Complex cardiac Nkx2-5 gene expression activated by noggin-sensitive enhancers followed by chamber-specific modules

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We previously reported that an Nkx2-5-GFP bacterial artificial chromosome in transgenic mice recapitulated the endogenous gene activity in the heart. Here, we identified three additional previously uncharacterized distal enhancer modules of Nkx2-5: UH6, which directed transgene expression in the right ventricle, interventricular septum, and atrial ventricular canal; UH5, which directed expression in both atria; and UH4, which directed transgene expression in tongue muscle. Nkx2-5 enhancers drive cardiogenic gene activity from the earliest progenitors to the late-stage embryonic heart, reside within its 27 kb of 5′ flanking sequences, organized in a tandem array. Nkx2-5 enhancers involved with stomach-, tongue-, and chamber-restricted expression displayed *lacZ* transgene activity and chromatin histone acetylation patterns consistent with tissue-specific expression. An examination of Nkx2-5 gene activity in murine embryonic stem cells converted to beating embryoid bodies showed that only the proximal active region 2 and GATA-Smad enhancers were chromatin-remodeled. Chromatin remodeling of active region 2 and GATA-Smad enhancers were blunted by noggin coexpression, which indicated dependence on bone morphogenetic protein signaling for their chromatin activation during activation of Nkx2-5 expression.

enhancer modules | heart development | Nkx2-5 gene regulation

Nkx2-5 (1), also called *Csx* (2), is a vertebrate homologue of the homeobox *tinman* gene (3), which is required for cardiac development in insects and vertebrates (3, 4). Nkx2-5 is expressed in early cardiac progenitor cells before cardiogenic differentiation and through adulthood (see ref. 5 for review) and demarcates the heart field (6). Nkx2-5 also is expressed to pharyngeal endoderm, spleen, distal stomach, and tongue muscle (1). Heart formation is dependent on signals from adjacent endoderm (7). Activation of *tinman* transcription in dorsal mesoderm is dependent on signaling by the bone morphogenetic protein (BMP) family member decapentaplegic (8). Schultheiss *et al.* (9) showed that ectopic application of BMPs to regions of chick embryos allowed for the induction of Nkx2-5. Activin, another TGF-β family member, also may induce cardiogenesis (10), whereas combined BMP2 and FGF4 signaling may be necessary to induce cardiogenesis in nonprecardiac embryonic mesoderm (11). Targeted disruption of *Nkx2-5* caused early embryonic lethality, with cardiac development arrested at the linear heart-tube stage (12, 13), similar to that seen in BMP2-null mouse embryos (14). Recent studies showed that mutations in *Nkx2-5* were responsible for congenital cardiac malformations and atrioventricular conduction abnormalities in humans (15).

The *Nkx2-5* gene has demonstrated surprising complexity, with multiple enhancers acting in distinct populations of cardiomyocytes during development (16–19). Schwartz and Olson (20) described a model for cardiac development based on the modularity of transcriptional units that control Nkx2-5 and suggest a potential role for this modularity in the evolution of the multichambered heart. Chi *et al.* (21) reported that transgenic mice harboring a GFP reporter gene under the regulation of the murine *Nkx2-5* genomic locus in a bacterial artificial chromosome (BAC) closely resembled the endogenous *Nkx2-5* gene activity. Thus, core cardiac regulatory regions were apparently circumscribed within this BAC. The present study revealed that all of the chamber-specific enhancers are organized in a distal tandem array, consistent with the idea that the multichambered heart evolved as a modular organ (22) under the control of individual regulatory modules (23, 24). Many of these distal *Nkx2-5* enhancer regions displayed cardiac-restricted histone acetylation patterns indicative of activated chromatin remodeling (25–27). In addition, among the many regulatory enhancers, only two enhancers were structurally remodeled by histone acetylation, which coincided and correlated well with *Nkx2-5* gene activation.

Experimental Procedures

BAC Clones. BAC clone RP23-130D16 was obtained from the BACPAC Resource Center at Children's Hospital Oakland Research Institute in Oakland, CA (http://bacpac.chori.org). RP23-130D16 was constructed from C57BL/6J mouse DNA in pBACe3.6 vector, and it contained ≈16 kb of 5′ flanking sequences and 180 kb of 3′ flanking sequences of *Nkx2-5* confirmed by PCR and BAC end-sequencing. The bacteria host strain for BAC clones was DH10B (*recA*$^-$, *recBC*$^+$). Retrofitting the Nkx2-5-GFP-BAC clone with *loxP* transposons was performed as described in refs. 28–31. BAC cloning was described by Chi *et al.* (21) and Muyrers *et al.* (32). SJ-1 cells were kindly provided by Bert O'Malley (Baylor College of Medicine), which was modified from the DY380 *Escherichia coli* cell line (33) by deleting β-galactosidase sequences from the bacterial genome by homologous recombination, which eliminated recombination between *lacZ* sequences in the bacterial genome with the targeting construct. Transgenic lines were maintained on C57/BL6 background.

Inserting *loxP*-Tn*10* into Nkx2-5 GFP BAC Clone. Retrofitting the Nkx2-5 GFP BAC clone with *loxP* transposons was performed in a manner similar to that described in ref. 29. A newly developed transposon plasmid, pTnMarkerless1 (31), was used to retrofit Nkx2-5 GFP BAC.

