Cx30.2 enhancer analysis identifies Gata4 as a novel regulator of atrioventricular delay

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The cardiac conduction system comprises a specialized tract of electrically coupled cardiomyocytes responsible for impulse propagation through the heart. Abnormalities in cardiac conduction are responsible for numerous forms of cardiac arrhythmias, but relatively little is known about the gene regulatory mechanisms that control the formation of the conduction system. We demonstrate that a distal enhancer for the connexin 30.2 (Cx30.2, also known as Gjd3) gene, which encodes a gap junction protein required for normal atrioventricular (AV) delay in mice, is necessary and sufficient to direct expression to the developing AV canal (AVC) system. Moreover, we show that this enhancer requires Tbx5 and Gata4 for proper expression in the conduction system, and Gata4+/– mice have short PR intervals indicative of accelerated AV conduction. Thus, our results implicate Gata4 in conduction system function and provide a clearer understanding of the transcriptional pathways that impact normal AV delay.

KEY WORDS: Cardiac conduction system, Arrhythmia, Atrioventricular Node, Cx30.2, Gata4, Mouse

INTRODUCTION

The cardiac conduction system coordinates each cardiac contraction and is essential for the maintenance of cardiac rhythm and blood flow. Even subtle dysregulation of this intricately orchestrated system can cause cardiac arrhythmias and sudden death (Rubart and Zipes, 2005). Thus, an understanding of the molecular basis of cardiac conduction system development and function is likely to reveal pathogenetic mechanisms underlying cardiac arrhythmogenesis. Especially given previous failures of anti-arrhythmic agents in humans (Echt et al., 1991), such insight also represents an important step in the development of novel pharmacological agents targeted at ameliorating cardiac arrhythmias.

The cardiac conduction system comprises a specialized tract of electrically coupled cardiomyocytes responsible for impulse propagation through the heart (Christoffels et al., 2004; Mikawa and Hurtado, 2007) (see Fig. 1A). The AV node (AVN) is a specialized structure within the conduction system responsible for coordinating the relative timing of atrial and ventricular contraction. Specifically, the AVN delays AV impulse propagation, protects the ventricles from elevated atrial rates and acts as a pacemaker in the event of sinoatrial node (SAN) dysfunction (Meijler and Janse, 1988). Interestingly, normal functioning of the AVN becomes particularly important for maintaining adequate cardiac output in the setting of certain arrhythmias (e.g. heart block and pre-excitation) and congestive heart failure (Meijler and Janse, 1988; Leclercq and Kass, 2002).

Recent studies have begun to dissect the developmental gene expression programs involved in the formation of the AVCS. Early histological studies elegantly demonstrated the existence of a definitive AVN by E11 of mouse embryogenesis and suggested that a rudimentary AVCS forms as early as E8 (Viragh and Challice, 1977). Initial studies focusing on the phenotypes of mice carrying mutations in Nkx2.5 and Tbx5, which are also mutated in humans with inherited forms of AV conduction block, demonstrated that both transcription factors are required for AVN morphogenesis and rapid AV conduction (Schott et al., 1998; Bruneau et al., 2001; Jay et al., 2004; Moskowitz et al., 2004; Pashmforoush et al., 2004). Additional studies have identified the T-box transcription factors Tbx2 and Tbx3 as bona fide markers of the developing central conduction system (Habets et al., 2002; Hoogaars et al., 2004). In particular, Tbx3, which has been implicated in the human unlar-mammary syndrome, is required for pacemaker development and AVCS specification (Hoogaars et al., 2007; Bakker et al., 2008). More recently, conditional deletion of Alk3 (Bmpr1a – Mouse Genome Informatics) in the developing AV canal (AVC) using cGATA6-Cre mice demonstrated that BMP signaling impacts AV impulse propagation by regulating the proper formation of the annulus fibrosus and the AVN (Gaussin et al., 2005; Stroud et al., 2007). Although these studies have provided valuable insights into the formation and function of the AVCS, many of the molecular details remain to be completely elucidated.

Several transgenic mouse lines that allow direct visualization of the developing conduction system have hastened studies aimed at analyzing the development of the AVCS (Kuperschmidt et al., 1999; Davis et al., 2001; Rentschler et al., 2001; Kondo et al., 2003). Recently, the gap junction protein connexin 30.2 (Cx30.2, Gjd3 – Mouse Genome Informatics) has emerged as another physiologically relevant marker of the AVCS in mice (reviewed by Kreuzberg et al., 2006a). Although expressed at low levels throughout the conduction system, Cx30.2 preferentially marks the adult SAN and AVN, demonstrated by colocalization with acetylcholinesterase activity, Cx40 (Gja5 – Mouse Genome Informatics) and Cx45 (Gjc1 – Mouse Genome Informatics) (Kreuzberg et al., 2005). Consistently, Cx30.2 forms gap junctions with the slowest conductance of all the connexins expressed in the heart (Kreuzberg et al., 2005). Furthermore, Cx30.2 null mice have accelerated conduction through the AVN with a concomitantly shortened PR interval compared with wild-type mice (Kreuzberg et al., 2006b), revealing a requirement for Cx30.2 in normal AV delay. Given that Cx30.2 is enriched in the...
proximal conduction system of adult mice and is required for normal AV delay, we reasoned that it might also serve as a marker of the AVCS developmental program, thus facilitating an in-depth analysis of the gene regulatory mechanisms required for normal AVCS formation and function.

