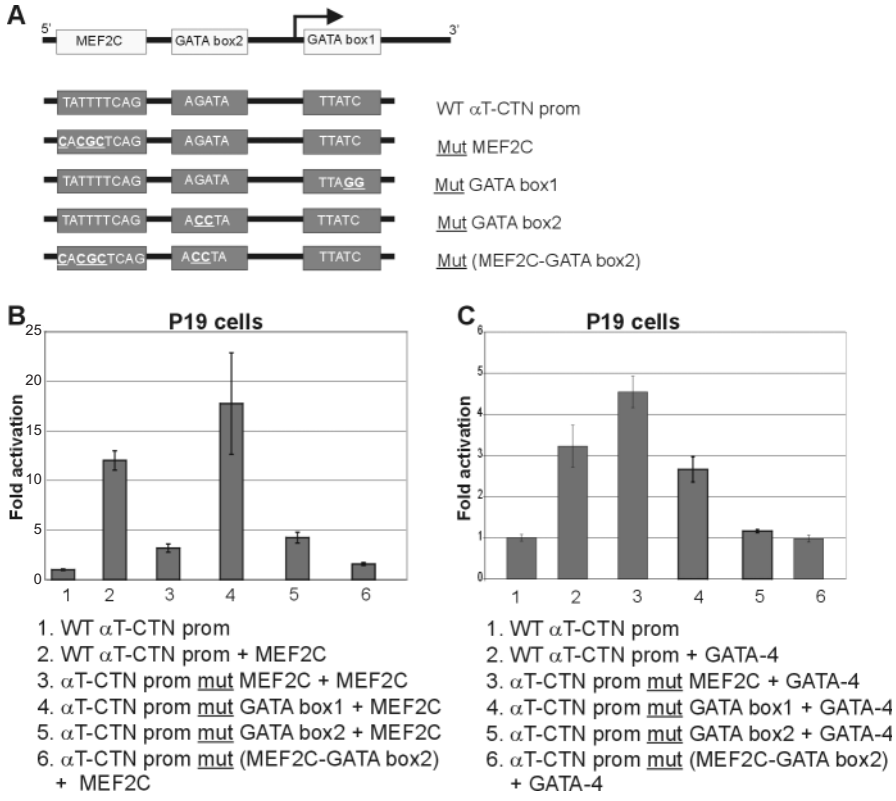

Figure 2. MEF2C and GATA-4 transactivate the αT-catenin promoter in P19 cells. Schematic diagram of the region of the αT-catenin gene near the start of transcription showing the wild-type and mutant constructs tested for promoter activity (**A**). Promoter constructs were co-transfected with MEF2C (**B**) and GATA-4 (**C**) transcription factors in P19 embryonal carcinoma cells. Values represent *x*-fold induction by MEF2C or GATA-4 and are the average of three independent experiments.

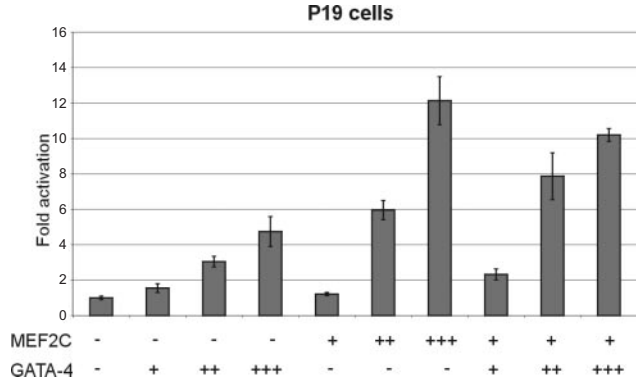

Figure 3. MEF2C and GATA-4 transcription factors transactivate the αT-catenin promoter synergistically. Different amounts of MEF2C and GATA-4 expression vectors were co-transfected with the αT-catenin promoter reporter construct into P19 cells.

binding could be detected to a distal αT-catenin promoter region.

In vitro binding assays were also performed to test binding of MEF2C to the αT-catenin promoter, but here we could not detect any specific shifted complexes (data not shown).