Construction of Targeting Vectors. The targeting vector was constructed on pKO1905 backbone (Stratagene), as described in ref. 21. A 13-kb SalI fragment was cloned into the SalI site of pBluescript-SK to generate pBS-Nkx2-5 clone 2. The *lacZ* cDNA from pPD46.21 was subcloned in-frame into the XhoI site of pBS-Nkx2-5 clone 2 after modification to generate pBS-Nkx2-5lacZ construct. A 7-kb fragment containing 3.4 kb of Nkx2-5 sequences upstream

Abbreviations: BAC, bacterial artificial chromosome; BMP, bone morphogenetic protein; E*n*, embryonic day *n*; LV, left ventricle; RV, right ventricle; IV, interventricular septum; AVC, atrioventricular canal: ChIP, chromatin immunoprecipitation; AR, active region.

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of the ATG site and *lacZ* cDNA from pBS-Nkx2-5lacZ generated by NotI and XhoI digestion was subcloned into NotI and XhoI sites of the targeting vector. A genomic clone of *Nkx2-5* containing 3′ flanking sequences was kindly provided by Katherine Yutzey (Children's Hospital, Cincinnati), and a 1.4-kb fragment of 3′ flanking sequences generated by BamHI and SmaI digestion was subcloned into the BamHI and HpaI sites of the targeting vector.

Assay of Enhancers in Transgenic Mice. Different enhancer regions were cloned into the Hsp68lacZ reporter gene (34). UH4 was subcloned by PCR using Pfx DNA polymerase (Stratagene) into ZeroBlunt TOPO vector (Invitrogen). UH6 is a 7.3-kb fragment from a BamHI library generated from the 112-kb BAC genomic DNA, and UH5 is a 2.6-kb fragment from a HindIII library generated from the 112-kb BAC genomic DNA (21). Transgenic embryos were identified by PCR analysis of yolk-sac DNA by using a pair of *lacZ* primers and confirmed by Southern blot analysis. At least three independent transgenic mouse lines were evaluated for each *lacZ* transgene.

Sequence Alignment and Visualization Using VISTA. The genomic sequences of Nkx2-5(mCG1575) and Csx (hCG41243) were obtained by using the Celera Discovery System (www.celera.com) The 112-kb Nkx2-5 BAC sequences from the mouse genome were aligned against an equivalent portion of the human Csx sequences by using VISTA software (www-gsd.lbl.gov/vista) and were used for comparisons of noncoding regions (35).

ES Cell Culture, Transfection, and Differentiation. AB2.2 ES cells were routinely grown on gelatinized plates in the presence of lymphocyte inhibitory factor. pNoggin-CS2+ (kindly provided by Richard Harland, University of California, Berkeley) and pCS2+ (kindly provided by Dave Turner, University of Michigan, Ann Arbor) were transfected into ES cells, respectively, by using the Effectene transfection reagent kit (Qiagen, Valencia, CA). Embryoid bodies were made by using the "hanging drop" method (36) using ES cells 24 h after transfection.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed as described in refs. 37 and 38. ES cell lysates were precleared by incubation with salmon sperm DNA/protein A agarose-50% slurry (Santa Cruz Biotechnology), and ChIP was performed with 10 μg of anti-acetylhistone H3 and H4 antibodies (Santa Cruz Biotechnology). DNA fragments were used for PCR amplifications with appropriate primers in 25-μl reactions containing 2 μl of sample DNA. Thirty cycles of amplification are typically used. The following primers were used: active region 2 (AR2) enhancer, 5′-CTGCTCATCCATCAGCCAGACGAAGA-3′ and 5′-GAAAGATAAGCTGCAACTATCACCCGG-3′, 357-bp product; G-S(-6211, -5974) enhancer, 5′-CAGTCTTGGGAGCTCAAGACTAACC-3′ and 5′-CAGATCCCCAAGCTTACTAGCAACTAC-3′, 255-bp product; active region 1 (AR1) enhancer, 5′-CTGGGTCCTAATGCGGGTGGCGTCTC-3′ and 5′-AACCCTCTGCTGTGTGGCCTTGTATCT-3′, 246-bp product; UH6-LV enhancer, 5′-CCCCACAGCACAGAAAGTTCAGAATCC-3′ and 5′-CACCCACCAAAACACAGCCCCAGGATAA-3′, 299-bp product; UH5-atria enhancer, 5′-CCCTGCTATTGAGGATGCTCTCTTATG-3′ and 5′-TGGCAAAGCAAGCAGAGCAGAGGGAGGA-3′, 274-bp product; and tongue enhancer, 5′-CCCTATCTAACCAGCCATCAGTGAG-3′ and 5′-CGGCCTCCCTGCTTCCTGTAC-3′, 248-bp product. The PCR products were analyzed on 2% agarose gels.

RNA Isolation and RT-PCR Analysis. Total RNA was isolated from ES cells or embryoid bodies by using TRIzol reagents (Invitrogen). Reverse transcription was performed by using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. Each reaction contained 200 ng of total RNA.

