Structural and Functional Analysis of Domains Mediating Interaction Between the *Bagpipe* Homologue, Nkx3.1 and Serum Response Factor

YAN ZHANG,* REBECCA A. FILLMORE,† AND WARREN E. ZIMMER*,‡,[1]

*Department of Systems Biology and Translational Medicine, College of Medicine, Texas A&M Health Science Center, College Station, Texas 77843; †Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, Alabama 36688; and ‡Center for Environmental and Rural Health, Texas A&M University System, College Station, Texas 77843

Nkx3.1 is a member of the NK2 class of homeodomain proteins and is expressed in development, being an early marker of the sclerotome and prostate gland. It has been shown to be a critical factor for prostate differentiation and function. Previous studies suggested that Nkx3.1 interacts with Serum Response Factor (SRF) to transactivate the Smooth Muscle γ-Actin (SMGA) promoter. In studies presented here, we examined the molecular mechanisms underlying the functional synergy of these factors upon SMGA transcription. We demonstrate that full length Nkx3.1 physically interacts with SRF in the absence of DNA and that these factors are able to co-associate in cellular context using a mammalian two-hybrid system. The segment of SRF responsible for Nkx3.1 interaction was mapped to a ~30 amino acid region (AAs 142–171) at the N-terminal segment of the MADS box. Two separate regions of Nkx3.1 were found to mediate interactions with SRF. Interestingly, recognized domains of NK2 proteins, namely the TN, homeodomain DNA binding segment, and the NK2-SD do not participate in SRF interactions. One of the Nkx3.1 SRF binding domains was mapped to the N-terminal of the protein consistent with recent studies of these proteins using NMR spectroscopy by Gelmann and colleagues (1). A second SRF binding region was mapped to amino acids C-terminal to the homeodomain. Structural predictions indicate that both of the SRF interacting segments are largely hydrophobic in character and β-strand in structure. With co-transfection transcriptional analyses we found that interaction between SRF and Nkx3.1 as well as DNA binding by both factors was required for the observed transcriptional synergy. Thus our studies have identified novel protein-protein interacting domains within Nkx3.1 and SRF that operate in concert with their respective DNA binding domains to mediate functional transcriptional synergy of these factors to regulate SMGA gene activation. Exp Biol Med 233:297–309, 2008

Key words: prostate; differentiation; cancer; serum response factor; Nk2 homeodomain; Nkx3.1; tissue-specific expression; transcription

Introduction

Nkx3.1 is a mammalian homolog of the *Drosophila* NK2 homeodomain gene *bagpipe* that is expressed in a variety of cells during early development. Expression is first observed in the ventral paraxial mesoderm of caudal somites (2) and later in development expression is detected in multiple tissues (3–8). In adult Nkx3.1 expression is predominant in the male urogenital system, including the testis, seminal vesicle, and the prostate (9, 10). Urogenital expression on Nkx3.1 initiates development of the prostate and accessory male reproductive organs, and in humans the gene is located within a segment of chromosome 8 that is frequently found deleted in prostatic intraepithelial neoplasias and carcinomas (11). In addition, targeted disruption of Nkx3.1 in mice leads to a non-lethal phenotype with defects in prostate development (2, 3, 10, 12). Thus, Nkx3.1 is a critical regulator of prostate differentiation, although its mechanism of action is not well defined. Several studies have implicated Nkx3.1 as a repressor of Prostate Specific Antigen (PSA) gene transcription (13, 14). This observed repressor activity appears to involve the modulation of other transcriptional activators on the PSA promoter. In cell transfection assays, the ETS factor Prostate-Derived Ets

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[1] To whom correspondence should be addressed at the Department of. Systems Biology and Translational Medicine, 310B Joe H. Reynolds Building, Texas A&M Health Science Center, College Station, TX 77843-1114. E-mail: wezimmer@medicine.tamhsc.edu