Transgenic mice show tissue-specific activity of the αT-catenin promoter

To test tissue specificity of the cloned αT-catenin promoter region *in vivo*, we constructed transgenic mice in which the αT-catenin promoter drives the expression of the LacZ reporter gene. Six founder lines were identified by PCR and Southern-blot hybridization. No rearrangements of the transgene were detected. Expression of the transgene was detected initially by soaking dissected tissues in solutions containing the β-galactosidase substrate X-gal. Blue staining was clearly detected in the brain, with prominent staining in the cerebellum, and testis, and also in discrete areas of the heart and skeletal muscle (Figure 6). In some transgenic lines the transgene was not present in the germline, while other lines did not show any expression of the transgene.

Transgenic line #10 showed high expression levels of the transgene and was further analyzed in detail. The tissue specificity of the reporter gene determined by a β-galactosidase enzyme activity assay is shown in Figure 7. High to moderate levels of enzyme activity were detected only in brain, heart, skeletal muscle and testis. In all other tissues tested, no activity could be detected above the background. This tissue specificity is fully concordant with that found for αT-catenin expression by RT–PCR analysis of mouse tissues (11). To test this further, transgene expression and αT-catenin expression were analyzed at the cellular level, by X-gal staining and immunohistochemistry on cryosections.

X-gal staining on 10 μm cryosections of testis showed that β-galactosidase localizes to peritubular myoid cells and to the Sertoli cells (Figure 8B). In the Sertoli cells, the β-galactosidase staining was seen to be perinuclear, resulting in a dotted staining pattern on the outside of the testis tubule. The small blue deposits seen more centrally in the

