The Cardiac Determination Factor, Nkx2-5, Is Activated by Mutual Cofactors GATA-4 and Smad1/4 via a Novel Upstream Enhancer*

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The mammalian homologue of Drosophila tinman, Nkx2-5, plays an early role in regulating cardiac genes and morphogenesis. Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β family of signaling molecules, are involved in numerous developmental processes. BMP signaling is crucial in the regulation of Nkx2-5 expression and specification of the cardiac lineage. Constitutively active BMP type I receptor or the downstream pathway components and DNA-binding transcription factors, Smad1/4 directly activated Nkx2-5 gene transcription. We identified and characterized a novel upstream Nkx2-5 enhancer, composed of clustered repeats of Smad and GATA DNA binding sites. This composite Nkx2-5 enhancer was a direct target of BMP signaling via cooperative interactions between the downstream transducers Smad1/4 and GATA-4. In mammalian two hybrid assays, Smad factors recruited the hybrid gene GATA4-VP16 to strongly drive transcription of a reporter gene containing multimerized Smad binding sites. These cofactors interacted through the second zinc finger and adjacent basic domain of GATA-4 and the N-terminal domain of Smads. Smad4 and GATA4 were also found to bind in vivo with the Nkx2-5 composite enhancer, as revealed by chromatin immunoprecipitation analysis of differentiated P19 cells. Finally, transgenic mice containing the Smad/GATA composite enhancer recapitulated early murine Nkx2-5 cardiac expression. Deletion of this enhancer within a 10-kb transgene pBS-Nkx2-5 LacZ significantly reduced expression in the cardiac crescent. Thus, integration of GATA transcription factors with BMP signaling, through co-association with Smads factors, may initiate early Nkx2-5 expression; suggesting a vital role for the combination of these factors in the specification of cardiac progenitors.

The homeobox gene, Nkx2-5 (also called Csx), a vertebrate homologue of Drosophila tinman, is one of the earliest known markers of mesoderm fated to give rise to myocardium (reviewed by Harvey, Ref.1). Nkx2-5, tinman, and other related factors recognize novel NKE sequence elements (2, 3) and may be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: BMP, bone morphogenetic protein; TGF-β, transforming growth factor β; SBE, Smad binding element; Alk, activin-like kinase; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; FL, full-length.
Smad or Smad8), which then interacts with the common partner Smad4. TGF-β and activin, through a different set of receptors, utilize Smad2 and Smad3 as their pathway-specific signals. The heteromeric Smad complex translocates to the nucleus, binds DNA and activates transcription (reviewed in Ref. 20). Smad proteins may interact with other transcription factors to assist in increasing DNA binding specificity or affinity (21–23), as well as with transcriptional coactivators (24). Smad transcription factors are also known to be involved in various stages of embryonic and cardiac development. Homozygous Smad4 (27, 28) and Smad2 (29–31) mutant mice displayed a block in early mesoderm formation, while Smad5 knockout mice eventually died at 9.5–11.5 dpc with cardiac defects similar to bmp-2 knockout mice (32). Although null mutants of relevant TGF-β family receptors have often displayed lethality at gastrulation stages (including Alk2, Alk3, Alk5, and BMPR2, see review, Ref. 33), conditional deletion of Alk3 in the epiblast or in cardiac myocytes revealed roles for BMP signaling in maintenance of cardiac genes and proper heart formation (see review by Schneider et al., Ref. 34). Interestingly, Smad sites in the proximal enhancer of Nkx2-5 (35, 36) were found to be required for its activity. These sites were also located near essential GATA sites mentioned previously (10). Thus, across insect and vertebrate evolution, BMPs and the Smad factors are involved in embryonic heart development, and it is possible this signaling pathway interacts with GATA factors to activate Nkx2-5 and other cardiac genes.

We found that BMP signaling activated Nkx2-5 directly through Smad1/4 transcription factors, and that there was co-dependence with GATA factors. We identified a novel upstream Nkx2-5 gene enhancer composed of highly repeated Smad and GATA binding sites. This Smad/GATA composite enhancer directed robust LacZ reporter transgene activity in the cardiac crescent and in the chambered heart; thus, it recapitulated nascent embryonic Nkx2-5 gene activity. Smad factors were also capable of recruiting GATA-4 to a synthetic Smad reporter and in a mammalian two-hybrid assay. In addition, mutal GATA-4 and Smad physical co-association was shown here to be important in their role as intermediaries of the BMP signaling pathway.

EXPERIMENTAL PROCEDURES

Expression Vectors—Smad5 expression vector was donated by Kohei Miyazono. Smad6 cDNA was a gift from Dr. Martin Matzuk (Baylor College of Medicine, Houston, TX) and subcloned into pcDNA3.1+ (Invitrogen) with a FLAG epitope tag. Mouse GATA-4 cDNA was obtained by RT-PCR from day 10.5 embryo total RNA, then cloned in-frame with the C-terminal epitope tags in pcDNA3.1-myct-His-A (Invitrogen) using EcoRI and Xhol sites. From this vector, N- and C-terminal serial deletions were constructed. Additionally, a double point mutation was made changing paired cysteines of the second zinc finger to glycines (C270G/C273G). All deletions and mutations of GATA-4 were made by PCR and verified by sequencing.