Here, we define an enhancer for the Cx30.2 gene that is necessary and sufficient to direct expression to the developing AVCS, including the presumptive AVN. The minimal conduction system enhancer contains conserved Tbx5 and Gata4 binding sites required for AVCS-specific Cx30.2 expression. Furthermore, Tbx5 and Gata4 are expressed in Cx30.2⁺ cells of the developing AVCS and are required for normal Cx30.2 expression in vivo. Consistent with the model that Gata4-dependent expression of Cx30.2 might contribute to AV delay, Gata4⁺⁺ mice have a shortened PR interval compared to wild-type littermates and proceeding dorsally to the level of the presumptive AVN (arrow).

MATERIALS AND METHODS

Constructs

Enhancer constructs for transient transgenic experiments were created by PCR amplification using high-fidelity Taq polymerase (Takara, Shiga, Japan) with appropriate primers containing KpnI and XhoI linkers at the 5’ and 3’ ends, respectively. PCR fragments were digested and cloned directly into pGH-lacZ, which contains the HSP68 core promoter upstream of the lacZ coding sequence (Creemers et al., 2006). Binding site mutations were constructed using a modification of megaprimer mutagenesis (Ke and Madison, 1997) with appropriate mutant primers. The Tbx5 and Gata4 prokaryotic expression constructs were generated by PCR amplification of the Tbx5 and Gata4 coding regions followed by their insertion into the pRSET-A vector (Invitrogen, Carlsbad, CA, USA) downstream of an in-frame 6-His tag. All constructs were verified by sequence analysis. Primer sequences will be provided upon request.

Transgenic mice

All embryonic procedures were performed by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center. Cx30.2 enhancer-lacZ constructs were digested with SacII to remove vector sequences and purified using a QIAquick spin column (Qiagen, Valencia, CA, USA). Purified DNA was injected into fertilized eggs from B63F1 female mice and implanted into pseudopregnant ICR mice as previously described (Creemers et al., 2006). In experiments describing the developmental expression of Cx30.2, Cx30.2-lacZ male mice were crossed to ICR female mice, and the day of the vaginal plug was considered to be 0.5 dpc. Similar methodology was used to generate Gata4⁺⁻;Cx30.2-lacZ⁺⁺ and Tbx5⁺⁻;Cx30.2-lacZ⁺⁺ mice. Gata4⁺⁻ mice have been previously described (Xin et al., 2006) and Tbx5⁺⁻ mice were kindly provided by Benoit Bruneau (Bruneau et al., 2001). Pups were collected at the indicated embryonic or postnatal day and stained for β-galactosidase activity as previously described (Creemers et al., 2006). Sectioning and histology were performed on embryos postfixed in 10% phosphate-buffered formalin. Genotyping was performed using standard PCR-based methods; genotyping protocols and primer sequences will be provided upon request.

In situ hybridization

Whole mount in situ hybridizations on E13.5 embryos were carried out as previously described (Molkentin et al., 1997). Briefly, fixed embryos were digested with proteinase K to facilitate probe penetration. After several washes, embryos were incubated with digoxigenin-labeled antisense RNA (Roche, Indianapolis, IN, USA) overnight at 70°C. Following several high stringency post-hybridization washes, the embryos were blocked for 2 hours at room temperature followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche). After extensive washes, the embryos were incubated with BM Purple (Roche) for colorimetric detection of labeled transcripts. Section in situ hybridizations were performed using riboprobes labeled with 35S-UTP (Perkin-Elmer, Waltham, MA, USA) generated with a labeling kit (Ambion, Austin, TX, USA) as previously described (McFadden et al., 2005). The lacZ riboprobe was cloned as a 1.3 kb subfragment (Hu et al., 1999); the mouse Tbx3 riboprobe was generated from the full-length mouse cDNA (Open Biosystems, Huntsville, AL, USA); the Anf riboprobe was used as previously described (McFadden et al., 2005); and the Tbx2 probe was a kind gift of Virginia Papaioannou (Columbia University, New York, NY, USA).

Protein purification

His-tagged proteins were purified according to the manufacturer’s instructions (Novagen, San Diego, CA, USA). Briefly, BL21 DE3 bacteria carrying a plasmid for either Tbx5 or Gata4 were inoculated into a 250 ml culture and induced with 1 mM IPTG for 2 hours at 37°C during log phase growth. The bacterial pellet was resuspended in Buffer A (10 mM Tris pH 8.0, 10% glycerol, 500 mM NaCl, 0.1% NP-40, 10 mM β-mercaptoethanol, 1 mM PMSF and 25 mM imidazole), sonicated, cleared and incubated with Ni-NTA agarose beads (Novagen) with 1% NP-40 for 2 hours. The beads were washed and eluted with Buffer A containing increasing amounts of imidazole. Fractions containing His-tagged protein, identified by using Coomassie gels, were pooled and used for further experiments.

Electrophoretic mobility shift assay (EMSA)

Experiments were performed using annealed double-stranded oligonucleotides derived from the indicated regions of the Cx30.2 minimal enhancer end-labeled with T4 Polynucleotide Kinase (NEB, Ipswich, MA, USA). Binding reactions were carried out with recombinant proteins and
labeled oligonucleotides in 5% glycerol, 10 mM Tris pH 7.9, 50 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.1% NP-40, 1 mg/ml BSA, 1 μg p gd–DC for 15 minutes at room temperature. DNA-protein complexes were resolved by native PAGE using gels containing 5% polyacrylamide at 4°C, dried and exposed to film overnight. Primer sequences used for EMSAs will be provided upon request.

