PITX2 Isoform-specific Regulation of Atrial Natriuretic Factor Expression

SYNERGISM AND REPRESSION WITH Nkx2.5

PITX2 and Nkx2.5 are two of the earliest known transcriptional markers of vertebrate heart development. Pitx2−/− mice present with severe cardiac malformations and embryonic lethality, demonstrating a role for PITX2 in heart development. However, little is known about the downstream targets of PITX2 in cardiogenesis. We report here that the atrial natriuretic factor (ANF) promoter is a target of PITX2. PITX2A, PITX2B, and PITX2C isoforms differentially activate the ANF promoter. However, only PITX2C can synergistically activate the ANF promoter in the presence of Nkx2.5. We further demonstrate that the procollagen lysyl hydroxylase (PLOD1) promoter is regulated by Nkx2.5. Mechanistically, PITX2C and Nkx2.5 synergistically regulate ANF and PLOD1 expression through binding to their respective DNA elements. Surprisingly, PITX2A activation of the ANF and PLOD1 promoters is repressed by co-transfection of Nkx2.5. Expression studies have shown that Pitx2 is essential for heart development. In the developing chick, asymmetric expression of Pitx2 was first detected in stage 7 embryos on the left-sided lateral mesoderm (11). The asymmetric expression of Pitx2 remains in the left-sided lateral mesoderm, including left-sided heart tube as the two primitive heart tubes fuse. Thus, Pitx2 exhibits a left-right asymmetrical expression prior to the appearance of morphological asymmetry (11). In early mouse embryos, Pitx2 expression was detected bilaterally in the head fold mesenchyme at 7.5 days postcoitum. Asymmetric expression of Pitx2 was first observed in the left lateral plate mesoderm at 8.0 days postcoitum, extending laterally along the anteroposterior axis, somewhat resembling that of lefty-2 and nodal (12). At 9.5 days postcoitum in addition to the lateral plate mesoderm, asymmetrical expression of Pitx2 can be seen in the left truncus arteriosus, left dorsal part of the common atrium, left common cardinal vein, left side of foregut primordia, and lung buds (12). More importantly to this report, it has recently been shown that differential expression of Pitx2 isoforms is a key mechanism for heart development (13–16). Epigenetic and genetic studies reveal that tissue and organ development are differentially regulated by Pitx2a and Pitx2c isoforms. Eloquent experiments in mice that were defective for Pitx2a and Pitx2b expression demonstrate that different organs have distinct requirements for Pitx2c dosage (16). Pitx2 appears to be a key gene for determination of leftness in heart formation, consistent with the fact that cardiac defects are among the associated defects of Axenfeld-Rieger syndrome.

Several laboratories have determined that signaling molecules play a role in Pitx2 gene expression (11, 12, 17). Although the mechanism of these interactions is unknown, several laboratories are currently working on the regulation of Pitx2 expression. However, the downstream targets of PITX2 in heart development are not known, except for our characterization of the procollagen lysyl hydroxylase 1 (PLOD1) gene as a target of PITX2 (18). The PLOD genes encode enzymes that hydroxylate lysines in collagen; the hydroxyl lysine resides act as attachment sites for carbohydrate units and provide stability to in-
termolecular cross-links. Point mutations and rearrangements in the human PDL1 gene are causative for Ehlers-Danlos syndrome, kyphoscoliosis type, type VI, characterized by ocular, muscular, skin, and aortic defects (19–21).

We have now identified the atrial natriuretic factor (ANF) promoter as a target of PITX2. The ANF gene is expressed early in embryonic development at the stage when cells are committed to the cardiac phenotype. ANF expression during embryogenesis characterizes both atrial and ventricular cells. Consistent with these observations it has been shown that the ANF gene is regulated by cardiac muscle-specific transcription factors (22, 23). ANF and Pitx2 are expressed in overlapping domains and developmental stages during heart morphogenesis (24, 25).

A general theme in developmental biology is the use of a combinatorial code of transcription factors to control gene expression. In the heart, Pitx2 is co-expressed with several transcription factors, including Nkx2.5 (23, 26–30). Nkx2.5 is a particularly strong candidate to interact with PITX2 because it has been shown to interact with other proteins expressed in the heart (22, 23, 26).