Transgenic—Transgenic mice were generated by pronuclear injection. Fragments from the upstream region of the Nkx2-5 gene (G-S; 5,857 to -5,638; GA; 5,857 to -5,729; and SB; 5,731 to -5,636) were amplified by PCR and cloned into the Hsp68-LacZ vector (37). To generate Nkx2-5/smad-GATA-LacZ reporter construct, a 5.9-kb Smad-Apal fragment from pBS-Nkx2-5 LacZ (8) containing the GATA-Smad enhancer was subcloned into Smad and GATA sites of pBluescript-SK to generate pBS-Nkx2-5 (Smad-Apal), from which a 450bp HindIII fragment containing the GATA-Smad enhancer and the construct relgiated to generate pBS-Nkx2-5/Smad-ApalΔH3. The Smad-ApalH3 fragment was then subcloned into Smad-Apal sites of pBS-Nkx2-5 LacZ to generate a pBS-Nkx2-5/GATA-Smad-LacZ construct. DNA was prepared for transgenesis by removal of the plasmid backbone by restriction digestion by SalI, and the insert was purified using the QiAEX II gel purification kit (Qiagen). Injected embryos were collected at various gestational time points or were allowed to generate stable transgenic lines. Harvested embryos were fixed and stained for β-galactosidase activity as previously described (8). Embryos were genotyped by PCR using the following primers from Hsp68 and LacZ regions: 5′-TCAGAAGCTTGCGACATTTG-3′ and 5′-AAGGGGGAATGTG-GTCGAGCGG-3′, respectively. Sections (6 μm) were cut from paraffin-embedded embryos.

Cell Culture, Transfections, and Reporter Assays—Cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics. 107/12, and CVI cells were transfected using FuGENE 6 (Roche Applied Science) or LipofectAMINE (Invitrogen, Life Technologies, Inc.). According to the product instructions, P19 CL6 cells seeded at the initial density of 8 x 105/10 cm dish were cultured in differentiation media (minimal essential a-media (Invitrogen) containing 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen) and 1% MeSO(Sigma) for 6 days and further cultured for 4 more days in media without MeSO. All transfections were done in triplicate. Cells were transfected using Lysis Buffer and chloramphenicol acetyltransferase luciferase activity was measured using a luminometer to detect activated substrates (Sigma and Tropix), then normalized to total protein, and relative activity was found by comparison to activity of control samples. Error bars represent standard deviation from the mean.

Protein Production and GST Pull-downs—GST and GST-Smad proteins were induced by isopropyl-1-thio-b-D-galactopyranoside in BL21/DE3 bacterial cells. Proteins were purified utilizing glutathione-Sepharose beads as described (5). GATA-4 was radioactively labeled by inclusion of [35S]methionine by in vitro translation (Promega T7 T7 kit). Pull-downs were performed by incubating radiolabeled GATA-4 proteins with bead-bound GST proteins in binding buffer (100 mM NaCl, 5 mM HEPES, pH 7.9, 24 mM KCl, 0.5 mM MgCl2, 0.05% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) for 1 h at room temperature, followed by washing five times in the same buffer. Proteins were eluted by boiling in SDS-sample buffer. Samples were run on 10% SDS-PAGE gels, which were dried and subjected to autoradiography.

Immunoprecipitations—COS cells were transfected with myc-GATA-4 and FLAG-Smad vectors and immunoprecipitations using anti-FLAG or anti-HA antibody were carried out as described (25, 38). The cells were lysed in a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and GATA-4 proteins were immunoprecipitated from the whole cell lysate using an anti-Myc antibody (Santa Cruz Biotechnology). Samples were run on a 10% SDS-PAGE gel and Western blot analysis was performed using anti-Myc or anti-FLAG antibodies (Sigma).