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Factor (PDEF) activates PSA promoter constructs, which is antagonized by Nkx3.1 (13). Nkx3.1 expression has also been shown to suppress the activity of the Sp-family of activators on PSA transcription (14). A segment of the protein just adjacent to the amino-terminus of the homeodomain interacts with SP-family proteins, while a carboxyl-terminal segment of the protein is required for PDEF interactions (13–15). Other studies have mapped a functional repressor activity to the extreme carboxyl-terminal segment of the protein (16). Thus, while the well-conserved TN domain at the amino terminus of Nkx3.1 shares homology with known repressor domains in other regulatory proteins, a similar activity has not yet been identified for this domain in the Nk transfactor family.

Recent studies have indicated that in addition to the repressor activity upon PSA promoter constructs, Nkx3.1 possesses the ability to activate gene transcription, depending upon cellular context. Conditional ablation of the murine Nkx3.1 gene results in mice that developed preinvasive lesions within the prostate (17). Magee et al. (18) demonstrated that the loss of a single Nkx3.1 allele in these mice extended the proliferative capacity of luminal cells that leads to prostate epithelial hyperplasia. Using microarray analyses, these studies revealed a number of potential target genes that were influenced both negatively and positively by the loss of Nkx3.1 expression, underscoring the critical regulatory activity of this factor in prostate differentiation. We have previously demonstrated that the smooth muscle γ-actin (SMGA) gene is a molecular target of Nkx3.1 activity (16). Moreover, we have shown that SMGA is expressed in prostate epithelia and that this expression was androgen responsive via an Nkx3.1 mechanism (19). Prostate epithelial cell specific SMGA expression was found to be dependent upon Nkx3.1 synergistic interaction with Serum Response Factor (SRF) through a novel cis-element within the initial ~100 bp of the SMGA promoter (19, 20). This synergism included DNA independent protein-protein interactions (16) and recent expression of Nkx3.1 protein fragments indicate that amino-terminal domains participate in this interaction (1). In the studies presented here, we have expressed native and mutant proteins to map the critical domains necessary for interactions between these two important transcriptional factors. We have found that the synergistic transcriptional activation requires both interactions at multiple protein-protein interfaces and protein-DNA interactions, which indicates that one mechanism of Nkx3.1 dependent, transcriptional activation in prostate epithelia requires combinatorial interactions with other factors expressed within these cells.

Materials and Methods

DNA Constructs. Plasmids encoding GST with human SRF full length and various mutant forms of SRF fusion proteins along with the pCGN-SRF and pCGN-Nkx3.1 CMV expression constructs have been described previously (16, 19). The firefly luciferase reporter genes driven by human SMGA proximal promoter sequences (HSMGA3, HSMGA5) and was used as described previously (19). The GST-Nkx3.1 fusion protein expression vectors (pGEX2X-Nkx3.1WT, pGEX2X-Nkx3.1N (1–87), pGEX2X-Nkx3.1N+ (1–128), pGEX2X-Nkx3.1ΔCT (1–216), pGEX2X-Nkx3.1HOM (129–216)) were kindly provided by Dr. Horowitz, North Carolina State University (14).

Recombinant Proteins. GST-SRF (WT and mutants) and GST-Nkx3.1 (WT and mutants) vectors were used to transform *E. coli*, BL21 cells. Freshly transformed BL21 cells were grown in 10 ml of LB broth containing 100 μg/ml ampicillin overnight at 37°C, diluted ten times in fresh LB broth and incubated for four hours (OD600: 0.6–1.0). IPTG (100 μM) was added for two hours to induce fusion protein expression. Induced *E. coli* were collected by centrifugation (5000 *g* for 5 minutes), washed with Phosphate Buffered Saline, (PBS), and then suspended in 1 ml of PBS, sonicated to lyse the cells, and had cellular debris removed by centrifugation (15,000 *g* for 15 minutes). Fusion proteins were purified by binding to GST/agarose-beads and eluted with Glutathione (10 nM) essentially as suggested by the supplier (Amersham Biosciences; Piscataway, NJ). The concentration of the fusion proteins was determined by Bradford protein assay, and purity was determined by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis (21).