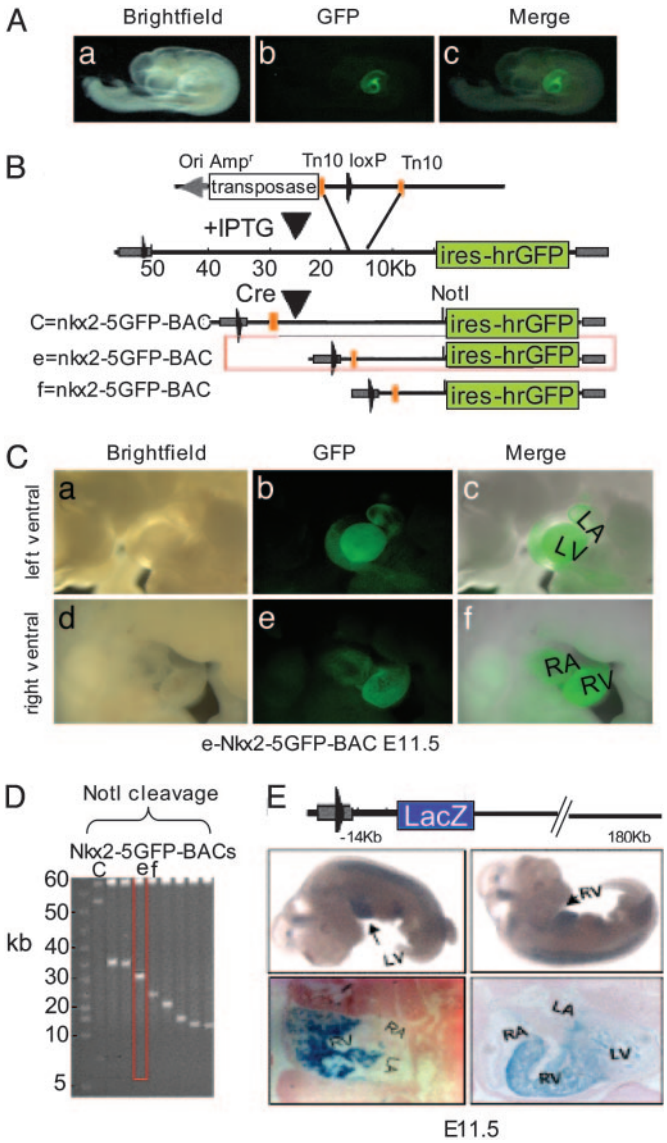

Fig. 1. Generation of nested deletions of Nkx2-5-GFP BAC identified the most distal 5′ border of the cardiac regulatory locus. (*A*) A green fluorescent image merged with a brightfield image of the Nkx2-5-GFP-BAC mouse embryo. (*B*) The strategy as a schematic diagram for generating gap deletions by retrofitting a *loxP* site by transposition into the Nkx2-5-GFP-BAC. Deletions were generated by Cre recombinase between the endogenous *loxP* site in the BAC vector and the transposed *loxP* site. (*C*) The expression pattern of e-Nkx2-5GFP-BAC transgenic mouse line. Shown are brightfield (*a*), green fluorescent (*b*), and merged (*c*) images of the left ventral view and the brightfield (*d*) green fluorescent (*e*), and merged (*f*) images of the right ventral view of an e-Nkx2-5-GFP-BAC transgenic embryo at E11.5, showing GFP expression in the left atrium (LA) and left ventricle (LV). (*D*) The series of gap deletion constructs run in a pulsed-field gel electrophoresis. The e-Nkx2-5-GFP-BAC construct (which has 27 kb on the 5′ flanking sequences and 60 kb on the 3′ flanking sequences) was selected for microinjection to make transgenic mouse lines. (*E*) A schematic diagram of Nkx2-5-LacZ-BAC construct, which has 16 kb on the 5′ flanking sequences and 180 kb on the 3′ flanking sequences. LacZ expression was observed in Nkx2-5-LacZ-BAC mice in the right ventricle (RV) and LV but was absent in both atria. LacZ expression also was observed in the distal stomach region. RA, right atrium.

PCR was performed by using Taq DNA polymerase (Invitrogen) with 25 cycles of amplification. Relative quantities between samples were normalized to GAPDH levels. The primers used for *Nkx2-5* were 5′-TCTCCGATCCATCCCACTTTATTG-3′ and 5′-TTGCGTTACGCACTCACTTTAATG-3′, 222-bp product.