Electrophoretic Mobility Shift Assays—GATA-4 gel shifts were performed as described (5) in 20 μl of GATA binding buffer (4 mM Tris, pH 7.9, 24 mM KCl, 0.4 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl2, 0.25 mM ZnCl2, 1% Ficoll). Smad gel shifts were performed as described (38) in 20 μl of Smad binding buffer (25 mM Tris pH 7.5, 80 mM NaCl, 35 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 50 μg/ml poly(dI-dC)). Oligonucleotide probes were synthesized and annealed, then radiolabeled using the Klenow Fragment to fill in 5′-overhanging DNA ends (BRL Ready-to-Transcribe). The sequences of the oligonucleotide probes were as follows: SBE-3′X, 5′-GTCGCCACAGACAGACAGG-3′ and 5′-GCCCT-GTCTGCTGCTGCTGGA-3′; GATA-3′X, 5′-GAAGATAGATAGATAGTA-3′ and 5′-GTTACTACTATCTATCTACT-3′. For competition, unlabeled annealed oligonucleotide pairs were used: VHC-GATA, 5′-TGATGGGACAGATGAGG-3′ and 5′-ATCTCCCTCTGTGCTAGG-3′; ANF-GATA, 5′-ATCTCCCTCTGCTAGGTAATTTAAA-3′ and 5′-TTTAAAGTTATCATCGCTCCCGAGAG-3′. Purified GST-Smad1/4 and in vitro translated GATA-4 were used for binding (see below).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assays were performed as described by Johnson and Bresnick (39). Briefly, ∼2 x 109 logarithmically growing or differentiated P19 CL6 cells were fixed with formaldehyde (final concentration 1% v/v) in growth media at room temperature for 10 min with gentle agitation. Glycine was added to a final concentration of 0.125 M to stop cross-linking. Fixed cells were pelleted by centrifugation and nuclei were isolated and sonicated to make soluble chromatin (fifteen times for 10 s each) and removal of 1% of total chromatin was the positive control in the PCR assays and denoted as input chromatin. The cell lysates were precleared by incubation with salmon sperm DNA/protein A agarose-50% slurry (Santa Cruz Biotechnology) and immunoprecipitation was performed with 2 μg of Smad4 antibody and/or a mixture of GATA4 antibodies (Santa Cruz Biotechnology). DNA-protein complexes were recovered with salmon sperm DNA/protein A agarose-50% slurry, followed by several rounds of washing. Bound DNA-protein complexes were eluted from the antibody/protein A agarose-50% slurry (1% SDS and 0.5 M NaCl), a second immunoprecipitation was carried out to increase specificity with the use of the same protocol. Formaldehyde cross-links were reversed by incubating the sample in the presence of 200 mM NaCl at 65°C. Samples were then extracted twice with phenol/chloroform and precipitated with ethanol. After ethanol precipitation and washes with ethanol, the immunoprecipitated DNA was eluted with Tris-EDTA and used in qPCR reactions using primers that are included in the Online Methods.
imated with ethanol in the presence of 20 μg of glycogen as a carrier. DNA fragments were recovered by centrifugation, resuspended in 100 μl of ddH₂O, and used for PCR amplifications. The primers used were as follows: composite enhancer, 5'-CAGTCTTGGGAGCTCAAGACTAACC-3' and 5'-CAGATCCCCAAGCTTACTAGCATA-3' (255bp product); AR2 enhancer, 5'-CTGCTCATCCATCAGCCAGACGAAGA-3' and 5'-GAAAGATAAGCTGCAACTATCACCGG-3' (357bp product); AR1 enhancer, 5'-CTGGGTCCTAATGCGGGTGGCTCTC-3' and 5'-AACCCTCTGCTGTGTGGCCTTGTATCT-3' (246bp product). The PCR products were fractionated on 2% agarose gels, stained with ethidium bromide, and photographed under UV light.

RESULTS

Nkx2-5 Upstream Enhancers Activated by BMP Signaling through Smad and GATA Transcription Factors—We wanted to identify BMP-responsive cis-acting sequences of Nkx2-5. Fig. 1A shows a map of 5'-flanking 10.5-kb fragment in the context of the Nkx2-5 gene (adapted from Ref. 7). Portions of the upstream regions of Nkx2-5 linked to a luciferase reporter (Alk3) in P19 EC cells. Activation levels are shown relative to control (empty expression vector), with error bars representing standard deviation from the mean. C, P19 cells were transfected with Nkx2-5 FL reporter and various Smad vectors, each with or without GATA-4. D, P19 cells transfected with FL reporter and either Smad1/4, Smad1C/4 (with an N-terminally truncated Smad1), or Alk3CA, along with GATA-4, GATA-6, or deletions of GATA-4 from the N and C termini. (GATA-4 full-length: 1–440; delN: 85–440; delC: 1–335.)

Downstream mediators of the BMP signaling pathway, the Smad factors, Smad1 and Smad4, were found to activate the FL reporter, alone and in combination maximally about 30-fold (Fig. 1C). Despite the known importance of GATA factors for expression in vivo, GATA-4 alone was found to weakly activate the FL reporter (3-fold), but synergized well with Smad1 and Smad4 alone and stronger in combination with the two Smad factors (51-, 63-, and 192-fold, respectively). Smad6, an inhibitory Smad, suppressed the activation by Smad1/4 with or without GATA-4 (decreased Smad1/4 activation by 50% and by 60% when GATA-4 was added). Thus, the candidate transfactor, GATA-4, acted as a Smad1 and Smad4 co-activator of Nkx2-5 gene activity. GATA-4 shows significant homology, functional redundancy and overlapping expression with GATA-5 and GATA-6.