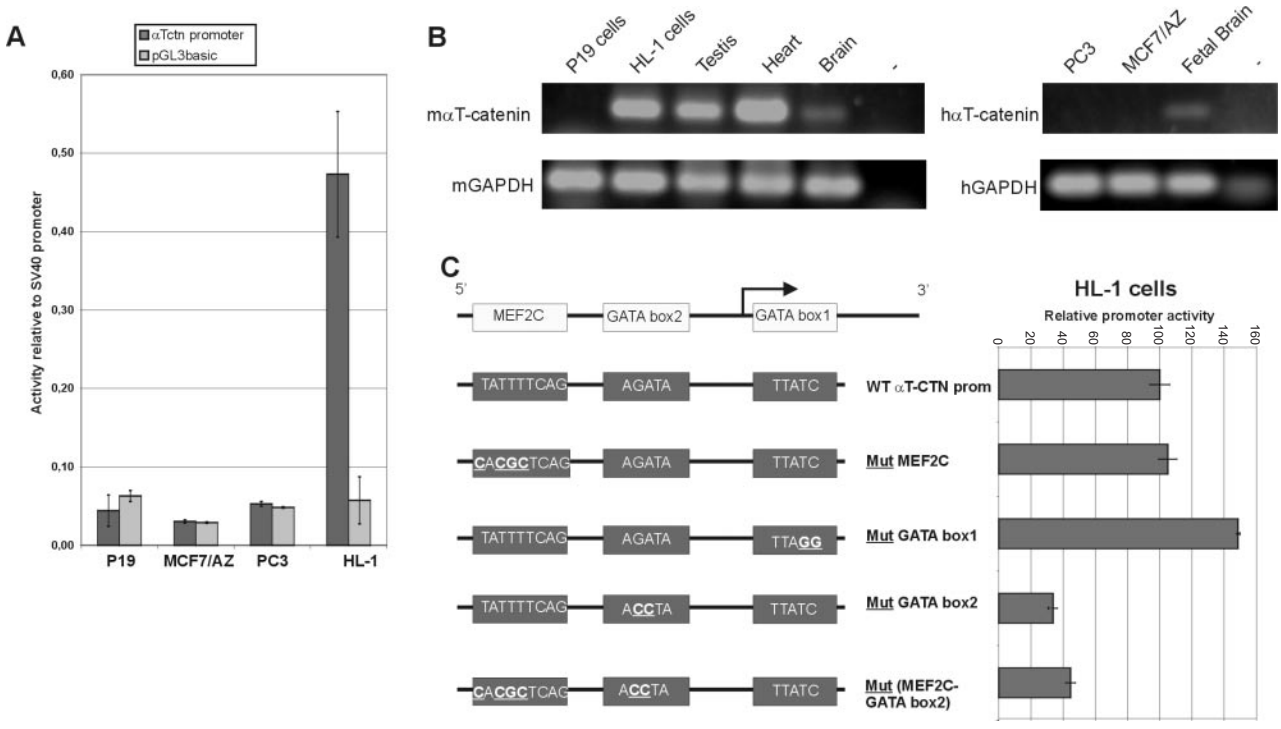

Figure 4. (**A**) Tissue specific activity of the αT-catenin promoter *in vitro*. An αT-catenin promoter construct was transfected in MCF7/AZ, PC3, P19 and HL-1 cells and its activity is represented as a fold of the SV40 promoter activity. The light gray bars represent background values measured by the empty pGL3Basic vector. (**B**) Tissue- and cell-line-specific expression of αT-catenin as shown by RT–PCR. (**C**) Functional role of αT-catenin promoter regulatory elements in cardiac muscle specific activity. Wild-type and mutant αT-catenin promoter constructs were transfected in cardiac HL-1 cells. Values represent percentage of activity compared to the promoter activity of the wild-type promoter construct (100%). The values are the average of three independent experiments.

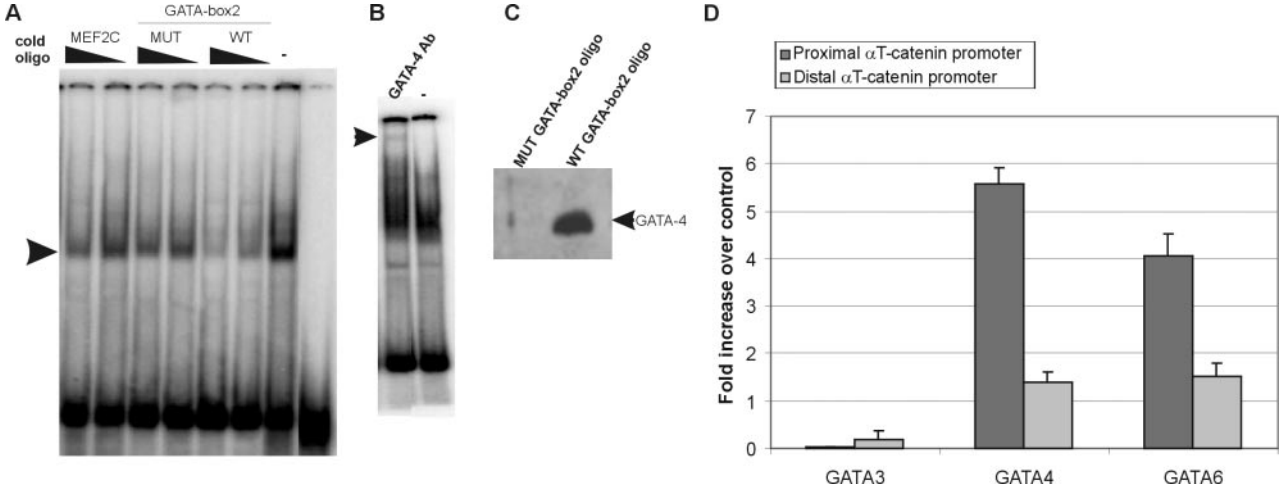

Figure 5. Site specificity of transcription factor binding to the GATA box2 site in the αT-catenin promoter in cardiac muscle cells. (**A**) Nuclear extracts from cardiac HL-1 cells were mixed with labeled double-stranded GATA box2 oligonucleotide (Table 1). One complex was shifted, as indicated by an arrow. This complex was removed by the addition of excess cold oligo, but not by an excess mutant oligonucleotide or MEF2C oligonucleotide. (**B**) Addition of a GATA-4 specific antibody supershifted the formed complex. (**C**) Wild-type and mutant biotin-labeled GATA box2 oligonucleotides were mixed with HL-1 nuclear extracts, and bound proteins were collected by streptavidin beads and analyzed on western blot with a GATA-4 specific antibody. (**D**) *In vivo* binding of proximal and distal αT-catenin promoter sequences to transcription factors GATA-3, GATA-4 and GATA-6 in HL-1 cells, as determined by ChIP analysis. Association of transcription factors was quantified by real-time PCR and is depicted as the fold increase in association of factors detected with specific anti-GATA antibodies, compared with the levels detected for negative control IgG antibody.