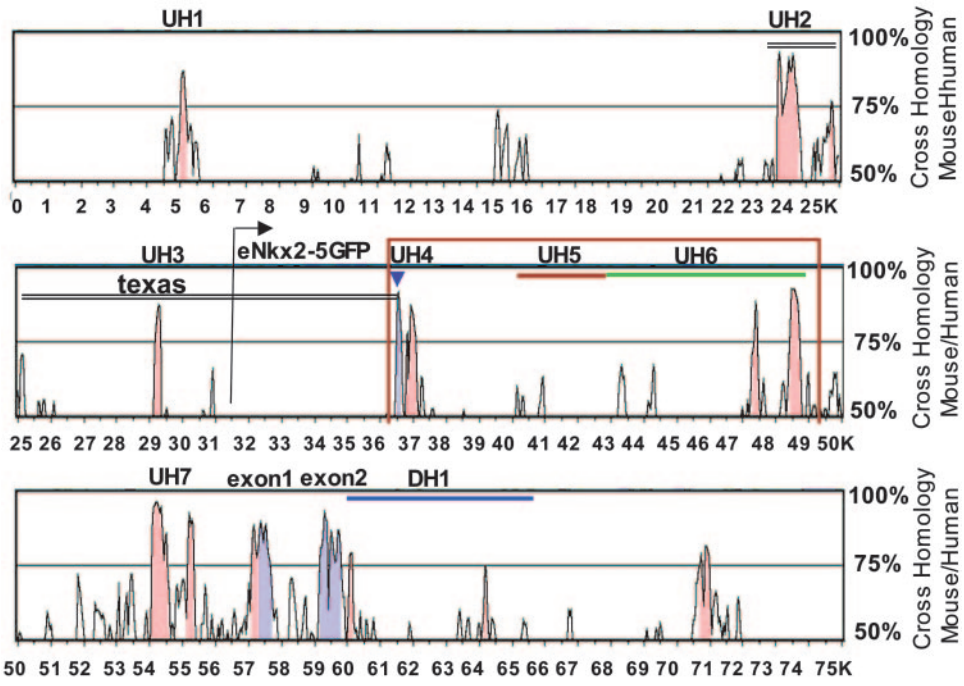

Fig. 2. Pairwise alignment of the mouse and human *Nkx2-5/Csx* loci revealed multiple upstream noncoding regions of high homology. Conserved sequences are shown relative to their position in the mouse (horizontal axes), and their percent cross-homology between mouse and human sequences (50–100%) are indicated on the vertical axes. The location of the gene *texas* is indicated by double underlines. Horizontal arrows indicate the direction of transcription for each gene. Peaks representing the noncoding sequences (red) fitting the criteria for conserved elements as well as coding sequences (blue) meeting the percentage criteria over their entire length are indicated. UH, upstream homology region. The arrow indicates the end of the eNkx2-5-GFP deletion construct on the 5′ flanking region. UH4, UH5, and UH6 above the profile indicate the location of the fragments for transgenic analysis.

Results

Generation of Nested Deletions of Nkx2-5-GFP-BAC Identified the Most Distal 5′ Border of the Cardiac Regulatory Locus. As shown in Fig. 1*A* and by Chi *et al.* (21), a BAC clone that contained ≈55 kb of both 5′ and 3′ sequences flanking the *Nkx2-5* coding region was capable of recapitulating the endogenous gene activity in the heart. We used Tn*10* random insertion mutagenesis to generate progressive deletions from one end in the BAC clone by Cre/*loxP* recombination in *E. coli* (Fig. 1*B*). This highly efficient method generated end deletions in the modified BAC clone and obviated the need to modify a number of overlapping BACs (39). A nested deletion series was generated in the Nkx2-5-GFP-BAC construct by random insertion of retrofited *loxP* sites. The deletion clone e-Nkx2-5-GFP-BAC was selected for microinjection, and all three transgenic founder mouse lines showed expression patterns similar to that of the full-length Nkx2-5-GFP-BAC line. GFP expression was observed in four chambers of the heart (Fig. 1*C*), in the pyloric sphincter, and in the spleen (not shown). GFP fluorescence was not observed in the tongue muscle. It is important to note that the 5′ flanking sequences in the serial deletions ranged from −38 kb to −15 kb (Fig. 1*D*), and the 3′ flanking sequences remained constant. The size of the clones in the deletion series is random, and transposition itself had no bias to any particular regions in Nkx2-5-GFP-BAC. We obtained two lines of Nkx2-5LacZ-BAC transgenic mice, and both lines have the same expression pattern as that shown with the 14-kb upstream region of Nkx2-5. LacZ expression was observed in the entire right ventricle (RV) and the trabeculated layer of the left ventricle (LV) but was missing from the compact layer of the LV and from both atria (Fig. 1*E*). Apparently, there were no additional regulatory elements in the extensive 3′ flanking regions that extended Nkx2-5 gene expression.

VISTA Revealed Multiple Noncoding Regions Containing High Levels of Cross-Species Homology. Comparison of genomic sequences for conserved noncoding sequences has proved fruitful for detecting conserved regulatory regions. Long-range genomic sequence alignment between mouse (*Nkx2-5*) and human (*Csx*) demonstrated multiple peaks of high levels of homology (Fig. 2), a subset of which corresponded precisely with known *Nkx2-5* enhancers and coding regions. DH1 matched to a RV enhancer (16). UH7 overlapped AR2, a proximal enhancer region responsive to BMP-Smad signaling (41, 42) that directed transgene expression in the cardiac crescent and later in the RV, stomach, and thyroid (17–20). The proximal peak of UH6 corresponded to another RV enhancer driven by GATA4 (19).