We investigated which domains of GATA-4 were necessary for Smad coactivation, and whether GATA-6 could substitute for GATA-4 as a Smad cofactor on Nkx2-5. In Fig. 1D, using a lower dose of Smad factors, a mild activation of the Nkx2-5 FL reporter by either Smad1/4 or Alk3CA was observed (3–4-fold). GATA-4 and GATA-6 were both potent coactivators of the Smads in the presence of Alk3CA. However, truncation of GATA-4 from either the N or C terminus, where both activation domains lie, abolished all activity, operating in a dominant negative fashion with respect to activation by the Smad factors. Further truncation of GATA-4 also eliminated activity (not shown). Additionally, Smad1C, an N-terminally truncated version of Smad1, substituted for the full-length Smad1 in all
cases, either because the N terminus of Smad1 is not involved in GATA co-activation, or more likely the Smad4 participation in the complex is sufficient for DNA binding and interaction with the GATA factors. It has been reported that this type of truncation actually increases the activity of a pathway-regulated Smad by relieving intramolecular inhibition between the N and C termini. We have shown the activation of the three major Nkx2-5 reporters (FL, US, and DS) by BMP signaling, and cooperative activation of the FL reporter by Smad and GATA transcription factors.

Further deletion analysis of the Nkx2-5 gene locus was performed in Fig. 2A, including deletions of the US reporter, using the alternative upstream promoter. In the US region, there was a loss of GATA-Smad cooperation between −10.5 and −8.5 kb, and a further loss of Smad activity between −8.5 and −5.2 kb (represented in Fig. 2B). On the primary promoter reporters (FL and DS and their deletions), there was a drop in cooperation between −10.5 and −5.9 kb, and a further drop in Smad and cooperative activity upon deletion to −4.8 kb. This reduction in responsiveness to GATA-Smad cooperation continued until deletion to the −1.6 kb, which still maintained a Smad response. Thus, a proximal Smad element was suggested, and a far distal and an intermediate region between −5 and −6 kb were important for combinatorial activity. Sites for cooperation could contain GATA or Smad elements, or both. The far distal site is most likely the GATA site identified by Lien et al. (9).

Identification and Characterization of a Novel GATA-Smad Enhancer in the Nkx2-5 Gene—We identified an intermediate region within the 10 kb 5′-flanking sequences that contained a cluster of GATA and Smad sites (Fig. 3, A and B). Within 175 bp, there were 27 consensus GATA sites (in bold) with 5 interspersed non-consensus sites (GGTA), followed by 9 consensus Smad boxes (AGAC, in italics), all in a tandem array, as shown in Fig. 3B. GATA sites were found often throughout the upstream region, including several previously identified sites. Isolated Smad boxes were also found elsewhere, but never in tandem repeats, and never directly adjacent to GATA sites. Since it is known that Smads bind efficiently to pairs or multimers (42–44), this Smad/GATA composite region was analyzed further for activation and binding by GATA and Smad factors.

The Smad/GATA cluster, and divided GATA and Smad portions, were cloned upstream of an Hsp68 minimal promoter linked to a LacZ reporter (HspLac). In Fig. 3C, these reporters, named G-S, GA (broken underline) and SB (underlined), were cotransfected into P19 cells with GATA and Smad factors and assayed for β-galactosidase activity. In Fig. 3C, GATA-4 and Smad1/4 coactivated on combined GATA and Smad sites (G-S). Transfected GATA-4 and Smad1/4 expression vectors alone weakly activated this reporter, while the combination strongly facilitated transcription activity (21-fold). The Smad reporter (SB) was only weakly activated by Smad transcription factors and moderately activated by GATA-4 alone or the combination, perhaps due to the presence of endogenous Smad activity in P19 cells. Surprisingly, GATA-4 could not activate through the multimeric GATA sites (GA), and the addition of Smad1/4 did not have any significant effect. These data may indicate that GATA-4 factors were attracted to repeated Smad sites via Smads factors, while conversely Smad1/4 were not recruited through repeated GATA sites. Apparently, the close juxtaposition of repeated Smad and adjacent GATA sites allowed for the highest level of co-activation. A fragment containing the previously described proximal cardiac and stomach enhancer, and shown to have critical GATA and Smad sites, was also tested for comparison (XB). This enhancer was not significantly activated by GATA-4, but it was Smad-responsive and it was also strongly co-activated by the combination of Smad and GATA factors (Fig. 3C).
GATA-4 and Smad1/4 Cooperate to Activate Nkx2-5