Chromatin immunoprecipitation assays
COS7 cells were transfected with the indicated Flag-tagged eukaryotic expression constructs and enhancer templates, and crosslinked, harvested and sheared according to a published protocol (Nelson et al., 2006). Sheared chromatin was immunoprecipitated with anti-Flag (Sigma, Milwaukee, WI, USA) or anti-β-galactosidase (Promega, Madison, WI, USA) antibodies and protein A sepharose beads (Upstate Biotechnology, Billerica, MA, USA). The immunoprecipitates were washed extensively, and associated genomic DNA was eluted and used as the template in PCR reactions using the indicated Cx30.2 enhancer primers for 30 cycles.

Gene expression profiling of Cx30.2* cells
Male Cx30.2-lacZ mice were crossed to female ICR mice to generate Cx30.2-lacZ hemizygous pups that were sacrificed at E17.5, and transgene-positive animals were identified by PCR genotyping using lacZ primers. Embryonic AV canals were dissected from the hearts of transgenic pups and placed into ice-cold PBS, minced extensively and digested in 0.25% trypsin (Invitrogen) for 30 minutes at 37°C, with gentle trituration every 10 minutes. Digested cells were strained and resuspended in a minimal volume of DMEM (Invitrogen) and incubated with an equal volume of 2 mM Fluorescein Digalactoside (Invitrogen) for 1 minute at 37°C. This cell suspension was diluted 10-fold with ice-cold DMEM and placed on ice for 1 hour prior to FACS analysis. FACS was performed at the UT Southwestern Flow Cytometry Core Facility, and Cx30.2-β-Gal and Cx30.2-β-Gal+ cells were sorted directly into TRizol (Invitrogen) for the purification of total RNA. RT-PCR analysis was performed with cDNA created by the oligo-dT method (Invitrogen) and the indicated sequencing primers using GoTaq Polymerase (Promega) in a Mastercycler EP gradient thermal cycler (Eppendorf, Westbury, NY, USA) for 30-35 cycles.

β-Galactosidase assays
β-Galactosidase assays were performed as described previously (Jay et al., 2004). Briefly, embryos were removed at E12.5 and placed into ice-cold PBS, and AVCs were microdissected and flash-frozen in liquid nitrogen. Embryonic AVCs were subsequently lysed in 10% β-mercaptoethanol, 67 mM Na3PO4 and 0.88 mg/ml ONPG for 1 hour at 37°C prior to the addition of 1 ml of stop solution (1 M Na2CO3) and the measurement of A420. Reactions were performed in triplicate and samples of the indicated genotypes were averaged together to generate final results. A sample of each lysate (10 μl) was used for the BCA assay (Pierce, Rockford, IL, USA) to determine protein concentrations to normalize the β-galactosidase activity of each sample.

Quantitative real-time PCR
Real-time PCR was performed as previously described (Liu et al., 2008) using gene-specific probes (ABI, Foster City, CA, USA) and cDNA generated using oligo-dT primers (Invitrogen) on RNA isolated from E12.5 AVCs. PCR reactions were performed in an ABI Prism 7000 Sequence Detection System (ABI) and analyzed using the ΔΔCt method according to the manufacturer’s instructions.

Electrocardiogram analysis
Electrocardiograms (EKGs) were performed on adult mice between the ages of 4 and 14 weeks that were anesthetized with 50 μg/kg of pentobarbital delivered by intraperitoneal (IP) injection. Mice were laid down on a heating pad to maintain core body temperature, and limb leads were placed subcutaneously. Lead II EKGs were recorded at a sampling rate of 4000 Hz (ADInstruments, Colorado Springs, CO, USA) both at rest and continuously for 30 seconds following IP injection with 375 μg isoproterenol. EKG intervals were measured using the LabChart software package (ADInstruments), by an investigator who was blinded to the genotypes of the mice, at baseline and every 10 seconds following isoproterenol injection. Comparisons in all experiments were made between heterozygous and wild-type age-matched littersmates with the same genetic background.

Statistical analysis
All data are expressed as mean±s.e.m. except where indicated. P values were generated using the Student’s two-tailed t-test, and statistical significance was considered for P<0.05.

RESULTS
A Cx30.2 enhancer drives expression in the developing AVCS
We sought to define the cis-regulatory sequences that direct transcription of the Cx30.2 gene to the proximal conduction system, and we were particularly interested in testing whether these regulatory sequences were active during embryogenesis. Therefore, the entire noncoding region 5’ of the Cx30.2 start codon until the next gene on chromosome 11 (Top2a) was cloned upstream of the HSP68-lacZ cassette and injected into mouse oocytes. Transgenic embryos were collected at E17.5 and stained with X-Gal, revealing expression in the AVC and the presumptive AVN by whole mount and serial sections (Fig. 1B,C) in a configuration reminiscent of the minK-lacZ and cGATA6-lacZ reporter lines (Kuperschmidt et al., 1999; Davis et al., 2001; Kondo et al., 2003). This experiment suggested that all of the information necessary to drive Cx30.2 expression within the AVCS resides in the 10.8 kb DNA fragment upstream of the Cx30.2 translational start site.