In vitro transcribed/translated proteins were produced using PCR-generated fragments of pCGN-Nkx3.1 (Nkx3.1ΔNT, ΔCT, ΔHD, HD, ΔH3) as templates and a coupled reticulocyte lysate system (TNT^{tm}, Promega; Madison, WI). Five μl of the PCR fragments containing a T7 promoter and HA-epitope were added to a TNT Quick Master Mix and incubated for 90 minutes at 30°C. This was followed by western blot detection using HA-antibody (Boehringer Mannheim, Roche; Nutley, NJ) to confirm the *in vitro* translated protein products.

SRF and Nkx3.1 fusion proteins with HA-epitope were also produced in COS7 cells by transfection of pCGN-SRF and pCGN-Nkx3.1 (WT, mutants) expression vectors. 48 hours after transfection, the cells were washed with cold PBS and collected by centrifugation. Cells were then re-suspended in 1× binding buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 5 mM $MgCl_2$; 1 mM dithiotheritol (DTT); 0.05% Nonidet P-40; and protease inhibitors), sonicated, and had cellular debris removed by centrifugation. The protein concentration of the cellular lysates was determined by Bradford Assay (BioRad; Hercules, CA) and they were then used for *in vitro* protein-protein assays.

GST Pull-Down Assay. Wild type, full length Nkx3.1 and various mutants were incubated with GST-fusion proteins of SRF (WT or mutants) that were pre-bound to glutathione-agarose beads in ice-cold binding

buffer, as above, essentially as described in Chen and Schwartz (22). Bound proteins co-associated with the SRF peptides were eluted by adding 2×SDS sample buffer and subjected to Western blotting using anti-HA antibody.

Electrophoretic Mobility Shift Assay. Electrophoretic Mobility Shift Assay (EMSA) was performed essentially as described previously (16) using bacterially expressed Nkx3.1 fusion proteins. Double-stranded oligonucleotides corresponding to the smooth muscle γ-actin promoter NKE/SRE1, NK-A, NK-B, and NK-C elements (Fig. 5) were generated by annealing of the individual oligonucleotides and used in the reactions as described previously (16, 19). Briefly, 23 μl containing 3 μg of poly(dI-dC), binding buffer (10 mM Tris-HCl, pH 8.0; 50 mM NaCl; 1 mM DTT; 1 mM sodium phosphate; 5% glycerol), and appropriate quantities of proteins were incubated 10 min on ice; the probe was then added (35,000–40,000 CPM/reaction) and the mixture then incubated for 30 min at room temperature. The reactions were then separated on a 5% polyacrylamide gel in 0.5 × TBE buffer and following electrophoreses for 1.5 hours at 180 V, the gel was dried at 80°C for 1 hour and binding complexes were visualized by autoradiography (16).

Cell Culture, Transient Transfection, and Luciferase Activity Assay. African Green Monkey kidney epithelial cells (CV-1, ATCC, CCL-70) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% P/S. CV-1 cells were plated at 50,000 cells per well in 12-well dishes and transfected 24 hours later with liposome/DNA mixtures containing 2 μg total DNA which included 1 μg of luciferase reporter vector and 1 μg of pCGN-derived expression vectors. 48 hours after transfection, cells were harvested and 20 μl of cell extract was mixed with 100 μl of luciferase substrate solution and the emitted luminescence was measured for 15 seconds. Protein concentrations were measured by the Bradford Method and used to normalize luciferase activities which were plotted as mean ± SEM as previously described (16, 19, 23).