Binding of GATA and Smad Transcription Factors to Multimeric Sites—To test for efficient binding of the composite enhancer by GATA and Smad transcription factors, electrophoretic mobility shift assays (EMSA) were performed using portions of the Smad/GATA composite enhancer as oligonucleotide probes shown in Fig. 3B. EMSA was tested on pairs of annealed oligonucleotides containing trimer repeats of GATA or Smad sites, as representative of the highly repeated sites found in the combinatorial enhancer. Fig. 3D shows the binding of bacterially expressed and purified Smad1 and Smad4 (as GST fusions) to the multimeric Smad sites. Binding by Smad4 was stronger than that of Smad1, and the combination of the two showed the strongest binding and stronger formation of higher order complexes. EMSA specificity was demonstrated by competition with the cold triplet Smad sites. Fig. 3E showed the binding of GATA-4-containing nuclear extracts to multimeric GATA sites. Competition by cold GATA triplets was stronger than that observed for a single GATA site from the αMHC or ANF promoters, illustrating the high affinity and specificity of binding of GATA-4 to direct repeated sites. Anti-GATA-4 antibody supershifted the binding complexes. Thus, the Smad/GATA composite Nkx2-5 enhancer contained consensus sequences that were strongly bound by GATA and Smad factors.

Smad Transcription Factors Recruited GATA-4 in Mammalian Two-hybrid Assays—Recently GATA factors were shown to recruit Smads to GATA sites (42), and we asked if the converse were true. We tested (SBE)4-Luc reporter gene which contains four (CAGACAG) repeats in inverted orientation and the converse were true. We tested (SBE)4-Luc reporter gene which contains four (CAGACAG) repeats in inverted orientation and a minimal promoter (42). This reporter gene has been well characterized in several cell types, including P19 cells. In Fig. 4A, Smad1 and Smad4 displayed recruitment of GATA-4 to the multiple SBEs. Smad1 and Smad4 showed similar activities on this reporter (12- and 14-fold), and their partnership was evident when combined (40-fold). Smad1C, despite having higher activity on its own, was a poor substitute for Smad1 in combinations with Smad4 and GATA-4. Vp16 transfected alone with the Smad reporter stimulated synthetic promoter but was minimal considering the exaggerated cooperation by the use of the VP16-GATA construct (up to 500-fold for the Smad1/4 combination).

In Fig. 4B, GATA-4 and various Smad factors were cotransfected with the Smad reporter. GATA-4 when cotransfected...
with Smad4 almost tripled the activity of Smad4 alone. Addition of Smad2 did not increase the activation by Smad4 with or without GATA-4. Smad1 and Smad5 were weakly coactivated by GATA4, while Smad3 displayed very strong cooperation with GATA-4 (18-fold over Smad3/4 alone). Smad2 has an insertion in the DNA-binding MH1 domain, preventing its binding to Smad sites, and activation of this reporter. In Fig. 4, Smad4 was cotransfected with either GATA-4 or a double point mutant that eliminates the secondary structure of the second zinc finger, and thus rendered unable to bind target sites. The GATA-4 zinc finger mutant was able to cooperate with Smad4, but to a lesser degree, about half as strongly as the wild type. Thus GATA-4 is recruited to the SBE reporter by virtue of interaction Smad transcription factors.

**Smad Proteins Co-associated with GATA Proteins**—Considering the functional interaction between Smad and GATA factors to coactivate different reporter genes, and the probable recruitment of GATA-4 to Smad sites by Smad proteins, we asked if they physically co-associate. We performed pull-down assays using bacterially expressed GST-Smads and radiolabeled in vitro translated GATA proteins. In Fig. 5, A and B, various full-length and truncated versions of GST-Smad proteins were assayed for association with GATA-4. Fig. 5A is an autoradiogram showing the GATA-4 protein compared with 20% of translation product input. All Smad proteins tested were able to interact with GATA-4. GATA-4 was not bound by GST-only. Full-length Smad1 bound weakly with GATA-4, but a truncated version missing the C terminus (Smad1NL, with the N-terminal MH1 and the non-conserved linker domain) bound much more strongly, possibly from intramolecular inhibition, in the absence of phosphorylation by BMP receptors. Full-length Smad4 also bound very well. Smad3 and Smad3N interacted about the same as Smad1NL (adjusting for less GST protein pulled-down), but Smad3NL-ΔLG (a construct with a 12-amino acid deletion in the MH1 domain abrogating DNA binding) interacted more weakly. Taken together, these results show that the DNA-binding N-terminal domain of Smad proteins was able to bind GATA-4, in a region possibly overlapping the DNA binding residues.

Next we wanted to determine which GATA factors and domains were involved in Smad protein interaction. We reasoned that since Smad4 interacted strongly with GATA-4 and it is the common partner for all Smad pathways, that it would be the best Smad protein to use for analyzing GATA interaction. Fig. 5B is a compilation of autoradiograms of the radiolabeled in vitro translated GATA proteins following pull-downs. Full-length GATA-4, -5, and -6 were all bound by GST-Smad4, but not by GST only. This indicated that the interacting domain would likely reside in a conserved region of the GATA family, possibly one of the zinc finger domains. Further analysis revealed that binding to Smad4 required the second zinc finger (ZF2) and adjacent basic domain of GATA-4, and that interaction did not occur with the N-terminal domains, extreme C-terminal domain, or the first zinc finger (ZF1). A full-length GATA-4 with mutations of the zinc-coordinating cysteines of the second zinc finger was bound by GST-Smad4 nearly as well as the wild type, indicating the binding is sequence-dependent, not structure-dependent.