In order to better define the embryonic pattern of Cx30.2-lacZ expression, we performed a detailed developmental analysis of its expression. The lacZ transgene was detected as early as E8.5, predominantly within atrial and ventricular myocytes in proximity to the developing AVC (Fig. 2A). The onset of lacZ expression occurs between E7.5 and E8.5, as X-Gal staining of E7.5 embryos was not detected (data not shown). Cx30.2-lacZ expression continued within the developing AVC as the embryo turned and the heart began to loop (Fig. 2B,C). At E10.5 to E12.5, lacZ expression remained within the developing AVC and presumptive AVN, but not in AV valve progenitor cells of the endocardial cushions, underscoring the specificity of transgene expression within the developing AVCS (Fig. 2D-F; see also Fig. S1 in the supplementary material). Extracardiac expression of the lacZ transgene only became evident at E12.5 in the central nervous system (Fig. 2F), consistent with previous studies (Kreuzberg et al., 2007). We verified that lacZ was expressed in a pattern consistent with endogenous Cx30.2 expression by performing whole-mount in situ hybridizations on hearts dissected from E13.5 embryos (Fig. 2G).

Although these data strongly suggested that cells expressing the Cx30.2-lacZ transgene contribute to the developing AVCS based on morphological criteria (Viragh and Challicke, 1977), we wished to confirm their identity using previously established molecular markers. Section in situ hybridizations on Cx30.2-lacZ E12.5 embryos revealed that cells expressing lacZ colocalize with those expressing the central conduction system marker Tbx3 (Hoogaars et al., 2004), but not the chamber-specific marker Anf (Nppa – Mouse Genome Informatics) (Fig. 2H); we obtained similar results using a Tbx2 riboprobe (data not shown). Taken together, these results suggest that the Cx30.2-lacZ transgene marks a subset of Tbx3+ Anf+ cells in the developing AVCS and provide further support for the existence of a functional AVCS at least as early as E8.5.

DEVELOPMENT
A 660 bp Cx30.2 enhancer is sufficient to direct AVCS-specific gene expression

To delimit the boundaries of the Cx30.2 enhancer, we generated a series of 5′ and 3′ deletion constructs cloned into the HSP68-lacZ cassette, which narrowed the minimal enhancer to a 1.2 kb region between 2.9 and 1.7 kb upstream of the Cx30.2 start codon (Fig. 3A; see also Fig. S2 in the supplementary material). Further mapping revealed that the region between 2.9 and 2.3 kb upstream of the start codon directed expression of Cx30.2-lacZ to the conduction system (Fig. 3A; Fig. S2 in the supplementary material). Although we attempted to further refine the boundaries of the enhancer, smaller genomic fragments were unable to fully recapitulate AVCS expression in transient transgenic assays (data not shown). Thus, the Cx30.2 minimal enhancer maps to a 660 bp fragment that is necessary and sufficient for Cx30.2 expression in the developing AVCS, including in the presumptive AVN.

Interestingly, bioinformatic analysis revealed that the 660 bp minimal enhancer contains several regions of ultraconservation (Fig. 3B). Two regions drew particular attention, given the presence of conserved putative binding sites for Tbx5 and Gata4, transcription...
Regulation of the Cx30.2 enhancer by Gata4

In order to test whether Tbx5 and/or Gata4 could indeed bind to the Cx30.2 enhancer in vitro, we performed electrophoretic mobility shift assays (EMSAs) using highly purified recombinant proteins and 32P-labeled enhancer DNA fragments. Recombinant Gata4 bound to the Cx30.2 minimal enhancer in a specific manner, as indicated by the fact that wild-type, but not mutant, unlabeled competitor oligonucleotide could inhibit DNA binding of Gata4 (Fig. 4A, left panel). Similarly, recombinant Tbx5 could bind to both putative T-box elements (TBE1 and TBE2) within the Cx30.2 minimal enhancer, and the specificity of DNA binding was demonstrated by competition with unlabeled wild-type, but not mutant, oligonucleotide (Fig. 4A, right panel). Next, chromatin immunoprecipitation (ChIP) was used to confirm that Tbx5 and GATA factors could indeed bind to the Cx30.2 enhancer in a cellular context. These experiments revealed that the anti-Flag antibody, but not an irrelevant antibody (β-Gal) or beads alone, could immunoprecipitate the Cx30.2 minimal enhancer only in the presence of transfected Flag-tagged Tbx5 or Gata4 (Fig. 4B). Given the ability of Gata4 and Tbx5 to physically interact (Garg et al., 2003) and the proximity of the TBE1 and GATA binding sites, we also tested whether they could form a ternary complex on the Cx30.2 minimal enhancer. The EMSA in Fig. 4C demonstrates that Gata4 and Tbx5 do indeed form a complex on the enhancer, but only in the presence of intact GATA and T-box elements, revealing that ternary complex formation requires the DNA binding activity of both transcription factors. Thus, the Cx30.2 minimal enhancer harbors one GATA element and two T-box elements that direct DNA binding and ternary complex formation of Gata4 and Tbx5, implicating these transcription factors in AVCS-specific gene expression in vivo.