tubule represent staining in the cytoplasm of the Sertoli cells. Localization of the endogenous αT-catenin was visualized by incubation with an αT-catenin-specific antibody. In contrast to previous findings where αT-catenin was only found localized to the peritubular myoid cells of the human testis (11), in mouse we also found αT-catenin expression inside the tubule, apparently on Sertoli-cell–germ-cell contacts (Figure 8C).

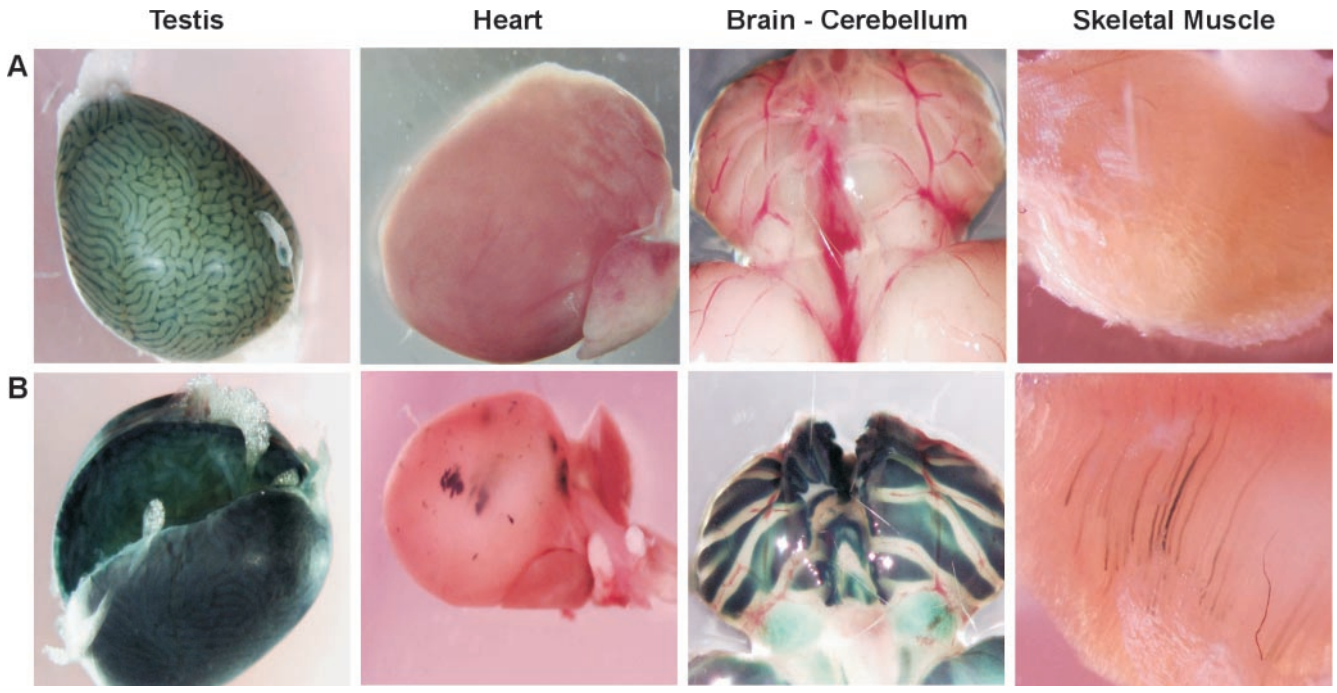

Figure 6. Wholemount X-gal staining of testis, heart, brain and skeletal muscle of wild-type (**A**) and transgenic (**B**) mice. Tissues were removed and stained overnight in X-gal solution. Note the variegated expression pattern in heart and skeletal muscle.