We were concerned that the GST pull-down experiments would not be representative of in vivo physiological conditions, so we performed co-immunoprecipitation from transfected cells. As shown in the first panel of Fig. 5C, Myc-tagged
GATA-4 proteins (FL or C-terminal) were immunoprecipitated (using an anti-Myc antibody) from whole cell extracts, bringing down the co-transfected FLAG-Smad proteins by virtue of physical interaction. The lower panels show the equal loading of Smad and GATA proteins in all reactions. In agreement with Fig. 5A, the full-length GATA-4 was able to strongly interact with Smad4, but more weakly with Smad1, 2, or 3. Surprisingly, the deletion of the N-terminal domain and the first zinc finger allowed strong interaction with all of the Smads, which suggested an intramolecular inhibition by those regions, and confirmed interaction of GATA-4 through its second zinc finger and other C-terminal residues. In summary, the interaction between GATA and Smad proteins occurs between the Smad N terminus and the GATA ZF2 and basic domains.

In Vivo Interaction between Smad4 and GATA4 with the Nkx2-5 Enhancer—To obtain direct evidence of in vivo interaction of Smad4 and GATA4 with the Nkx2-5 composite enhancer identified in this study, we used chromosome immunoprecipitation analysis (ChIP) on both logarithmically growing and differentiated cells, as shown in Fig. 6. After formaldehyde cross-linking and precipitation of the chromatin with Smad4- and GATA4-specific antibodies, the precipitated DNA was subjected to PCR amplification with the use of specific primers for the Smad/GATA enhancer region, as well as primers for the Nkx2-5 AR2 enhancer region, and AR1 enhancer region. It has been shown that a single Smad site within AR2 is required for the enhancer activity at early and late stages of heart development in vivo (10); therefore the AR2 region can serve as a positive control for Smad4 immunoprecipitation. The distal enhancer AR1 was shown to be regulated by GATA4 (9). In Smad4 immunoprecipitation with differentiated P19 CL6 cells, a strong AR2 signal and a weaker composite enhancer signal were observed, whereas no amplification of AR1 enhancer sequences was detected. In Smad4 immunoprecipitation with logarithmically growing P19 cells, none of the above three enhancer sequences could be amplified, demonstrating the Smad4 binding to AR2 and the Smad/GATA enhancer regions were dependent upon cardiac differentiation. As a negative control, the chromatin was immunoprecipitated without antibodies. In these precipitates none of the above three enhancer sequences were detectable, demonstrating the specificity of the procedure. Also, GATA4 was observed to bind to the Smad/GATA composite enhancer and AR2 but not to the AR1 enhancer by the in vivo ChIP assays.

Mouse Smad/GATA Composite Enhancer Is Required for Early Nkx2-5 Embryonic Expression—The GATA and Smad
cluster was then evaluated for biological activity in transgenic mice generated with the G-S Hsp68-LacZ reporter gene. In Fig. 7, A and B, two independent F0 transient transgenic embryos harvested at about E8 for analysis of β-galactosidase activity showed similar patterns of expression. The embryo in Fig. 7B displayed staining only in the cardiac crescent, as previously shown for the endogenous Nkx2-5 gene at this stage, while that in Fig. 7C has staining which also extended through the lateral plate mesoderm. These transgenic embryos were representative of several others at this stage, whose expression patterns are summarized in Fig. 7A. The isolated halves of the enhancer were unable to induce significant expression of LacZ (GA and SB, also in Fig. 7A), indicating a requirement for cooperativity between Smad and GATA sites.

In addition, a transgenic line containing the composite enhancer (G-S) was generated, and F1 embryos were analyzed at various stages. Staining was observed throughout the heart tube (E8.5, Fig. 7E) and in the looped heart (E9.5, Fig. 7F). Sporadic and much weaker LacZ staining was also observed in the neural tube, notochord and somites. Sections from E9.5 embryos showed staining in both the myocardium (Fig. 7F) and in the umbilical vein and the floor of the forebrain (not shown). Heart staining was found in both conotruncal and ventricular myocardies, and in the A-V junction, during chamber specification. Later (E11.5), LacZ staining faded in these sites, and was observed to be parrially much lower than that from the wild type transgene. Thus in the presence of other well defined Nkx2-5 enhancers its natural promoters within the 10-kb DNA fragment, the GATA-Smad composite enhancer revealed a clear obligatory role for driving early Nkx2-5 expression in the cardiac crescent.