To determine whether any of the GATA or T-box elements are required for Cx30.2 expression in vivo, we created transgenic constructs harboring single mutations in each of the binding sites in the context of the 1.2 kb minimal enhancer. Mutation of either T-box element individually (-2.9/–1.7 TBE1mut, –2.9/–1.7 TBE2mut) or the context of the 1.2 kb minimal enhancer, and the specificity of DNA binding was demonstrated by competition with unlabeled wild-type, but not mutant, oligonucleotide (Fig. 4A, right panel). Next, chromatin immunoprecipitation (ChIP) was used to confirm that Tbx5 and GATA factors could indeed bind to the Cx30.2 enhancer in a cellular context. These experiments revealed that the anti-Flag antibody, but not an irrelevant antibody (β-Gal) or beads alone, could immunoprecipitate the Cx30.2 minimal enhancer only in the presence of transfected Flag-tagged Tbx5 or Gata4 (Fig. 4B). Given the ability of Gata4 and Tbx5 to physically interact (Garg et al., 2003) and the proximity of the TBE1 and GATA binding sites, we also tested whether they could form a ternary complex on the Cx30.2 minimal enhancer. The EMSA in Fig. 4C demonstrates that Gata4 and Tbx5 do indeed form a complex on the enhancer, but only in the presence of intact GATA and T-box elements, revealing that ternary complex formation requires the DNA binding activity of both transcription factors. Thus, the Cx30.2 minimal enhancer harbors one GATA element and two T-box elements that direct DNA binding and ternary complex formation of Gata4 and Tbx5, implicating these transcription factors in AVCS-specific gene expression in vivo.

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Fig. 4. Tbx5 and GATA binding sites are required for Cx30.2 expression in the conduction system. (A) Electrophoretic mobility shift assay (EMSA) demonstrating specific binding of Gata4 and Tbx5 to oligonucleotides derived from the Cx30.2 minimal enhancer. The probes are shown at the bottom of the gel and the indicated recombinant proteins (Gata4 or Tbx5) and 100× wild-type (w) or mutant (m) cold competitors are shown at the top of each gel. The dHAND GATA probe corresponds to a previously characterized GATA binding site from the dHAND cardiac enhancer (McFadden et al., 2000). The oligonucleotides were mutated as follows: TBE1mut, ACACCT→ATTTCT; TBE2mut, AGGGTG→AGAAAAT; GATAmut, TATC→TTTT. (B) Chromatin immunoprecipitation (ChIP) experiments were performed to confirm that Tbx5 and GATA factors bind to the Cx30.2 enhancer in a cellular context. Sheared chromatin was prepared and immunoprecipitated with the indicated antibody or with beads alone, and PCR was performed on the associated genomic DNA. Flag antibody immunoprecipitated Cx30.2 enhancer DNA only when transfected with Flag-tagged Gata4 or Tbx5 but not with Flag alone. (C) EMSA demonstrating that Gata4 and Tbx5 can form a complex on the composite TBE1/GATA element derived from the Cx30.2 minimal enhancer. As shown, complex formation only occurs in the presence of both transcription factors and with intact DNA binding sites. Each element was mutated as shown in A. (D) The Cx30.2 TBE1, TBE2 and GATA elements are required for expression of a Cx30.2 minimal enhancer-lacZ transgene in vivo. The indicated DNA binding site mutations used in EMSAs were introduced in the context of a Cx30.2 minimal enhancer, injected into fertilized mouse oocytes, harvested and stained for X-Gal activity at E17.5. LacZ transgenic hearts were scored as positive based on whether the staining pattern was similar to that observed for the wild-type –2.9/+1.7 construct by comparing whole-mount hearts and equivalent serial sections as shown. (E) Hearts from E17.5 Cx30.2-lacZ transgenic embryos were harvested and AVCs were microdissected as shown in the schematic diagram. Pooled AVCs were digested and sorted by FACS. Cx30.2-β-Gal+ and Cx30.2-β-Gal− AVCs cells were then used to isolate RNA. The FACS plot indicates the gates used to purify Cx30.2-β-Gal+ (R2) and Cx30.2-β-Gal− (R1) cells from E17.5 AVCs. RT-PCR analysis of the indicated genes in Cx30.2-β-Gal+ and Cx30.2-β-Gal− AVCs cells demonstrates that Tbx5 and Gata4 are expressed in Cx30.2-β-Gal− AVCs cells.