Mammalian Two-Hybrid Assays. Analyses of protein interactions in a cellular context were performed by mammalian two-hybrid experiments using the CheckMate™ System from Promega (Madison, WI). Sequences encoding human SRF were isolated as an XbaI restriction fragment from pCGN-SRF and the purified DNA ligated into like digested pBIND vector, forming a vector expressing a fusion protein of the DNA binding segment of GAL4 connected to the SRF. Nkx 3.1 (WT and various mutants) coding sequences were generated from pCGN-Nkx 3.1 vectors (16, 19) by PCR which introduced an XbaI site at the 3′ segment and a Bam HI site at the 5′ end; the fragments were then cloned into a like digested pACT vector of the CheckMate system which would express fusion proteins of the Nkx3.1 fragments and the activation domain of VP16. The expression of appropriate proteins was verified by analysis of lysates generated from CV-1 cells transfected with singular vectors using SRF or VP16 specific antibodies. These vectors were then used to transfect CV-1 cells in concert with a luciferase vector that has binding sites for the GAL4 protein (pG5-Luc) and, following transfection, luciferase activity was measured in cell lysates as described above. As controls, each experiment also contained cells transfected with MyoD and Id expression vectors supplied by the manufacturer. The luciferase activities were averaged over 3–5 experiments and the averages plotted ± SEM.

Protein Structural Analyses. Nkx3.1 potential structural conformations were approximated by using the Self-Optimized Prediction Method (SOPMA) computer-based analysis package. Amino acid sequences were submitted to the program via the Pole BioInformatique Lyonnais Web site (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_sompa.pl) as described in Geourjon and Deleage (24).

Statistical Analyses. Luciferase data was analyzed using the Statistix for Windows (Analytical Software; Tallahassee, FL) or Excel (Microsoft) software. Data was examined using an independent sample *t* test to analyze the Luciferase activity measurements (expressed as mean ± SEM). Where the variance was not equal between the populations, the Smith-Satterthwait's *t* test was applied. The difference between observations was considered significant when $P < 0.05$.

Results

SRF Interaction with Nkx3.1. We have previously demonstrated that SRF and Nkx3.1 synergistically activate SMGA gene transcriptional activity in prostate epithelia (19). In a similar manner, α-cardiac actin gene transcription in heart cells is dependent upon SRF and an Nk2 homeodomain, Nkx 2.5, and this synergic activity was dependent upon specific protein-protein associations (22). To evaluate if SRF directly interacts with Nkx3.1, we used the GST-pulldown assay as described by Shore and Sharrocks (25, 26). Fusion proteins of SRF, full length and various domains, combined with glutathione–S–transferase (GST) were expressed in bacteria, purified and immobilized on glutathione-agarose beads. The beads containing SRF fragment-GST or GST only as a control were incubated with cellular lysates prepared from CV-1 cells transfected with pCGN-Nkx3.1 (16). This vector expressed Nkx3.1 fused with an HA-tag epitope which is used to detect the Nkx3.1 protein by western blotting using an anti-HA antibody as described previously (16, 19). As shown in Figure 1A, Nkx3.1 was bound by native SRF (Lane 2) and a variety of the other SRF protein domains (Lanes 3–7). Although the different SRF domains bound the Nkx3.1 with various apparent affinities, it was not retained by the GST only (Lane 9) or by the GST–SRF protein in which the MADS box domain of SRF was deleted (Δ 46–244, Lane 8). The results from the GST-SRF pull down