**DISCUSSION**

Cooperative Activation of Nkx2-5 by GATA-4 and Smad1/4 — The Nkx2-5 promoter provides an excellent model for studying the regulation of cardiogenesis and cardiac gene expression. Nkx2-5 is the earliest marker for the developing heart field, and its expression persists throughout the myocardium in the fully formed heart. Multiple enhancers and repressors have been identified in the Nkx2-5 upstream regulatory region orchestrating expression in several tissues. These results illustrate the tightly controlled, multistep process of gene regulation throughout heart development. Several lines of experimentation have shown that BMP signaling activates the expression of Nkx2-5, but it has not yet been shown to do so directly. We provide in this paper a clear demonstration of Nkx2-5 activation by BMP. This pathway participates in a cross-talk with cardiac GATA factors through Smad-GATA physical interaction and transcriptional cooperation; it is probable that other pathways and transcription factors would also coordinate with these signals, positively or negatively, to specify the cardiac lineage and yield the narrow expression pattern of Nkx2-5. Activation by BMP signaling was most notable in multipotent P19 EC cells as opposed to other more fibroblastic lines. Recently published work from our laboratory has shown that 10T1/2 fibroblasts can be differentiated into smooth muscle by addition of transcription factor combinations (including GATA-6, Ref. 45), while P19 cells are more cardiogenic in

**Fig. 6.** Smad4 and GATA4 bind in vivo to the enhancer region (−6211 to −5974) in differentiated P19 cells. A, in vivo binding of Smad4 and GATA4 to the AR (−6211, −5974) enhancer and AR1, AR2 enhancer regions, as shown was assessed by ChIP. Schematic presentation of the locations of AR1, AR2, and AR (−6211, −5974) enhancer regions in the Nkx2-5 gene locus. 1a, 1b, 1c, and 2 represent the exons of Nkx2-5. B, an input control to show that DNA-protein complexes from undifferentiated (−DMSO) and differentiated (+DMSO) P19 cells that contained equivalent levels of enhancer target sequences. PCR was performed directly on dilutions of chromatin from complexes not subjected to immunoprecipitation. C, DNA-protein complexes were immunoprecipitated without antibodies (No Ab) or with anti-Smad4 antibodies. The PCR primers were specific for AR (−6211, −5974) enhancer (255 bp), AR2 enhancer (357 bp), and AR1 enhancer (246 bp), respectively. ChIP analysis showed that in differentiated P19 cells, Smad4 binds specifically to the GATA-Smad (G-S) enhancer region (−6211, −5974) and AR2 enhancer, but not to AR1 enhancer. Smad4 doesn’t bind to any of the above enhancer regions in undifferentiated P19 cells. GATA4 was observed to bind to the Smad-GATA enhancer and AR2 but not to the AR1 enhancer.
nature (40, 41), with a clear involvement of BMP signaling and the Smads as well as Nkx2-5 and GATA factors (18, 19). Thus we believe that cell context is vital for activation of Nkx2-5, emphasizing the input of multiple cues in cell fate determinations.

Although GATA sites in Nkx2-5 have been shown to be required for its expression in the heart, in our hands GATA-4 was a poor activator of Nkx2-5 reporter genes. Only when GATA-4 and Smad1/4 were both present was strong activation observed. Interestingly, in transfections either Smad1 or

**Fig. 7.** The GATA-Smad composite region acts as a cardiac enhancer in transgenic mouse embryos. Transgenic mouse embryos were made by injection of fertilized mouse embryos with the GATA-Smad combinatorial element, or deletions containing either half, placed upstream of a Hsp68 minimal promoter driving the LacZ reporter gene (G-S, GA, and SB reporters). *A,* table summarizing staining found in F<sub>0</sub> transgenic embryos. (*, this embryo had very weak staining in the crescent at E7.5.) *B* and *C* are micrographs of two independent E8 F<sub>0</sub> transgenic embryos after fixation and staining for β-galactosidase activity (blue staining). Expression of transgene is seen in the cardiac crescent of both embryos. *D–G* are micrographs of embryos of several stages from a single transgenic line containing the G-S reporter. *D* is from E8.5, *E* and *F* are from E9.5, and *G* is from E11.5. CC, cardiac crescent; LPM, lateral plate mesoderm; HT, heart tube; Ht, heart; NT, neural tube; Nc, Notochord; AV, atrio-ventricular junction; A, atrium; V, ventricle; OFT, outflow tract, T, telencephalon.

**Fig. 8.** GATA-Smad composite enhancer is essential for early Nkx2-5 expression. *A,* this panel shows a schematic diagram of a 10-kb upstream region of the Nkx2-5 gene that contains the GATA-Smad enhancer located between −6211 and −5974 nucleotides. *B,* this panel shows robust LacZ staining in the cardiac crescent of 3 independent transgenic mice that contain the 10-kb DNA insert of pBS-Nkx2-5, as shown by PCR assays, and displayed strong LacZ staining pattern, similar to that observed in Reecey et al. (8). *C,* this panel shows a lack of staining in 3 of 4 PCR-positive transgenic embryos containing a pBS-Nkx2-5/GATA-Smad/LacZ construct. In comparison, 1 of 4 PCR-positive transgenic embryos showed much weaker LacZ staining in the cardiac crescent region, than observed in the wild type transgenic mice shown in *panel B.*
GATA-4 and Smad1/4 Cooperate to Activate Nkx2-5