Because PITX2 and ANF are expressed early during cardiogenesis we asked whether PITX2 could activate the ANF promoter. Sequence analysis of the ANF promoter revealed several PITX2 binding elements. The ANF promoter has been shown to contain NKE elements (CAAGTG) or Nkx2.5 binding sites (23, 31). Sequence analysis of the PLOD1 promoter has identified several NKE elements, and we demonstrate that Nkx2.5 regulates PLOD1 expression. Our studies reveal that PITX2 isoforms differentially activate the ANF promoter. PITX2C and Nkx2.5 synergistically activate the ANF and PLOD1 promoters, whereas Nkx2.5 negatively regulates PITX2A activation of these promoters in a cell-dependent manner. We provide a molecular mechanism for the regulation of heart development through the activation of the PLOD1 and ANF promoters by PITX2 isoforms and Nkx2.5.

MATERIALS AND METHODS
Expression and Purification of GST-PITX2 Fusion Proteins—The PITX2 isoforms were PCR-amplified from a cDNA clone as described (6, 32). All pGST-PITX2 plasmids were confirmed by DNA sequencing. The Nkx2.5 cDNA was PCR-amplified and cloned into the pGexS-P2-GST vector (Amersham Biosciences) using primers containing Nkx2.5 sequences and unique restriction enzyme sites. The pGex-Nkx2.5 plasmid was confirmed by sequencing. The plasmids were transformed into BL21 cells. Proteins were isolated as described (6, 32). Proteins were cleaved from the GST moiety using 80 units of PreScission protease (Amersham Biosciences) and incubated in 60-mm dishes and transfected by electroporation. CHO and C3H10T1/2 cells were mixed with 2.5 × 10^5 transfected CHO cells. Transfected cell cultures were incubated for 24 h, lysed, and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity. Expression of transiently expressed PITX2 proteins in CHO cells has been previously demonstrated (32).

RT-PCR Assays—C3H10T1/2 and CHO cells were harvested, and the polyadenylated mRNA was extracted from tissue culture cells using the PolyATract System 1000 (Promega, Madison, WI). RT-PCR was performed using the TAKARA RNA PCR kit (Panvera/Takara, Madison, WI). The RT and PCR cycles were performed on an Eppendorf Mastercycler gradient thermocycler.

RESULTS
PITX2C and Nkx2.5 Synergistically Regulate the ANF Promoter—Pitx2c and Nkx2.5 synergistically regulate the ANF promoter in CHO cells. We analyzed whether PITX2 isoforms could specifically activate the ANF promoter containing a bicoid and NKE element (same as used for EMSA) into the BomH1 site upstream of the thymidine kinase (TK) promoter in the TK-luc plasmid (35). The TK-Pitx2 promoter contains three NKE oligonucleotides and one bicoid oligonucleotide. The ANF-638 promoter has been previously described (22). Mutation of the Pitx2 binding site (bicoid site) in the ANF promoter was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The 5′-AAATCC-3′ bicoid site was changed to 5′-AAATCCC-3′ using the following sense primer (5′-GGGAGAGAGGACCTTGAAGGCGAGCGC-3′) and antisense primer (5′-GGGCTCCCTCCATCAAG TTCTTCTCTCCTCC-3′). The mutations are underlined, and the new promoter was named ANF-638 ΔBic. Construction of the PLOD1 promoter plasmids has been previously described (18, 33). The TK-PLOD1 promoter was constructed by ligation of the PLOD1 oligonucleotide containing a bicoid and NKE element (same as used for EMSA) into the BomH1 site upstream of the TK promoter in the TK-luc plasmid. This new promoter (TK-PLOD1) contains two PLOD1 oligonucleotide sequences. All constructs were confirmed by DNA sequencing.