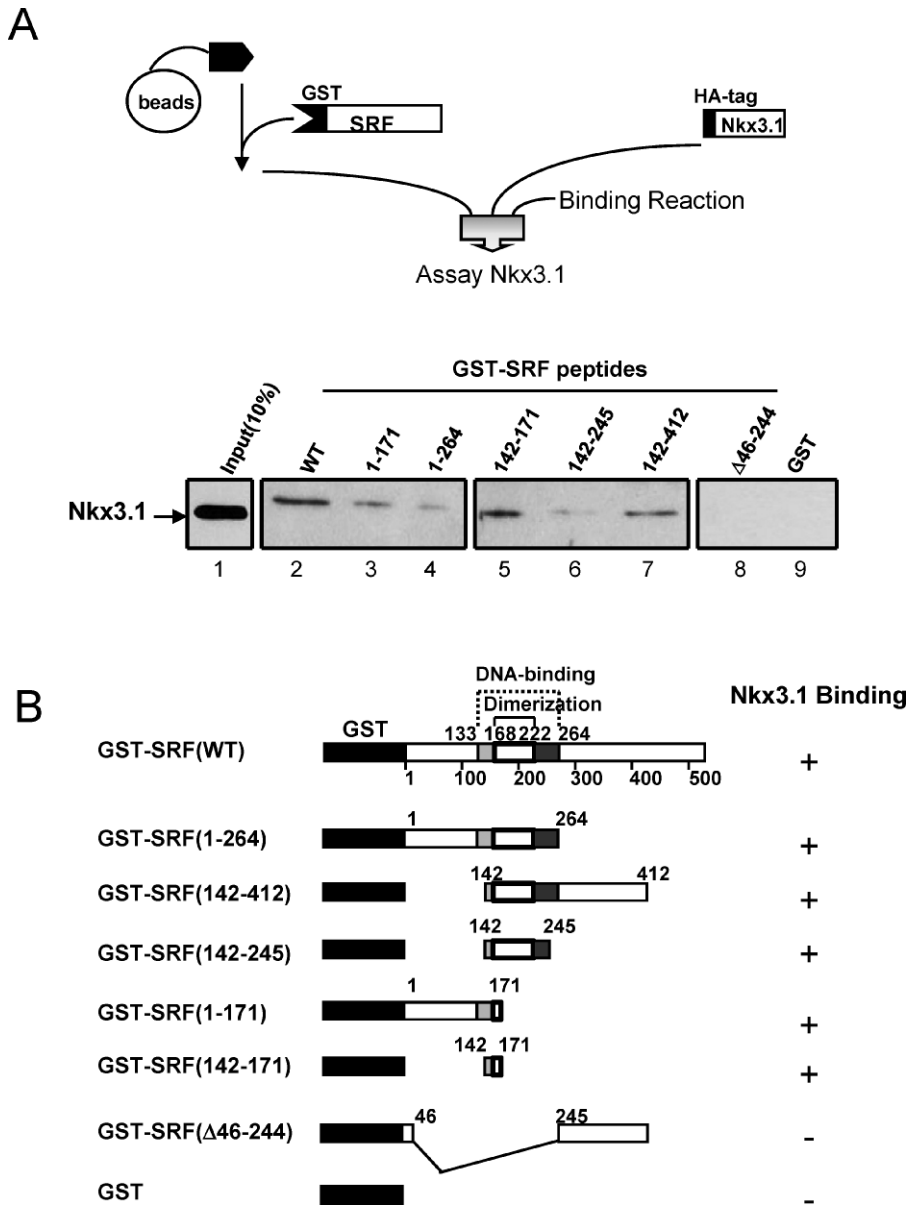

Figure 1. Mapping the domain of SRF required for interaction with Nkx3.1. (A) The upper section diagrams the experiment in which SRF peptides fused with GST are coupled to glutathione-agarose beads, and mixed with cellular lysates expressing Nkx3.1 fused to an N-terminal HA epitope tag. Thus the Nkx3.1 which bound to the GST-SRF peptides could be identified by western blotting using an HA-tag specific monoclonal antibody as detailed in Methods. Below the diagram are representative western blots from experiments using full-length (Lane 2) and different segments of SRF (Lanes 3–8). Lane 1 is an input control to demonstrate expression of the HA-Nkx3.1 protein, and Lane 9 is a control probing the HA-Nkx3.1 cell lysate with GST alone. (B) A summary of the GST-SRF pull down analysis of Nkx3.1 is shown. A diagram of the GST-SRF fusion peptides is shown and the ability of a particular GST-SRF fusion peptide to bind Nkx3.1is indicated by the + (positive binding) and – (no binding observed) symbols.