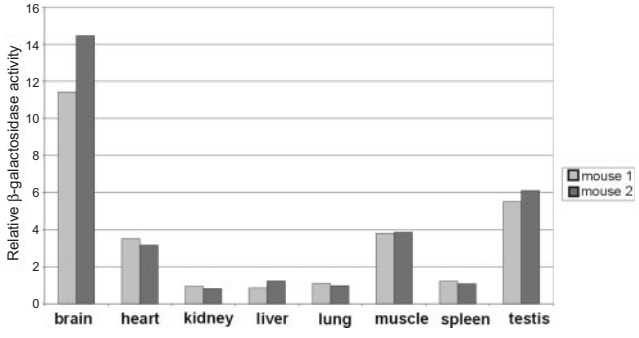

Figure 7. The αT-catenin promoter shows tissue-specific activity *in vivo*. Extracts were prepared from tissues from two transgenic and two wild-type mice and assayed for β-galactosidase activity. Values for each tissue represent the β-galactosidase activity in transgenic mice compared to wild-type mice, and are the means of three measurements.

In the brain, β-galactosidase staining mainly localized to the cell bodies of neurons in the granular cell layer of the cerebellum (Figure 8E). In this cerebellum, αT-catenin localized in the molecular layer, adjacent to the granular layer, probably in the axons of the neurons whose cell bodies are housed in the granular layer of the cerebellum (Figure 8F).

In heart and skeletal muscle, myocytes expressed β-galactosidase, but only in a variegated pattern reflecting that the transgene was expressed in some but not all cells.

DISCUSSION

To understand the mechanism of tissue-specific expression of the αT-catenin gene in testis, brain, heart and skeletal muscle, we cloned the 5′ flanking promoter region of the human gene *CTNNA3*. Sequence alignments of the human, rat and mouse promoter regions show very high sequence conservation of the first 300 bp upstream of the transcription initiation site. Within these short 5′ flanking sequences, a number of conserved regulatory elements were identified, including binding sites for GATA factors, MEF2C and E47/HAND. These binding sites are conserved in both sequence and mutual spacing. We demonstrated that these transcription factors readily transactivate the αT-catenin promoter in a specific manner, as mutation of the core binding site interfered with the transactivating potential of the respective transcription factors.

Further, we showed that GATA-4 is directly involved in regulating αT-catenin expression in cardiac muscle cells. The GATA-4 transcription factor specifically binds the GATA box2 sequence in the αT-catenin promoter as demonstrated by EMSA and DNA precipitations. Furthermore, ChIP analysis showed the *in vivo* binding of GATA-4 only to a proximal αT-catenin promoter region that contains the GATA box2 sequence, while no binding was observed to a distal promoter region. This GATA box2 sequence is critical for cardiac expression of the αT-catenin gene, as mutation of this GATA box2 sequence completely abolished αT-catenin promoter activity in transfected cardiac HL-1 cells. All these data together show that GATA-4 is an essential factor in regulating αT-catenin expression. GATA-4 is a member of the GATA family of zinc finger transcription factors and shows high expression in both the atrium and the ventricle throughout development [for review see (20)]. This GATA-4 transcription factor is known to regulate the expression of a number of cardiac-specific genes, among them cardiac troponin (32), corin (33), atrial natriuretic factor (34), cardiac sarcolemmal Na^{+}–Ca^{+} exhanger (35) and B-type natriuretic peptide (36).