**Diminished Cx30.2 enhancer activity in Tbx5 and Gata4 mutant mice**

In order to directly test whether Tbx5 and Gata4 factors regulate Cx30.2 gene expression in vivo, we crossed Cx30.2-lacZ transgenic mice with Gata4+/− and Tbx5+/− mice (Bruneau et al., 2001; Xin et al., 2006). As predicted by our transient transgenic experiments, lacZ expression was diminished at E12.5, detected by whole-mount X-Gal staining, in both heterozygous backgrounds (Fig. 5A). Furthermore, histological sections through the caudal AVN revealed that, although lacZ expression was diminished in the Cx30.2+ AVN of Gata4+/− mice, the overall size of the Cx30.2+ AVN did not appear significantly smaller compared with wild type (Fig. 5B); similar results were obtained with Gata4+/−; Cx30.2-lacZ/+ mice sacrificed at E10.5 and P3 (data not shown). By contrast, Tbx5+/− mice had diminished lacZ expression in the Cx30.2+ AVN, in addition to an apparent reduction in the overall size of this structure (Fig. 5B), consistent with the crucial function of Tbx5 in AVN morphogenesis (Moskowitz et al., 2004). In order to provide a more quantitative assessment of lacZ expression in the AVCS, we performed β-galactosidase assays on microdissected AVCs from E12.5 embryos to anatomically enrich for AVCS cells. As predicted by the whole-mount staining, this analysis revealed a quantitative decrease in β-galactosidase activity in both Gata4+/− and Tbx5+/− mice (Fig. 5C). Moreover, endogenous Cx30.2 gene expression was diminished in parallel with β-galactosidase activity, as assessed by quantitative real-time PCR analysis (Fig. 5D).
was quantitatively and qualitatively similar between statistically significant at were assayed in triplicate for each genotype, and all comparisons were demonstrated by quantitative RT-PCR analysis. At least three hearts decrease in endogenous Cx30.2 were statistically significant at hearts were assayed in triplicate for each genotype, and all comparisons expression in the AVC of E12.5 hearts as demonstrated by liquid lacZ Interestingly, these experiments demonstrated that Cx30.2 expression in the developing AVCS (arrowhead), observed in all mutants tested. (B) E12.5 embryos of the indicated genotype were subjected to histological analysis, and sections at the level of the caudal AVN are shown. Note the decreased lacZ expression in both heterozygous backgrounds, with reduced size of the Cx30.2-lacZ AVN only in the Tbx5+/- background. (C) There is a quantitative decrease in lacZ expression in the AVC of E12.5 hearts as demonstrated by liquid β-galactosidase (β-Gal) assays on the indicated genotypes. At least three hearts were assayed in triplicate for each genotype, and all comparisons were statistically significant at P<0.001. (D) There is a quantitative decrease in endogenous Cx30.2 expression in the AVC of E12.5 hearts as demonstrated by quantitative RT-PCR analysis. At least three hearts were assayed in triplicate for each genotype, and all comparisons were statistically significant at P<0.005.

In order to further test the notion that Gata4+/– mice have normal AVN morphogenesis, we performed in situ hybridization on E12.5 Gata4+/– embryos and wild-type siblings, using a Tbx3 riboprobe as a marker of the entire AVCS (Hoogaars et al., 2004). Interestingly, these experiments demonstrated that Tbx3 expression was quantitatively and qualitatively similar between Gata4+/– and wild-type mouse hearts (see Fig. S3A in the supplementary material). By contrast, Tbx3 expression was diminished in Tbx5–/– mice (see Fig. S3A in the supplementary material), consistent with previous results demonstrating that Tbx5 affects overall AVN morphogenesis (Moskowitz et al., 2004). Moreover, quantitative real-time PCR analysis confirmed that Tbx5–/– mice had diminished Tbx3 expression in the AVC, whereas the levels of Tbx3 in Gata4–/– mice were not significantly altered compared with wild type (see Fig. S3B in the supplementary material). Taken together, these experiments demonstrate that Cx30.2 is a direct transcriptional target of Gata4 and Tbx5 in the developing AVCS. Moreover, Gata4 can activate Cx30.2 expression without affecting the morphology of the Cx30.2 AVN, whereas Tbx5 appears to affect Cx30.2 expression in addition to AVN morphogenesis, suggesting that these two transcription factors might play unique roles in regulating AVN formation and function.

Gata4–/– mice have a shortened PR interval
Given that Cx30.2 decelerates impulse propagation through the AVN (Kreuzberg et al., 2006b), the identification of the Cx30.2 minimal enhancer should provide a valuable tool for dissecting the genetic circuitry underlying AVN gene expression and its role in establishing normal AV delay. Our experiments thus far provide further support for the crucial role of Tbx5 in AVN formation and suggest an unanticipated role for Gata4 in regulation of key AVCS-specific target genes such as Cx30.2. Since Tbx5 and Gata4 can form a ternary complex on the Cx30.2 minimal enhancer (Fig. 4C), both expressed in embryonic Cx30.2-lacZ AVCS cells (Fig. 4E) and genetically cooperate in cardiac morphogenesis (Garg et al., 2003; Maitra et al., 2008), we wished to specifically test whether Gata4–/– mice had functional defects in AV conduction. Thus, we recorded surface EKGS on Gata4–/– mice and compared them with their wild-type littermates. Remarkably, Gata4–/– mice had a shortened PR interval compared with wild-type (41.0±0.5 versus 44.0±0.8 milliseconds, P<0.01), although heart rate (HR), QRS interval and QT interval were similar between the two groups (Fig. 6A-B). Interestingly, the shortened PR interval in Gata4–/– mice partially phenocopies Cx30.2 knockout mice (Kreuzberg et al., 2006b), suggesting a genetic connection between Gata4 and Cx30.2 in vivo. In order to exclude the possibility that the observed differences in the PR interval of Gata4–/– mice were a result of differences in HR, we performed EKG analysis following isoproterenol infusion. As shown in Fig. S4 in the supplementary material, this data demonstrated that Gata4–/– mice had shortened PR intervals irrespective of heart rate. Interestingly, the accelerated AV conduction observed in Gata4–/– mice was not a general property of all GATA factors expressed in the heart, as Gata6+/– mice had normal PR intervals, even over a wide range of heart rates (see Fig. S5A,B in the supplementary material). Taken together, these experiments demonstrate that Gata4 contributes to the deceleration of AV impulse propagation and suggest that the Cx30.2 minimal enhancer can be utilized to identify other potential regulators of normal AV delay.