analyses are summarized in Figure 1B. As shown by these data, any GST-SRF fusion peptide containing the MADS box domain was capable of binding with the native Nkx3.1. Fine mapping analyses narrowed the Nkx3.1 binding ability to a section of the SRF MADS box domain, to 30 amino acids at the amino–terminus of this domain, amino acids 142–171 (Lane 5). The specificity of binding is shown by the abrogation of binding when this domain is deleted (Δ 46–244, Lane 8). The same results were obtained with Nkx3.1 proteins produced by *in vitro* translation, indicating that post-translational modification of the Nkx3.1 was not necessary to facilitate interactions (data not shown). Thus, these data show the interactions of Nkx3.1 and SRF in the absence of DNA-binding and defined the domain of SRF necessary for this binding as the initial 30 amino acids of the MADS box, amino acids 142 to 171 of this protein.

Multiple Domains of Nkx3.1 Facilitate Interactions with SRF. Having mapped the binding domain

upon SRF, we then asked which segment(s) of the Nkx3.1 were required for this interaction. In the initial set of experiments, we utilized GST-SRF proteins, GST-SRF wild type (full length) and GST-SRF 14–171. Each of these was found to bind Nkx3.1 (Fig. 1). The SRF fusion peptides were incubated with lysates generated from CV-1 cells transfected with pCGN–Nkx3.1 vectors encoding native protein, and various mutant proteins fused with an NH2-terminal HA-tag as illustrated in Figure 2A. Confirming the data shown in Figure 1, both the full-length SRF and SRF 142–171 peptides bound wild type, full-length Nkx3.1 (Lanes 1, 2; Fig. 2A). Deletion of sequences amino-terminal (AAs 1–124, Δ NT) or carboxy-terminal (AAs 184–237, Δ CT) to the homeodomain did not appreciably alter the binding, suggesting that either the homeodomain segment, present in both mutant proteins, directed interactions with SRF or there may be multiple SRF interacting sites on the Nkx3.1 molecule. Multiple SRF binding sites were indicated when the a mutant peptide lacking a segment of the homeodomain (AAs 167–188, Nkx3.1 Δ H3, Lanes 9 and 10) or the entire domain (Nkx3.1 Δ HD, Lanes 7 and 8) bound SRF and the SRF 142–171 peptide. However, for the Nkx3.1 homeodomain only, amino acids 125–184, did not exhibit strong SRF binding and did not bind with the SRF 142–171 peptide (Lanes 11 and 12), further indicating that multiple segments of Nkx3.1 are responsible for SRF interactions.

In order to demonstrate that these proteins interact within the context of a cell, we performed a mammalian two-hybrid experiment, where SRF was fused to a GAL–4 DNA binding segment and the Nkx3.1 peptides fused to a VP–16 transcriptional activation domain (27) as shown in Figure 2B. Co-expression of the SRF and Nkx3.1 fusion proteins in CV–1 fibroblast stimulated transcription from a luciferase reporter gene under the control of a GAL-4 DNA segment, when expression of the single proteins exhibited no transcriptional activation and reporter gene expression. Although none of the Nkx3.1 mutant proteins stimulated reporter expression to the extent of the full-length protein, all of the mutants except the Nkx3.1 homeodomain alone (Nkx3.1HD) stimulated transcription when coexpressed with the SRF–GAL4 fusion proteins. Taken together, these data indicated that there are two separate domains of Nkx3.1 which are capable of binding with SRF, one within sequences amino-terminal to the homeodomain and one within sequences carboxy-terminal to the homeodomain.