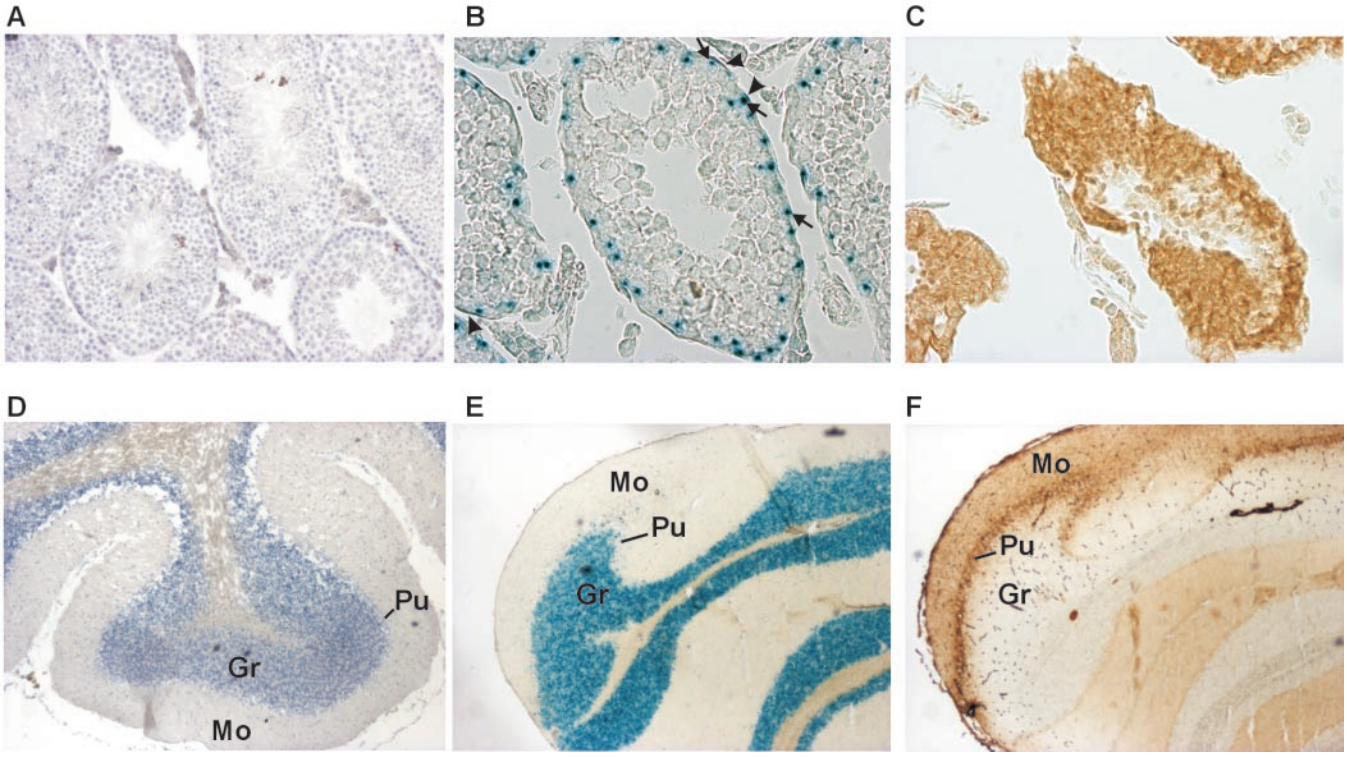

Figure 8. Cellular localization of β-galactosidase and αT-catenin expression in testis (**A**–**C**) and cerebellum (**D**–**F**). Cryosections from wild-type mice were stained with X-gal followed by counterstaining with hematoxylin to reveal tissue details (A and D). Cryosections of transgenic mice were stained with X-gal (B and E) or immunostained with an αT-catenin specific antibody (C and F). (B) Blue deposits are found in Sertoli cells (arrows) and peritubular myoid cells (arrowheads). Mo, Molecular layer; Pu, Purkinje cell layer; Gr, Granular cell layer.

Interestingly, GATA-4 also emerged as a transcriptional activator of the N-cadherin gene in cardiac muscle cells (37). It thus appears that one of the roles of GATA-4 in cardiac morphogenesis is the establishment of a functional cadherin/catenin complex in cardiomyocytes.

Expression analysis of other GATA factors in HL-1 cells revealed that in addition to GATA-4 there is also expression of GATA-3, GATA-5 and GATA-6 in this cardiac muscle cell line. We assessed in particular the activating role of GATA-4 on the αT-catenin promoter as this is the GATA factor expressed most prominently in HL-1 cells. But other GATA factors could have similar effects, as they all seem to recognize the same target sequence. Indeed, we showed in HL-1 cells that in addition to GATA-4, GATA-6 also binds the endogenous αT-catenin promoter, whereas GATA-3 does not. GATA-6 is known to colocalize with GATA-4 in the nucleus of cardiac myocytes (32) and hence can together with GATA-4 play a role in activation of transcription from the αT-catenin promoter in cardiac muscle cells. Cooperation of GATA-4 and GATA-6 transcription factors in controlling tissue-specific transcription has been reported previously (38).