Smad4 alone were sufficient for activity, although in the conventional model a BMP stimulus through the receptor and complex formation between the receptor-activated and common partner Smad proteins would be required. Although there may be endogenous BMP signaling and ample amounts of the requisite Smad in P19 cells, it has been seen that when overexpressed singly, the Smad factors become localized to the nucleus and transcriptionally active, and thus override or bypass the normal constraints on the pathway. Finally, deletion analysis of the Nkx2-5 5′-flanking region revealed multiple sites for BMP activation and GATA-Smad co-activation in both proximal and distal loci, roughly mapping to the previously reported enhancers (7, 35, 36). That a 200+ base pair region containing primarily GATA and Smad binding sites was able to direct such strong and specific staining as shown in Fig. 7 was somewhat surprising. We expected that there might be expression everywhere TGF-β family signaling or GATA factors existed, though the results were more restricted. Certainly, the removal of the enhancer, as shown in Fig. 8, indicates the biological functional importance of this combined GATA-Smads sequences, even in the presence of other regulatory enhancers, for driving early Nkx2-5 expression in the cardiac crescent. It is possible that the presence of the GATA sites gave specificity to the BMP/Smad signal, and *vice versa*. In fact, a GATA repeat site similar to the one we identified has been shown to act as a silencer or insulator in the β-globin locus (46). This is consistent with the inability of multiple GATA sites to be activated in transgenic mice or by GATA-4 in tissue culture. The combinatorial enhancer resides in or near an inhibitory region (8), and it may be that the GATA sites are themselves inhibitory in the absence of activated Smads. Despite possible negative effects, the combined sites were clearly effective in activating gene expression during early heart development.

Our results show that Smad4 binds in vivo to the Smad/GATA composite enhancer region in differentiated P19 CL6 cells, but not in logarithmically growing P19 CL6 cells. Also, consistent with previous studies (2, 3), our results showed that Smad4 bound strongly to AR2 enhancer region, but not AR1 enhancer region despite the presence of several reasonably good Smad binding sites in AR1 enhancer, suggesting that GATA binding to the different enhancer regions of Nkx2-5 is both spatially and temporally regulated. The results that GATA4 binds to Smad/GATA enhancer reflects the ability for adjacent GATA and Smad sites influence their mutual ability to co-activate transcription.

**GATA and Smad Proteins Are Mutually Interacting Cofactors**—Functional and physical interaction between GATA-3 and Smad3 was shown to be important during lymphoid cell development (42). Together, these factors cooperated to activate an IL-5 reporter in HepG2 and Jurkat cells, employing the recruitment of Smad3 to a critical GATA site. Physical interaction was reported to be through the N termini of both Smad3 and GATA3. We demonstrated in Fig. 5 the GATA4 interaction with Smad factors occurred through the second zinc finger and adjacent basic region of GATA-4, although both extreme N- and C-terminal domains may be necessary for transcriptional cooperation on Nkx2-5. We have shown cooperation between different classes of Smad and GATA factors in two-hybrid recruitment assays. GATA-4 was recruited to Smad sites of the SBE-Luc reporter and may enhance the binding of the Smad proteins to their sites, in addition to lending an activation function.

Smad4 was seen to interact equally well among the GATA-4/5/6 subfamily, and the interacting region previously identified is poorly conserved between these and all GATA factors, so we believe that the interaction through the highly conserved zinc finger region is a more relevant one. It has also been shown that Smad proteins interact with other zinc finger proteins, including Sp1, Evi-1, and OAZ2 (21, 25, 47), and GATA proteins are often seen to interact with other factors through their zinc fingers. We consider the mutual interaction between GATA and Smad factors as a general one, which applies to all of the GATA family members and at most or all Smads. Although we mostly focused on GATA-4, GATA-6 could functionally substitute for GATA-4 in Smad cooperation. The promiscuity of the interaction between the families may reflect a commonly used convergence of signaling and tissue-specific factors involved in many developmental processes.

Therefore, it is possible that other TGF-β family members besides BMPs can signal through the combinatorial enhancer in Nkx2-5, including activin, nodal, or TGF-β itself. Significantly, nodal and activin have important roles in formation of precardiac mesoderm (48, 49). Moreover, Smad factors might interact with other proteins containing GATA-like zinc fingers. We hypothesize that the GATA homologue in *Drosophila*, pannier, might interact with Mad and Medea to regulate *timman*. Interestingly, inhibition of the BMP pathway in Xenopus had a similar outcome as null mutations of GATA-4/5/6 in mouse, indicating a role of BMP signaling in the function of GATA factors in gut and heart development (50). We have shown involvement of GATA transcription factors in BMP signaling through association with Smads, with direct effects on Nkx2-5, suggesting a vital role in forming mesoderm and specification of cardiac progenitors.

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