To further define the SRF binding domains of Nkx3.1, we obtained a set of vectors encoding Nkx3.1 protein domains fused to GST (14). The Nkx3.1 GST fusion protein was expressed in BL21 bacteria, purified from the bacterial cell lysates and then utilized in pulldown assays as described for the SRF–GST fusion peptides (Fig. 1). For these assays we expressed SRF proteins, both native and mutants, in CV–1 fibroblast using the pCGN cytomegalovirus promoter system. Figure 3A illustrates the binding of two SRF protein domains with the Nkx3.1 GST fusion protein. As shown in Figure 3A a fusion protein containing the initial 266 amino acids of SRF bound with the full-length Nkx3.1, confirming that the SRF MADS box is required for the protein-protein interactions (Fig. 1). The importance of the MADS box for Nkx3.1 binding is further demonstrated by the observation that no binding to any Nkx3.1 peptides was observed with an SRF protein in which this domain was deleted (SRF Δ MADS, Fig. 3). No binding was observed when the SRF 1–266 lysate was probed with an Nkx3.1 protein domain consisting of the first 87 amino acids (Nkx3.1 N) or the Nkx3.1 homeodomain only (Nkx3.1 HD). The data from these experiments are summarized in Figure 3B, and indicate that a segment of the Nkx3.1 protein from amino acid 87 to amino acid 128 constitutes a protein domain important for interacting with SRF. Since the Nkx3.1 fragment containing the homeodomain plus approximately 30 amino acids did not exhibit SRF binding (Nkx3.1 HD), these data taken with the study shown in Figure 2, indicate that the two Nkx3.1 domains for SRF interaction are found at the amino terminus (near amino acids 87–128) and the carboxy terminus (AAs 216–234).

DNA Binding Is Required for the Nkx3.1/SRF Activation of the SMGA Promoter. We and others have demonstrated that the carboxy terminal segment of Nkx3.1 contains a repressor–like activity (1, 16, 23). That is, when it is deleted from the Nkx3.1 molecule there is an enhancement of transcriptional synergism with SRF as shown in Figure 4A. We performed the same co-transfection analysis using mutant Nkx3.1 lacking the entire homeodomain (3.1 Δ HD) or the third helix of this domain (3.1 Δ H3) and found that neither demonstrated synergistic transcriptional activation of the human SMGA promoter construct (Fig. 4A). As shown by EMSA analysis, neither the Nkx3.1 Δ HD nor the Nkx3.1 Δ H3 proteins were capable of binding to a consensus Nkx3.1 DNA element found within the prostate specific antigen (PSA) promoter (13). However, the Nkx3.1 Δ CT and Nkx3.1 HD (homeodomain only) proteins exhibited binding to the PSA, Nkx3.1 element; this binding was effectively blocked using unlabeled DNA containing the NKE/SRE segment identified as an Nkx3.1 binding site on the chicken SMGA promoter (16). Thus, Nkx3.1 DNA binding is important for functional synergy between it and SRF to activate SMGA transcription. It has been shown that SRF DNA binding is required for it to work with the cardiac Nkx specific factor, Nkx 2.5, to activate cardiac–specific gene transcription (22). We tested whether SRF DNA binding was required for it to work with Nkx3.1 by expressing the DNA-binding deficient SRF mutant, SRF PM1, in our co-transfection assay (Fig. 4C). When the PM1 dominant–negative SRF is expressed in our assays, we found no transcriptional activation from the SMGA promoter. EMSA analysis confirmed that the expressed SRF–PM1 (Lane 1) did not bind with the NKE–SRE of the SMGA promoter (Lane 5). Taken together, these data show that DNA-binding by both