Expression and Reporter Constructs—Expression plasmids contain the cytomegalovirus (CMV) promoter linked to the PITX2 DNA were constructed in pcDNA 3.1 MycHisC Invitrogen (32, 34); the Nkx2.5 plasmid has been previously described (35). The TK-Bic and TK-NKE (TK-Hmx) promoter constructs have been previously described (35). The TK-Bic/NKE promoter was constructed by cloning bicoid and NKE oligonucleotides into the BomH1 site upstream of the thymidine kinase (TK) promoter in the TK-luc plasmid (35). The TK-Bic/NKE promoter contains three NKE oligonucleotides and one bicoid oligonucleotide. The ANF-638 promoter has been previously described (22). Mutation of the Pitx2 binding site (bicoid site) in the ANF promoter was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The 5′-AAATCC-3′ bicoid site was changed to 5′-AAATCCC-3′ using the following sense primer (5′-GGGAGAGAGGACCTTGAAGGCGAGCGC-3′) and antisense primer (5′-GGGCTCCCTCCATCAAG TTCTTCTCTCCTCC-3′). The mutations are underlined, and the new promoter was named ANF-638 ΔBic. Construction of the PLOD1 promoter plasmids has been previously described (18, 33). The TK-PLOD1 promoter was constructed by ligation of the PLOD1 oligonucleotide containing a bicoid and NKE element (same as used for EMSA) into the BomH1 site upstream of the TK promoter in the TK-luc plasmid. This new promoter (TK-PLOD1) contains two PLOD1 oligonucleotide sequences. All constructs were confirmed by DNA sequencing. A CMV β-galactosidase reporter plasmid (Clontech) was co-transfected in all experiments as a control for transfection efficiency.

Expression and Luciferase Assays—CHO cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and penicillin/streptomycin in 60-mm dishes and transfected by electroporation. CHO and C3H10T1/2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin in 60-mm dishes and fed with 5% fetal bovine serum and Dulbecco’s modified Eagle’s medium. Electroporation of CHO cells was at 380 V and 950 microfarads (Bio-Rad) and C3H10T1/2 cells at 380 V and 1,200 microfarads; cells were fed 24 h prior to transfection. Transfected cells were incubated for 24 h, lysed, and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity. Expression of transiently expressed PITX2 proteins in CHO cells has been previously demonstrated (32).

RT-PCR Assays—C3H10T1/2 and CHO cells were harvested, and the polyadenylated mRNA was extracted from tissue culture cells using the PolyATract System 1000 (Promega, Madison, WI). RT-PCR was performed using the TAKARA RNA PCR kit (Panvera/Takara, Madison, WI). The RT and PCR cycles were performed on an Eppendorf Mastercycler gradient thermocycler.

PCR of the cDNA product was performed using primers specific to Pitx2 isoforms. The antisense primer used in these studies was (5′-GAGGGAGGaGCAAAAGAAAAG-3′) and was complementary to the homeodomain of Pitx2. The sense primer for Pitxzc2a was (5′-GGGGGCCTCGGCTGAGCACG-3′) and was complementary to the homeodomain of Pitx2. The sense primer for Pitxzb was (5′-GGCGCGTTGAATGCTCTCC-3′) and the sense primer for Pitxzc2 was (5′-GACCTCCCTAAAACATGACT-3′). All of these sense primers are specific to the unique N terminus of each PITX2 isoform. The PCR products were resolved on a 1% agarose gel, stained with ethidium bromide for appropriate size. CHO cells provided a negative control. Sequencing reactions were performed on samples, and sequences were analyzed on an Applied Biosystems 373 sequencer (PerkinElmer Life Sciences). All RT-PCR products were sequenced to confirm their identity.
638 promoter. Surprisingly, the PITX2A isoform activates the ANF promoter at higher levels (22-fold) than either PITX2B (10-fold) or PITX2C (8-fold) (Fig. 1A). Because PITX2C is the major PITX2 isoform expressed in the heart, this was unexpected and PITX2B although not expressed in the heart was shown to activate the ANF-638 promoter in CHO cells. Similar to previous studies using a proximal ANF promoter in HeLa cells (23), we observe low activation of the ANF-638 promoter by Nkx2.5 at 3-fold (Fig. 1B). Co-transfection of Nkx2.5 with PITX2A and PITX2B did not result in synergistic activation. However, expression of Nkx2.5 and PITX2C resulted in a 45-fold synergistic activation of the ANF-638 promoter in CHO cells (Fig. 1B). The PITX2 isoforms differ only in their N termini, and it appears from these data that the N terminus of PITX2C may facilitate the synergistic effect in combination with Nkx2.5. All PITX2 isoforms have been previously shown to be stable and equally expressed in CHO cells (32).