Transgenic Analysis of the Conserved Noncoding Regions Revealed Previously Uncharacterized Enhancers. The ability of each of these highly homologous regions, UH4, UH5, and UH6, to drive *lacZ* expression was tested in transgenic mice by using the Hsp68LacZ expression vector. UH6-Hsp68LacZ directed *lacZ* expression at embryonic (E) days 10.5 and 12.5 in the entire RV and the interventricular septum (IVS) (Fig. 3 *Aa–Af*). LacZ staining also was observed in the trabecular layer of the LV (Fig. 3 *Ab*, *Ae*, and *Af*), indicating that there was a weak LV enhancer within this transgene, in addition to the known RV enhancer within the UH6 region. In the atrial region, there was a subset of LacZ-positive cells in the interatrial groove. There also was a cluster of LacZ-positive cells in the IVS. The pattern of LacZ expression remained constant until E12.5 (Fig. 3 *Ac–Af*) and later (data not shown).

The UH5-Hsp68lacZ transgene directed LacZ expression throughout the looping heart at E9.5 (Fig. 3*Ba*), in the common atria, the common ventricle, and the aortic sac (Fig. 3*Bb*). LacZ expression also was observed in the foregut at E9.5 (Fig. 3*Ba*). By E12.5, the LacZ expression was restricted to both atria, AVC, IVS, and a subset of cardiomyocytes in the LV but was absent from the RV (Fig. 3 *Bc–Bg*). LacZ expression also was observed at E12.5 in the distal stomach region (Fig. 3*Bd*, black arrow), which is the site of foregut-derived pyloric sphincter and the spleen, that express Nkx2-5. *In situ* hybridization using antisense BMP2 probe showed that BMP2 gene was expressed in the myocardial layers overlaying the AVC (Fig. 3*C*), which colocalized with the LacZ expression pattern of both UH5 and UH6-Hsp68lacZ in the AVC.

Identification of the Tongue Enhancer and *texas*. UH4-HSP68LacZ transgene was expressed specifically in the tongue primordia at E11.5 (Fig. 4 *a* and *b*) and strongly in the tongue muscle at E12.5 (Fig. 4 *c* and *d*) and after (Fig. 4 *e–h*), whereas weaker expression was noted in the somites and body wall muscle (Fig. 4*f*). Surprisingly, tongue muscle expression was not observed in either the