The MEF2C transcription factor was able to transactivate the αT-catenin promoter in co-transfection studies. Involvement of this factor in regulating cardiac expression of αT-catenin expression is less clear. Mutation of the MEF2C site had no effect on αT-catenin promoter activity in cardiac HL-1 cells. However, it has been described that MEF2C can exert its effect via GATA boxes as well (23). This is also clear from our co-transfection studies in P19 cells: mutation of the MEF2C site only partially abolished MEF2C transactivation and mutation of the GATA box2 site was also necessary to completely destroy the activating effect of MEF2C on the αT-catenin promoter. On the other hand, we have been unable to detect MEF2C in a complex bound to the GATA box2 sequence by DNA precipitations or EMSA supershift analysis (data not shown). So far, we have no evidence for a direct role for MEF2C in cardiac expression of αT-catenin, but we certainly cannot exclude it.

Another major finding of this study is that the cloned αT-catenin promoter region can direct tissue-specific expression of a reporter gene both *in vitro*, and *in vivo* in transgenic mice. In the transgenic mice the β-galactosidase transgene showed expression in heart, testis, brain and skeletal muscle, which exactly concurs with endogenous αT-catenin expression. Apparently most information required to direct spatial αT-catenin gene activation is comprised within the cloned promoter sequence. Our *in vitro* studies have shown that GATA-4 is involved in activating the αT-catenin promoter in cardiac muscle cells, but this transcription factor is also a key regulator of gonadal gene transcription (24). In testis GATA-4 is expressed in the Sertoli cells (39) and here it transactivates a number of testis-specific genes (24). Our *in vivo* studies with transgenic mice showed αT-catenin promoter activity in Sertoli cells, strongly suggesting that GATA-4 is also involved in regulating testis-specific expression of αT-catenin. αT-catenin expression in Sertoli cells was also somewhat less restricted than in previous findings, where αT-catenin was only detected in the peritubular myoid cells of human testis (11).

This difference in localization may be due to species differences between man and mouse. Nonetheless, we successfully used the human αT-catenin promoter for mouse transgenesis as the human and mouse promoters show very high sequence identity and conservation of the regulatory elements.

The αT-catenin promoter is also highly active in the brain *in vivo*, which is consistent with endogenous αT-catenin protein expression. The factors involved in regulating brain-specific expression of αT-catenin could not be studied *in vitro*, as we found no *in vitro* system for culturing neurally derived cells expressing αT-catenin. Our *in vitro* data showed that MEF2C can transactivate the αT-catenin promoter. Apart from its expression in the heart, the MEF2C transcription factor also shows high expression in postmitotic neurons in different regions of the brain (40), and is therefore a good candidate for regulating brain-specific αT-catenin expression. Future experiments will aim at verifying this.

In heart and skeletal muscle we could detect only a variegated expression of the *LacZ* transgene. Variegated expression is frequently observed in transgenic mice and reflects transgene silencing in a fraction of cells [for review see (41)]. The mechanism is poorly understood, but it is believed to involve epigenetic modification of the transgene due to chromosomal position effects (42). A remarkable finding is that the position effect variegation in our transgenics was only evident in cardiac and skeletal muscle cells, while in brain and testis transgene expression was apparently not influenced by its chromosomal position. The 2917 bp αT-catenin promoter region used for transgenesis may lack an insulator element or locus control region that mitigates variegating position effects in heart and skeletal muscle. Altogether, our studies show that transgene expression driven by the 2917 bp αT-catenin promoter sequence occurs exclusively in the heart, testis, brain and skeletal muscle.

In conclusion, we report on the cloning of the promoter region of the αT-catenin gene and show that it is responsible for the observed tissue-restricted expression of this α-catenin. We demonstrate a functional role for the GATA-4 transcription factor and a possible role for MEF2C in this tissue-restricted expression of αT-catenin.

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