The PITX2 Binding Site in the ANF-638 Promoter Is Required for Activation and Synergy with Nkx2.5—The PITX2 binding sequence 5'-GAATCC-3' is changed to 5'-GAACCTT-3' to determine whether it was functional and required for PITX2 activation (Fig. 2A). As expected, all three major PITX2 isoforms were unable to activate the ANF-638 promoter in transfected CHO cells (Fig. 2B). Although Nkx2.5 activation of this promoter was unaffected, co-expression with PITX2C did not result in synergy (Fig. 2B).

Nkx2.5 Regulates the PLOD1 Promoter in CHO Cells—PLOD1 expression has been implicated in heart development, and we have previously shown that PITX2 regulates PLOD1 expression (9, 10, 18, 32, 36). Sequence analysis of the PLOD1 261 full-length promoter revealed seven NKE elements. Because it is expressed in the heart we asked whether Nkx2.5 regulated PLOD1 expression (Fig. 3A). Transfection of CHO cells with the PLOD1 261 promoter and Nkx2.5 resulted in a modest 3-fold activation (Fig. 3B). We observed only additive transcriptional activation of the PLOD1 261 promoter upon co-transfection of PITX2A and PITX2B isoforms with Nkx2.5 (Fig. 3B). However, as observed with the ANF promoter, PITX2C and Nkx2.5 synergistically activated the PLOD1 full-length promoter (compare 33- to 12-fold for PITX2C alone; Fig. 3B). As controls we show that the minimal PLOD1 2561 promoter is not activated by each PITX2 isoform and Nkx2.5 and only slightly activated by co-transfection experiments (Fig. 3B). Interestingly, the minimal PLOD1 2561 promoter contains two NKE elements but is not activated by Nkx2.5. Because the full-length PLOD1 promoter demonstrates only a 3-fold activation, it appears that the location of the two NKE sites in the proximal promoter are not sufficient for activation by Nkx2.5. Sequence analysis of the PLOD1 2561 promoter reveals other factor binding sites. Thus, the lack of other factors binding in this region may affect Nkx2.5 activation of the proximal promoter.

PITX2 Isoforms and Nkx2.5 Form a Ternary Complex with the PLOD1 Bicoid NKE—EMSA experiments were used to determine whether a physical interaction occurred between PITX2C and Nkx2.5 dependent upon one or the other binding to DNA. We first tested whether bound PITX2 isoforms could interact with Nkx2.5. Addition of 80 ng of PITX2 isoforms with 80 and 160 ng of Nkx2.5 to the labeled bicoid probe did not reveal a slower migrating band indicative of a PITX2/Nkx2.5 protein complex on the gel (data not shown). Thus, bound PITX2 is not capable of interacting with Nkx2.5. We next asked whether PITX2 could interact with Nkx2.5 when it was bound to DNA. Addition of 80 ng of Nkx2.5 and 40, 80, and 160 ng of PITX2 isoforms to the labeled NKE probe did not produce a slower migrating band indicative of a PITX2/Nkx2.5 protein complex on the gel (data not shown). Thus, bound Nkx2.5 was unable to interact with PITX2 isoforms. We have performed GST-Nkx2.5 and GST-PITX2C pull-down experiments using pure PITX2 and Nkx2.5 proteins and were unable to demonstrate a specific physical interaction between Nkx2.5 and PITX2C (data not shown). These data and transfection...
results with the ANF-638 ΔBic promoter suggest that the PITX2C and Nkx2.5 synergistic effect on the ANF promoter is because of the independent binding of each factor to its preferred DNA element. Using a DNA probe derived from the PLOD1 promoter with a bicoid site (TAATCC) on one strand and 3 base pairs flanking this element, a NKE site (AAAGTG) on the opposite strand we demonstrate independent binding by PITX2 and Nkx2.5 (Fig. 4, A and B). Thus, these proteins can bind independent of each other and in close proximity to form a ternary complex. Most of the bicoid and NKE elements are spaced at least 50 bp apart in the ANF and PLOD1 promoters. However, this promoter sequence demonstrates the ability of these two homeodomain transcription factors to bind 3 base pairs apart and on opposite strands.