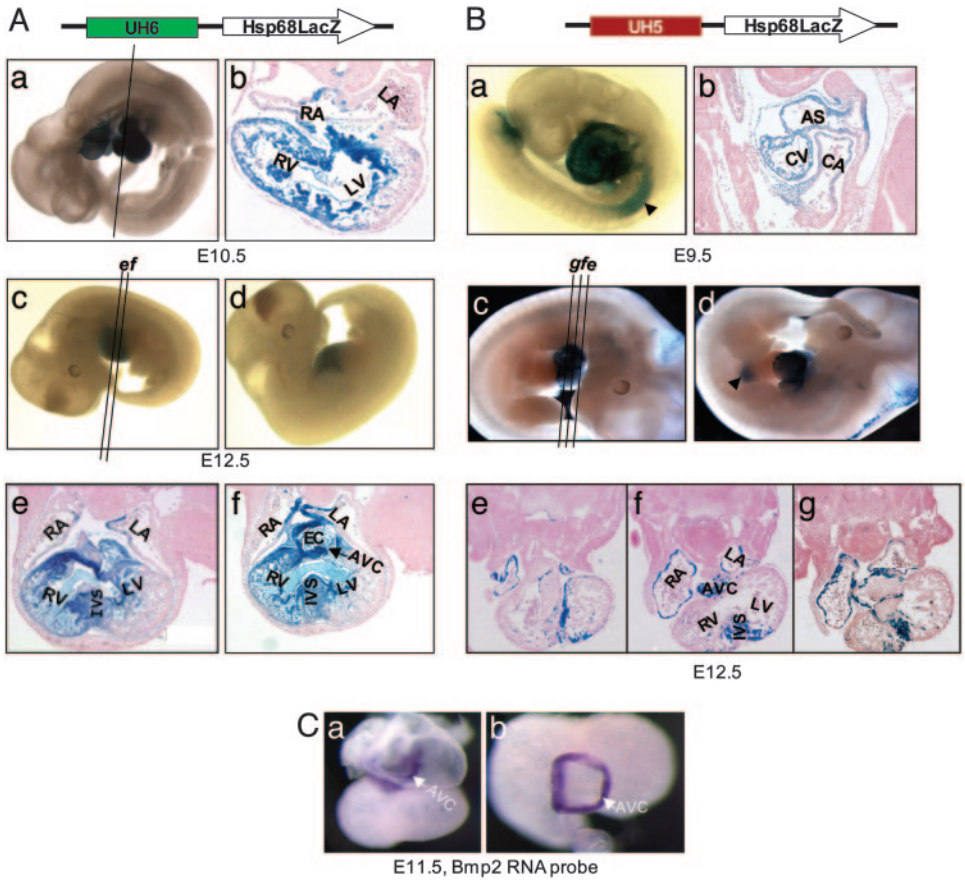

Fig. 3. Transgenic analysis of the conserved noncoding regions functioned as previously uncharacterized enhancers for expression in the interventricular septum (IVS), atrial ventricular canal, and atria in mouse embryos. (*A*) Whole-mount X-gal staining (*a*) and transverse sections (*b*) of an E10.5 embryo carrying the UH6-Hsp68lacZ transgene are shown. LacZ expression was observed uniformly in the entire RV and in a subset of cells in the trabecular layer and septal wall of the LV. There was a cluster of LacZ-positive myocardial cells in the IVS and in the atrioventricular canal (AVC). (*c* and *d*) Whole-mount X-gal staining (*c*) and transverse sections (*d*) of an E12.5 embryo carrying the UH6-Hsp68lacZ transgene are shown. Transgene expression was similar as that in E10.5. (*B*) Whole-mount X-gal staining (*a*) and transverse sections (*b*) of an E9.5 embryo carrying the UH5-Hsp68lacZ transgene, in which the LacZ expression is homogenous in the looping heart at E9.5. LacZ also is expressed in the foregut at this stage (as indicated by an arrow in *a*). *Bb* shows the transverse section of the embryo in *a* stained with nuclear fast red, revealing LacZ expression in the aortic sac, the common atria, and the common ventricle. *Bc* (right ventral view) and *Bd* (left ventral view) show the whole-mount staining at E12.5, with LacZ expression restricted to the RA and LA, AVC, IVS, and a subset of cells in the LV, also in the distal stomach region (as indicated by an arrow in *d*). *Be–Bg* show the transverse sections of the embryo in *c*. (*C*) *In situ* hybridization using antisense Bmp-2 mRNA on E11.5 embryonic heart, showing the intense staining in the myocardial layer of the AVC. *Ca* is the whole-heart view, and *Cb* is a heart cut in the AVC region and viewed from the above.

Nkx2-5-GFP-BAC transgenic mice or the transgenic mice generated from the genomic region encompassing 22.7 kb surrounding the *Nkx2-5* gene. Complicating matters is the fact that the first peak of UH2 (arrow in Fig. 2) corresponds precisely to exon 1 of an EST from a differentiated C2C12 myotube library (GenBank accession no. AA597013; IMAGE clone ID, 1059294 5′), and the EST contains two other exons with 100% homology to the more upstream genomic sequences (12480–12622 and 263–407) in the BAC. The corresponding gene lies on the 5′ side of *Nkx2-5* and is transcribed in the opposite orientation and was named *texas* for the 5′-terminal-expressed *NKX2-5*-associated skeletal muscle gene.

Histone Acetylation Pattern on Nkx2-*5* Enhancers Correlated Well with Their Activation both Temporally and Spatially. Histone acetylation increases the accessibility of nucleosomal DNA to sequence-specific DNA-binding proteins and is associated with transcriptional activation (25, 26). Fig. 5 shows a schematic diagram of the enhancers analyzed by a ChIP assay (27, 37, 38) with anti-acetylated histone H4 antibodies. Liver tissues do not express Nkx2-5 and served as negative controls. Three primary conclusions were drawn from this analysis (Fig. 5*A*). First, histone acetylation patterns showed tissue specificity: cardiac enhancers as indicated by the detection of AR2, G-S, AR1, UH6-LV, and UH5-atria, and UH4-tongue PCR-amplified DNA fragments were retrieved from a ChIP assay with anti-acetylated histone H4 antibodies from E12.5 and neonatal hearts. Enhancers that direct expression in the stomach and spleen, AR2 and AR1 sequences, were immunoprecipitated with anti-acetylhistone H4 antibodies from E12.5 neonatal stomach. The tongue enhancer (UH4) was immunoprecipitated only from E12.5 and neonatal tongue tissues and not from cardiac tissue. Second, the anti-acetylhistone H4 ChIP pattern correlated well with the activation of these enhancers, as determined by transgenic analysis (Figs. 3 and 4). In addition, antibodies directed to acetylhistone H3 and anti-acetylhistone H4 had similar superimposable ChIP patterns on these different Nkx2-5 enhancers (data not shown).

Which of these modular enhancers were activated during the earliest appearance of the *Nkx2-5* gene activity? As shown in Fig. 5, Nkx2-5 started to be expressed on day 6 in embryoid bodies when embryoid bodies were used as an *in vitro* system to mimic cardiac differentiation (36) until day 8. Noggin expression in embryoid bodies, which blocks BMP signaling (43), significantly diminished the earliest Nkx2-5 induction (Fig. 5*B*). Third, only two cardiac enhancers, AR2 and G-S, were PCR-amplified from ChIP from embryoid bodies at days 6 and 8 transfected with the control plasmid pCS2+ yet were not immunoprecipitated from embryoid bodies transfected with noggin at the same time points (Fig. 5*C*). None of the other *Nkx2-5* enhancers assayed here, AR1, UH6-LV, UH5-atria, and UH4-tongue, were immunoprecipitated with anti-acetylhistone H4 antibodies at the same time points in embryoid bodies. In addition, Fig. 5*D* shows a whole-mount X-gal staining of an E7.5 embryo carrying the G-S-Hsp68lacZ transgene in the early cardiac crescent, consistent with our previous observations (44) that the GATA-Smad-enriched enhancer is activated early in cardiogenesis.