**DISCUSSION**

Here we describe a detailed analysis of the transcriptional control of gene expression within the developing AVCS, including the AVN. We define an enhancer that is necessary and sufficient to direct specific expression of Cx30.2 within this specialized region of the heart as early as the looping heart tube stage. The Cx30.2 minimal enhancer requires Tbx5 and Gata4 for its activity and reveals an
unanticipated role for Gata4 in normal AV conduction, suggesting that the enhancer might serve as a valuable tool for dissecting the logic underlying AVCS-specific gene expression.

The Cx30.2 minimal enhancer marks a subset of developing AVN cells

Early histological studies had suggested the existence of a rudimentary AVCS as early as E8 (Viragh and Challice, 1977); our results provide additional evidence for the early establishment of the AVCS between E7.5 and E8.5. Since the first observed cardiac contraction occurs around E8.5, our developmental analysis of Cx30.2-lacZ expression suggests that a primitive, functional AVCS probably emerges coincident with a functional SAN. Given the recent demonstration that Anf-expressing cells do not give rise to the SAN (Hoogaars et al., 2007), it is tempting to speculate that the AVN is also specified early during embryogenesis. Nevertheless, future studies will need to determine whether AVN morphogenesis involves early specification or continuous recruitment, two models that are not necessarily mutually exclusive.

The complexity of AVN structure is highlighted by the presence of at least six distinct electrophysiological cell types within the AVN (Meijler and Janse, 1988), implying that formation of this crucial structure is likely to be at least as complicated. Previous studies have demonstrated that Tbx5 and Nkx2.5 function in part by regulating morphogenesis of the AVN (Jay et al., 2004; Moskowitz et al., 2004; Pashmforoush et al., 2004; Ismat et al., 2005; Moskowitz et al., 2007). Although Gata4 is required for heart tube formation and ventral morphogenesis (Kuo et al., 1997; Molkentin et al., 1997), the early lethality of Gata4 null mice has hampered an analysis of the potential function of Gata4 during cardiac lineage commitment later in development. Recent studies have clearly established that Gata4 is a dosage-sensitive regulator of heart formation, with important functions in diverse aspects of cardiac morphogenesis (Kuo et al., 1997; Molkentin et al., 1997; Pu et al., 2004; Xin et al., 2006). Here we show that Gata4 is also required for the expression of Cx30.2 in the developing AVCS and contributes to normal AV delay. Given that transcription factor knockouts typically have pleiotropic effects, there is a high likelihood that Gata4 regulates additional genes that impact AV delay, and future studies will be aimed at exploring these complexities in more detail. The pleiotropy of transcription factor knockouts is illustrated in Tbx5+/– mice (Bruneau et al., 2001), which have a prolonged PR interval despite the reduced expression of Cx30.2 (Fig. 5C,D). Since Tbx5 regulates target genes with opposing functional consequences on AV conduction (e.g. Cx30.2 and Cx40; see Fig. 5D and Fig. S6 in the supplementary material), we believe that the PR prolongation in Tbx5+/– mice results from the dominant effect of Tbx5 on the expression of genes required for rapid AV conduction (see Fig. 7).

Despite widespread expression throughout the myocardium, Gata4 appears to have a very specific effect on AV conduction (Fig. 6). We suggest that this observation might be explained by the following: (1) dosage-dependent effects of Gata4; (2) interaction between Gata4 and other cofactors; and/or (3) region-specific redundancies with other GATA factors. Similar to the rheostat mechanism proposed for Tbx5 (Mori et al., 2006), Gata4 might affect subsets of genes depending on their sensitivity to Gata4 concentration, which in turn stems from the nature of specific GATA elements within their enhancer or promoter regions. By this reasoning, perhaps genes involved in slow AV conduction are particularly sensitive to a 50% reduction in Gata4 levels. In addition, the interaction of Gata4 with other cofactors might dictate regional implications for the role of Gata4 in normal AV conduction

Several transcription factors that have been implicated in conduction system development play additional vital roles in cardiac development and organogenesis (Schott et al., 1998; Bruneau et al., 2001; Jay et al., 2004; Moskowitz et al., 2004; Pashmforoush et al., 2004; Ismat et al., 2005; Moskowitz et al., 2007). Although Gata4 is required for heart tube formation and ventral morphogenesis (Kuo et al., 1997; Molkentin et al., 1997), the early lethality of Gata4 null mice has hampered an analysis of the potential function of Gata4 during cardiac lineage commitment later in development. Recent studies have clearly established that Gata4 is a dosage-sensitive regulator of heart formation, with important functions in diverse aspects of cardiac morphogenesis (Kuo et al., 1997; Molkentin et al., 1997; Pu et al., 2004; Xin et al., 2006). Here we show that Gata4 is also required for the expression of Cx30.2 in the developing AVCS and contributes to normal AV delay. Given that transcription factor knockouts typically have pleiotropic effects, there is a high likelihood that Gata4 regulates additional genes that impact AV delay, and future studies will be aimed at exploring these complexities in more detail. The pleiotropy of transcription factor knockouts is illustrated in Tbx5+/– mice (Bruneau et al., 2001), which have a prolonged PR interval despite the reduced expression of Cx30.2 (Fig. 5C,D). Since Tbx5 regulates target genes with opposing functional consequences on AV conduction (e.g. Cx30.2 and Cx40; see Fig. 5D and Fig. S6 in the supplementary material), we believe that the PR prolongation in Tbx5+/– mice results from the dominant effect of Tbx5 on the expression of genes required for rapid AV conduction (see Fig. 7).