**Transcriptional Synergism by PITX2C and Nkx2.5 Requires Independent DNA Binding**—Another possible explanation for transcriptional synergism by PITX2C and Nkx2.5 could involve the interaction of a cellular factor acting as a linker between the two proteins. To address this possibility in functional assays we used two artificial promoters containing either four bicoid elements or three NKE elements upstream of the TK promoter. We have previously shown that the TK-Bic promoter is responsive to PITX2A and that TK-NKE is responsive to Nkx2.5 in transfected COS-7 cells (6, 35). We reasoned that if a linker protein were present in CHO cells then co-transfecting PITX2 and Nkx2.5 would result in a synergistic activation,
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because only one of these factors can bind to the promoter. However, although the TK-Bic promoter (which can only bind PITX2) is activated by both PITX2A and PITX2C isoforms at 3- and 4-fold respectively, co-transfection with Nkx2.5 did not result in synergistic activation (Fig. 5). Furthermore, Nkx2.5 activated the TK-NKE promoter (which can only bind Nkx2.5) at 4-fold in CHO cells but co-transfection of PITX2A and PITX2C did not result in synergistic activation (Fig. 5).

Conversely, we constructed two artificial promoters containing combinations of bicoid and NKE elements upstream of the TK promoter. The TK-Bic/NKE promoter (three NKE elements and one bicoid element) was activated by PITX2 isoforms and Nkx2.5 independently as expected but was not synergistically activated by co-expression of PITX2C and Nkx2.5 in CHO cells (data not shown). We observed only additive activation by co-expression of PITX2C and Nkx2.5. The second promoter contained two PLOD1 oligonucleotides upstream of the TK promoter. The TK-PLOD1 promoter was also independently activated by PITX2 isoforms and Nkx2.5. However, we were unable to observe synergism of this promoter by PITX2C and Nkx2.5 co-expression in CHO cells (data not shown). We observe only additive activation by co-expression of these factors. These experiments with artificial promoters demonstrate the importance of evolutionarily conserved natural promoter sequences and spacing of binding sites in the regulation of gene expression. Clearly, independent binding of transcription factors in an artificial context is not sufficient for synergistic activation of these promoters. It appears that PITX2 and Nkx2.5 binding sites must reside in the context and spacing of naturally occurring gene sequences to be regulated through synergistic activation. Furthermore, although these experiments may seem simplistic they demonstrate that other factors in CHO cells do not facilitate a PITX2/Nkx2.5 interaction as a mechanism for synergy.

Nkx2.5 Acts to Inhibit PITX2A Activation of the ANF and PLOD1 Promoters in the Embryonic C3H10T1/2 Cell Line—We have previously demonstrated that PITX2 isoform transcriptional activities are regulated in a cell-dependent manner (32). The C3H10T1/2 embryonic cell line has been used previously to demonstrate ANF promoter regulation by Nkx2.5 (22), and we demonstrate in this report that these cells endogenously express Pitx2a and Pitx2c isoforms. Similar to the expression pattern seen in heart tissues we find a 3-4-fold increase in Pitx2c expression compared with Pitx2a (Fig. 6A). As in heart tissues, Pitx2c is the major Pitx2 isoform and Pitx2b is not detected in C3H10T1/2 cell (Fig. 6A). Because Pitx2a and Pitx2c isoforms are expressed in this embryonic cell line, we reasoned that Pitx2 co-factors may also be expressed. Similar to CHO cells we find that PITX2A activates the ANF promoter at higher levels (14-fold) than PITX2B (3-fold) and PITX2C (7-fold) (Fig. 6B). Nkx2.5 only minimally activates the ANF promoter at 3-fold. However, co-transfection of Nkx2.5 with PITX2A resulted in a 3-fold inhibition of PITX2A activity in this cell line from 14- to 4-fold (Fig. 6B). Co-transfection of Nkx2.5 did not affect PITX2B activity. However, it resulted in synergism of the ANF promoter with PITX2C at 19-fold activation (Fig. 6B). The synergistic activation by PITX2C and Nkx2.5 was similar to that observed in CHO cells, but the inhibition of PITX2A activity was very surprising. These data would suggest that a factor in these cells interacts with the PITX2A isoform to cause a repressive effect in combination with Nkx2.5.