Discussion

Organization of *Nkx2-5* Enhancers: Proximal vs. Distal Enhancers. Schwartz and Olson (20) described a model for cardiac development based on the modular nature of transcriptional units that

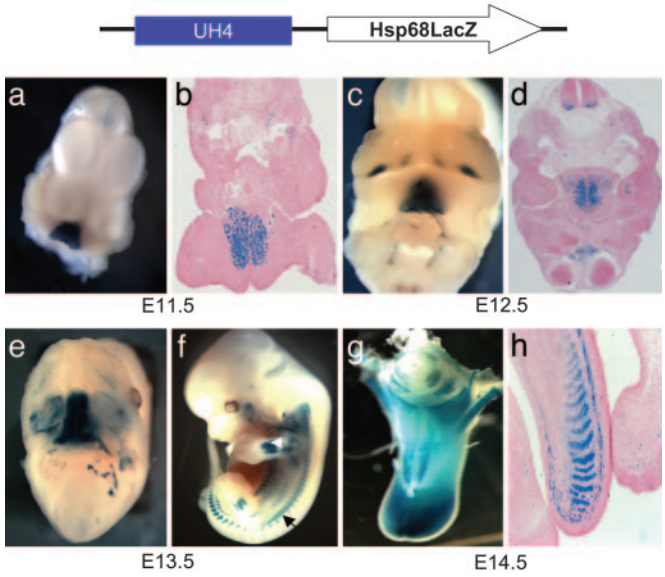

Fig. 4. Identification of the tongue enhancer identified by UH4-Hsp68lacZ transgene expression patterns. Whole-mount X-gal staining (*a*, *c*, and *e–g*) and transverse sections (*b*, *d*, and *h*) of embryos carrying the UH4-Hsp68lacZNkx2-5 transgene. Transgene expression was observed specifically in the tongue primordia at E11.5 (*a* and *b*) and in the tongue muscle at E12.5 (*c* and *d*) and after (*e–h*). LacZ staining also was seen after E12.5 in the somites (*f*, small arrow) and the muscle in the limb (*f*, arrowhead).

control Nkx2-5 expression at a time when the LV and atrial enhancers remained unidentified. Three additional previously uncharacterized enhancer regions of *Nkx2-5* were identified, and LacZ expression patterns observed in the heart strongly supported the "modular and combinatorial genetic pathway hypothesis" for the formation of vertebrate heart. In addition, congenital cardiac malformations associated with human *Nkx2-5* mutations suggest a role for *Nkx2-5* at later stages of cardiogenesis. Chien and colleagues (45) recently reported that a ventricular-restricted knockout of Nkx2-5 results in massive trabecular muscle overgrowth and conduction system defects and suggested that a dysregulation of the Nkx2-5-BMP-10 axis was responsible for the defects. These findings indicated that besides the critical role for Nkx2-5 in early cardiac specification and commitment, it was being used again in later elaboration of the chambers, septation, and the conduction system. Possibly, the dosage of Nkx2-5 is tightly regulated in different chambers/regions of the heart by each of the enhancer modules to achieve its physiological functions.

Interestingly, UH4 corresponded to a highly conserved enhancer that drove LacZ expression strongly in the tongue muscle. The finding was especially intriguing because tongue muscle expression was not observed in either the Nkx2-5 GFP BAC transgenic mice or the transgenic mice generated from the genomic region encompassing 22.7 kb surrounding the *Nkx2-5* gene. This observation suggests that there are strong repressor regions residing around UH4 and that additional activating regions must reside outside the BAC for expression in tongue muscle. We speculate that the tongue enhancer was used primarily to drive *texas* in the tongue and skeletal muscle but also served as an *Nkx2-5* enhancer in the tongue; such as the intragenic or shared enhancers for muscle-specific transcription factors Myf5 and Mrf4 (39).

Histone Acetylation Plays a Role in Establishing Long-Range Transactivation of *Nkx2-5* on the Enhancers. We showed that the histone acetylation patterns of the recently identified *Nkx2-5* enhancers were developmentally regulated and tissue-specific. Histone acetylation patterns correlated well with spatial and temporal activation of these enhancer elements on the chromosome and in agreement with the transgenic study; thus, histone acetylation played a role in regulating *Nkx2-5* expression by modulating the activation status of its enhancers. We also showed that AR2 and G-S enhancers were chromatin remodeling-dependent on BMP-Smads signaling. This observation also was in agreement with previous studies showing that both AR2 and G-S enhancers are essential for *Nkx2-5* expression in the cardiac crescent (41, 42, 44). A single Smad site in AR2 and a cluster of Smads sites in G-S enhancers were required for enhancer activity at early stages of heart development *in vivo*. In fact, deletion of the G-S enhancer, which contains multiple arrays of Smad and GATA DNA-binding sites, blocked the appearance of reporter gene activity in the cardiac crescent (44). Point mutations of GATA and Smad sequences in AR2 also blocked expression during cardiogenesis (41, 42). Also, the multimeric G-S enhancer activated early in the cardiac crescent was later down-regulated in E11 transgenic mice (44) and showed a loss of acetylated histones by E12.5 (Fig. 5), perhaps through enhanced BMP-induced inhibitory Smad6 expression (46). In addition, Lassar and colleagues (47)