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Fig. 7. Model for transcriptional regulation of normal AV conduction. Tbx5 and Gata4 cooperatively regulate the expression of myocardial genes (Anf) that are required for normal cardiac formation and function. Within the AVCS, Tbx5 and Gata4 control distinct transcriptional networks required for normal AV conduction. Although Tbx5 activates gene expression programs with opposing functional properties, its dominant effect (wide arrow) is regulating rapid AV conduction. By contrast, Gata4 is envisioned to predominantly affect the slow-conduction program with an unknown role (dashed arrow) in the regulation of fast-conduction genes. In this simplified diagram, Cx40 and Cx30.2 are depicted as representatives of the fast-conduction and slow-conduction gene expression programs, respectively.

effects on gene expression and cardiac function, as exemplified by the recent demonstration that interaction between Gata4 and Tbx5 is required for human atrial septal morphogenesis (Garg et al., 2003). Similarly, despite the widespread myocardial expression of Tbx5 and Nkx2.5, mice heterozygous for either transcription factor have similar PR prolongation (Schott et al., 1998; Bruneau et al., 2001; Jay et al., 2004; Moskowitz et al., 2004), underscoring the functional importance of their physical interaction and coexpression in the AVN. In addition, we speculate that a working myocardium-enriched repressor or conduction system-enriched co-activator restricts Cx30.2 expression to the developing AVCS. Finally, although Gata4 and Gata6 are clearly redundant in some aspects of cardiac morphogenesis (Xin et al., 2006), it remains a distinct possibility that they might have nonredundant functions in specific tissues within the heart. Consistent with this idea, we showed that Gata4+/– mice have a shortened PR interval, although Gata6+/– mice have PR intervals that are not significantly different from their wild-type littermates (Fig. 6 and Fig. S5 in the supplementary material).

Although Gata4 and Tbx5 cooperatively regulate myocardial genes (e.g. Anf) required for cardiac morphogenesis (Garg et al., 2003), our experiments suggest that they also impact AVCS-specific gene expression. Within the developing AVCS, we envision two distinct programs of gene expression that mediate fast and slow conduction, as depicted in Fig. 7. As shown, whereas Tbx5 impacts both gene expression programs, with a dominant effect on rapid conduction, Gata4 predominantly affects slow conduction, with an unknown role in rapid AV conduction. Although Gata4 has been implicated in Cx40 regulation (Linhares et al., 2004), we have been unable to detect a reduction in Cx40 transcript levels in Gata4+/– mice (see Fig. S6 in the supplementary material). Thus, we hypothesize that by regulating slow AV conduction target genes, Gata4 might fine-tune normal AV delay, especially given the relatively modest phenotypic effect on the PR interval observed in Gata4+/– mice. Furthermore, although Cx30.2 and Cx40 serve as crucial markers of the slow- and fast-conducting elements that contribute to normal AV conduction, the analysis of Cx45 regulation will also provide valuable insights, given that Cx30.2/Cx40 double knockout mice have normal PR intervals (Schrickel et al., 2009). In order to explain how Tbx5 might influence the expression of genes required for both fast and slow conduction, two potential models can be envisioned. First, cellular heterogeneity within the AVCS might allow Tbx5 to regulate specific subsets of genes in different cell types depending on the transcriptional milieu of a particular cell. Second, target gene sensitivity might be influenced by the number and/or affinity of Tbx5 binding sites or by the presence or absence of additional transcription factor binding sites (e.g. TBE/NKE versus TBE/GATA). Nevertheless, determining the individual contributions of Gata4 and Tbx5 to each gene expression program will further our understanding of the genetic circuitry underlying normal AV conduction and will probably require the deletion of individual transcription factors within specific subsets of AVN cells.

The AVCS helps to maintain cardiac output by delaying AV impulse propagation for adequate ventricular filling prior to systole and protects the ventricles from the potentially catastrophic effects of an elevated atrial rate (Meijler and Janse, 1988). In addition to Cx30.2 expression, recent studies have implicated BMP signaling in mice (Gaussin et al., 2005; Stroud et al., 2007) and Neuregulin and Notch signaling in zebrafish (Milan et al., 2006) in establishing normal AV delay, although the downstream targets of these pathways have not been characterized. Here we have focused on Cx30.2, a crucial gene involved in establishing normal AV delay, and worked upstream to identify Gata4 as a potential nodal point for regulating other genes dedicated to slow AV impulse propagation. Further detailed analysis of these pathways might some day shed light on the pathophysiology of clinical arrhythmias arising from abnormal AV conduction.

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Supplementary material
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References


Milan, D. J., Giakos, A. C., Serrafi, F. C., Peterson, R. T. and MacRae, C. A. (2006). Notch1b and neuregulin are required for specification of central cardiac conduction tissue. Development 133, 1125-1132.


Pu, W. T., Ishiwata, T., Juraksc, A. L., Ma, Q. and Izumo, S. (2004). GATA4 is a major cardiac transcriptional regulator in the developing heart. Circ. Res. 95, 235-244.