To demonstrate equal expression of our PITX2 isoforms, we performed a Western blot analysis of our transfected C3H10T1/2 cells (Fig. 6C). The Western blot demonstrates that the PITX2 isoforms are stable and expressed in equal amounts. Thus, differences in PITX2 isoform transcriptional activities are not because of unequal expression.

Similar to CHO cells, transfection of C3H10T1/2 cells with the ANF-638 ∆Bic promoter demonstrated that PITX2 binding was required for activation. All PITX2 isoforms were unable to activate this promoter in these cells (Fig. 7, data not shown for PITX2B). As expected, co-transfection of PITX2C and Nkx2.5 did not synergistically activate the ANF-638 ∆Bic promoter (Fig. 7). Furthermore, because PITX2A cannot activate this promoter independently, co-transfection with Nkx2.5 failed to repress its activity. PITX2 binding to the ANF promoter is required for the synergistic and repressive activities regulated by Nkx2.5 in the C3H10T1/2 cell line.

Co-transfection of Nkx2.5 with PITX2A also causes a repression of PITX2A activation of the full-length PLOD1 261 promoter in C3H10T1/2 cells (Fig. 8). The PITX2 isoforms and Nkx2.5 by themselves activate the PLOD1 promoter similar to the ANF promoter in these cells. The repression of PLOD1 promoter activity by PITX2A and Nkx2.5 and synergism by PITX2C and Nkx2.5 is not as pronounced as observed with the ANF promoter. However, these data indicate that this effect is not promoter-specific (Fig. 8). It appears to be specific to the C3H10T1/2 cell line because we do not observe this effect in transfected HeLa, GH3, or LS8 cell lines (data not shown). The major difference in these cell lines compared with C3H10T1/2 is that the C3H10T1/2 cell line is of embryonic origin.

Cell-specific Repression of PITX2A Activity by Nkx2.5 Requires Both Factors to Bind the Promoter—We have shown that PITX2A and PITX2C do not interact with Nkx2.5 in a functional assay using the TK-Bic and TK-NKE promoters in CHO cells. However, the results using the C3H10T1/2 cell line and...
ANF and PLOD1 promoters indicate that cellular factors may be interacting with PITX2A, which results in a repression of PITX2A-activated transcription by Nkx2.5. Again our rationale was that if a cellular factor interacts with PITX2A to cause this repression we might be able to demonstrate this effect using a promoter construct that binds either PITX2 isoforms or Nkx2.5 but not both. Interestingly, PITX2A activates the TK-Bic promoter ~3-fold more than PITX2C in C3H10T1/2 cells; compare 16-fold activation by PITX2A to 5-fold activation by PITX2C (Fig. 9). In CHO cells the transcriptional activities of the two PITX2 isoforms were similar (Fig. 6). Thus, there could be a cellular factor in these cells that increases the transcriptional activity of the PITX2A isoform but not PITX2C. However, cotransfection of Nkx2.5 did not affect the activities of either PITX2A or PITX2C using the TK-Bic promoter (Fig. 9). However, compared with CHO cells transfected with the PITX2 isoforms, Nkx2.5, and the TK-NKE promoter, in C3H10T1/2 cells Nkx2.5 is minimally active although we observe a slight activation of the TK-NKE promoter by the PITX2 isoforms that is increased upon co-transfection of PITX2 and Nkx2.5 (Fig. 9).

We asked whether the artificial TK-BicNKE and TK-PLOD1 promoters would demonstrate synergism or repression of PITX2 isoform activity with Nkx2.5 in C3H10T1/2 cells. Similar to the results in CHO cells we were unable to observe PITX2C and Nkx2.5 synergism of these promoters (data not shown). However, PITX2A activation of TK-Bic/NKE and TK-PLOD1 was repressed by co-transfection with Nkx2.5 (data not shown). Thus, although the synergistic effect between PITX2C and Nkx2.5 appears to be promoter-specific the repression of PITX2A activity by Nkx2.5 is cell-dependent. Synergism and repression require independent DNA binding of both factors.

Although we do not yet understand the mechanism of these interactions in heart development, we do provide the first link of PITX2 involvement in heart development through the identification of ANF as a downstream target. We further provide a molecular mechanism for the major role the PITX2C isoform plays in heart development as reported by several laboratories. Our research demonstrates that only the PITX2C isoform can synergistically activate the ANF and PLOD1 promoters in the presence of Nkx2.5. We also provide a new mechanism for Nkx2.5 in that it can repress the activation of the ANF and PLOD1 promoters by PITX2A in C3H10T1/2 cells.