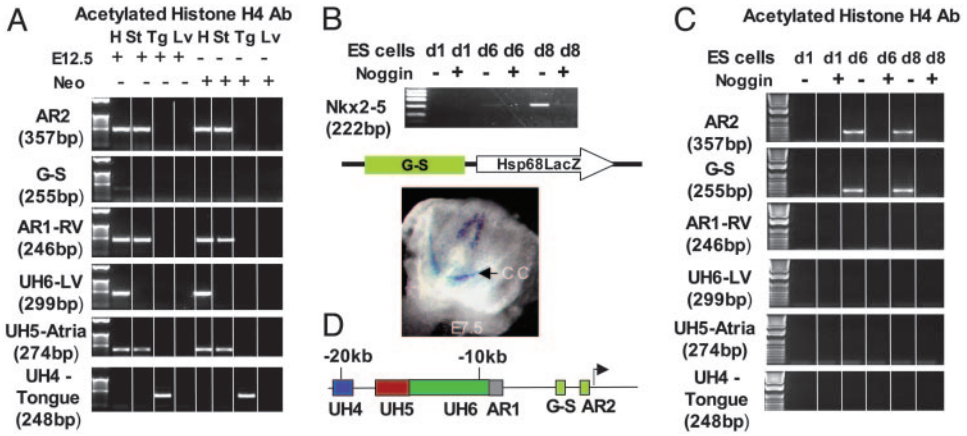

Fig. 5. Histone H4 acetylation patterns correlated well with temporal and spatial activity of *Nkx2-5* distal enhancers and the activation of the proximal AR2 and G-S modules during ES cell induced cardiogenesis. (*A*) The anti-acetylhistone H4 pattern of *Nkx2-5* enhancers from embryonic and neonatal tissues. (*B*) The RT-PCR analysis of ES cells and embryoid bodies at different time points after aggregation, with and without noggin. (*C*) The histone H4 acetylation pattern of AB2.2 ES cells and embryoid bodies at days 6 and 8 of differentiation. pNoggin-CS2+ expression plasmid was transfected into ES cells to block BMP signaling. pCS2+ vector served as a transfection control. (*D*) Whole-mount X-gal staining of an E7.5 embryo carrying the G-SHsp68lacZ transgene and a schematic diagram of the enhancers assayed by ChIP for histone acetylation patterns.

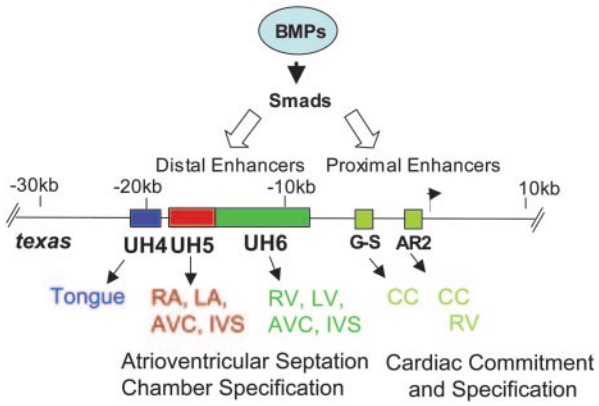

Fig. 6. A general model for murine embryonic *Nkx2-5* transcriptional regulation. *Nkx2-5* is driven by two groups of enhancers: proximal and distal, both of which are downstream of the BMP-Smad signaling pathway. Earlier on, at the cardiac crescent stages, secreted BMPs from pharyngeal endoderm stimulate *Nkx2-5* gene transcription by chromatin remodeling of the proximal enhancers G-S and AR2 in the cardiac crescent (CC) and are required for cardiac commitment and specification. The G-S enhancer is not active in later stages of heart formation, whereas AR2 provides RV expression. Later, during chamber specification, enriched BMP-Smad signaling may activate the distal enhancers, UH5 and UH6, that direct *Nkx2-5* gene activity in the atria, ventricles, IVS, and AVC during chamberization.

identified three cardiac-activating regions that surround the chick *Nkx2.5* gene, similar to its murine counterpart AR2 (17, 18, 20), capable of driving transgene expression in the cardiac crescent in a BMP-responsive fashion and dependent on GATA- and SMAD-binding sites.

A Model for *Nkx2-5* Transcriptional Regulation. We showed that the earliest *Nkx2-5* cardiac enhancers were AR2 and G-S, both of which are BMP-dependent and proximal to the promoter region. The previously uncharacterized distal enhancer regions identified in this study were responsible for chamber-/tissue-specific expression of *Nkx2-5* at later stages of cardiogenesis, such as the UH5 enhancer, which may be regulated by Bmp2/4, as a signal from the myocardium directly mediating atrioventricular septation (48). The model proposed here is one of *Nkx2-5* being a tightly regulated gene, with its regulation having two stages that coincide with its functions (Fig. 6): (*i*) during early cardiac commitment and specification, there were at least two cardiac enhancers that ensured its uniform expression in the cardiac crescent; (*ii*) multiple tandem arrayed enhancers were then activated during chamber specification, and these distal enhancers restrict their domains of influence to different regions/chambers of the heart. This observation was similar to a *cGATA6* enhancer that is activated early and then selectively marked the atrioventricular conduction system (49). This report on the chamber-/region-specific enhancer analysis of *Nkx2-5* strongly supported the modular and combinatorial genetic pathway hypothesis for the formation of vertebrate heart.

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