**DISCUSSION**

*Pitx2* is co-expressed with the transcription factors Nkx2.5 and GATA-4 in the heart (23,26–28,30). Nkx2.5 and GATA-4 synergistically activate the heart ANF gene, whereas Nkx2.5 and serum response factor co-activate the cardiac α-actin promoter (22, 23, 26). More recently it has been shown that Nkx2.5 in combination with dHAND is required for cardiac ventricle formation and with Tbx2 inhibits ANF expression in the atrioventricular canal (24, 37). Furthermore, mutations in Nkx2.5 have been shown to cause congenital heart disease (38–40). Pitx2 and Nkx2.5 are two transcription factors that represent the earliest markers of precardiac cells, and both play major roles in vertebrate cardiogenesis (1, 3, 13–16). The identification of cardiogenic target genes for these transcription factors presents a major challenge for those studying their functional activities. We have previously shown Pitx2 protein expression in the developing mouse heart. At E9.5 Pitx2 is expressed in the wall of the left atrium, at E11.5 it is seen in the septum primum, and at E12.5 in the left atrium, septum primum, and wall of the right atrium (25). Interestingly, ANF transcripts have been detected in these same regions and at identical stages of mouse development (24). The overlapping expression patterns of these two genes would suggest that PITX2 could regulate ANF expression. The data presented in this report demonstrate that PITX2 regulates ANF expression and that PLOD1 is a target gene for Nkx2.5. Because PITX2 activates ANF, this would place the role of PITX2 in the commitment stage of heart precursor cells required to convert cardioblasts into cardiomyocytes and suggests a role for PITX2 in cell proliferation and migration.

**PITX2 Isoform-specific Transcriptional Activation of ANF and PLOD1 Modulated by Nkx2.5—**PITX family members (PITX1 and PITX2) have been shown to interact with other transcription factors (41–43). PITX2 can physically interact with POU homeodomain protein Pit-1 to synergistically regulate prolactin expression (6). All PITX2 isoforms can physically interact with each other to regulate gene expression (32).
Nkx2.5 has been shown to physically interact with GATA-4 to synergistically activate ANF expression (22, 23). Our results demonstrate that ANF is a downstream target of PITX2 and that only the PITX2C isoform can synergistically activate the ANF promoter in the presence of Nkx2.5. We further demonstrate that the PITX2C isoform and Nkx2.5 synergistically activate PLOD1. The transfection results initially suggested that PITX2C and Nkx2.5 might physically interact; however, we were unable to demonstrate a physical interaction between these two proteins. Our results are similar to a recent report demonstrating the cooperative action of Tbx2 and Nkx2.5 in inhibiting ANF expression (24). Although Tbx2 and Nkx2.5 do not physically interact they bind independently to their respective DNA elements and form a ternary complex. In this report we demonstrate a cooperative action of PITX2 and Nkx2.5 in regulating ANF expression. We expanded on this observation by using a TK minimal promoter containing either upstream bicoid or NKE sites or combinations of them in transfection assays. These artificial promoters demonstrate that separate independent binding of PITX2 and Nkx2.5 is required for synergism or PITX2A repression. We speculate that independent DNA binding by PITX2C and Nkx2.5 causes DNA bending, which results in bringing these two factors into close proximity where they can regulate transcription. DNA bending by homeodomain transcription factors has been previously reported (44–46), and recently it was shown that PITX1 can bend DNA

Fig. 6. Nkx2.5 represses PITX2A activation of the ANF promoter in C3H10T1/2 embryonic cells. A, RT-PCR of mRNA isolated from C3H10T1/2 cells showing the expression of Pitx2a and Pitx2c isoforms. Primers specific to the N-terminal sequences, which differ in each isoform, were used in combination with an antisense homeodomain primer. Pitx2a and Pitx2c isoforms were detected using specific sense primers. Markers for conformation of band sizes were 1 Kb ladder on the left and PCR markers (Promega) on the right. A positive control RT-PCR was done as a method to confirm the RNA isolation procedure. The RT-PCR experiment was repeated six times using three independent cell RNA preparations. All products were confirmed by sequencing the amplified bands. B, C3H10T1/2 cells were transfected with the ANF-638 luciferase reporter gene (5 µg). The cells were co-transfected with the CMV-PITX2 isoform and/or CMV-Nkx2.5 expression plasmids or the CMV plasmid without PITX2 (−) (2.5 µg). To control for transfection efficiency, all transfections included the CMV β-galactosidase reporter (0.5 µg). Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with the ANF promoter plasmids without PITX2 expression and normalized to β-galactosidase activity (±S.E. from seven independent experiments). The mean ANF promoter luciferase activity with PITX2 expression was about 150,000 light units per 15 µg of protein, and the β-galactosidase activity was about 100,000 light units per 15 µg of protein. C, Western blot of transfected C3H10T1/2 cell lysates using the P2R10 PITX2 antibody. C3H10T1/2 cell lysates from transfection experiments (B) (20 µg) were tested for PITX2 isoform expression. As a control, 200 ng of bacterial expressed PITX2 isoforms were used to show the correct migration of the transient expressed PITX2 isoform proteins. C3H10T1/2 cells co-transfected with the ANF promoter construct and empty expression vector were used as a mock control.

Fig. 7. The bicoid element in the ANF promoter is required for Nkx2.5 repression of PITX2A activity in C3H10T1/2 cells. C3H10T1/2 cells were transfected with the ANF-638 ΔBic luciferase reporter gene (5 µg) as described in Fig. 6 (±S.E. from three independent experiments).
by binding to a bicoid site (47). PITX2 can interact with factors through its C-terminal tail (34), and Nkx2.5 can interact with factors through its homeodomain (22). However, because the three major PITX2 isoforms only differ in their N-terminal residues we speculate that the N terminus may be interacting with cellular factors to regulate their activities. We have recently demonstrated cell-specific differences in the activities of the three major PITX2 isoforms, PITX2A, 2B, and 2C (32). It appears that these differences are because of cell-specific factors binding to their N-terminal residues. Similarly, the N terminus of Nkx2.5 may be interacting with cellular factors to regulate its transcriptional activity (48). Thus, the N terminus of PITX2C may be involved in the ability of this isoform to synergistically activate the ANF promoter in cooperation with Nkx2.5. This differential interaction of PITX2 isoforms with

Is Nkx2.5 a Repressor of PITX2A Transcriptional Activation?—Nkx2.5 is the vertebrate homolog of Tin, a transcription factor required for Drosophila heart formation (49, 50). Tin has been shown to be both an activator and a repressor of transcription (48, 51, 52). However, Nkx2.5 has not been shown to act as a repressor, which may be because of the differences in the N-terminal residues between Tin and Nkx2.5 (48). Clearly, we do not demonstrate that Nkx2.5 independently represses transcription; however, we have observed a unique activity of Nkx2.5 in combination with PITX2A expression. We use a variety of cells and cell lines to assay for PITX2-interacting factors and how they affect PITX2 transcriptional activity,
which can provide insights to PITX2 isoform-specific regulation of tissue and organ development. Surprisingly, we found that PITX2A transcriptional activation of the ANF and PLOD1 promoters was repressed by co-transfection with Nkx2.5 in the C3H10T1/2 cell line but not in CHO cells. This repressive effect was specific for the PITX2A isoform. We speculate that the N-terminal sequences of PITX2A differentially interact with cellular factors to govern its activity in the context of the general transcriptional machinery complex. Although Pitx2a is expressed in the vertebrate heart, the general consensus suggests that Pitx2c is the major effector of heart formation (14–16). Because we have now identified two cardiogenic genes regulated by PITX2 isoforms it seems possible that a mechanism for increased PITX2C involvement in heart development is the major effector of heart formation (14–16). Because we have now identified two cardiogenic genes regulated by PITX2 isoforms it seems possible that a mechanism for increased PITX2C involvement in heart development is cause there are combinations of other factors that act in concert to regulate ANF and PLOD1 expression. This effect is clearly speculative, because there are combinations of other factors that act in concert to regulate ANF and PLOD1 expression. However, it does provide an intriguing possibility for the regulation of PITX2 isoform activities.

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