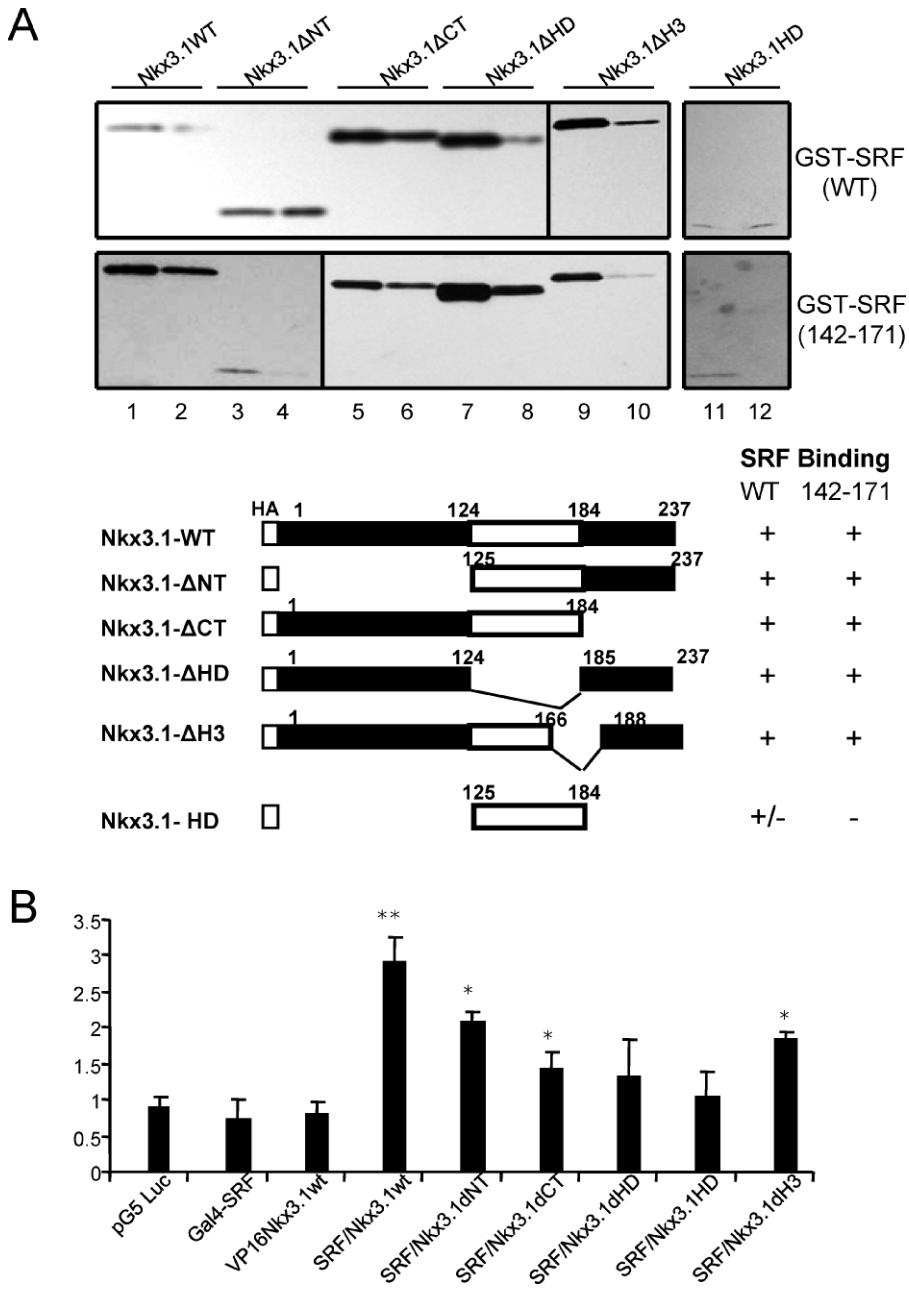

Figure 2. Mapping of Nkx3.1segments that interact with SRF. (A) The segment of Nkx3.1 responsible for SRF association was mapped by GST pull down analysis using full length SRF (GST-SRF, WT) or the minimal binding SRF region (GST-SRF, 142–171). Lysates were derived from cells expressing full length and mutants lacking the N-terminus (Nkx3.1 ΔNT), the C-terminus (Nkx3.1 ΔCT), the homeodomain (Nkx3.1 ΔHD), the DNA-binding third helix of the homeodomain (Nkx3.1 ΔH3), or the homeodomain alone (Nkx3.1 HD) as shown in the diagram below. Following pull down with GST-SRF peptides, Nkx3.1 association was measured by western blotting using an HA-specific antibody (Lanes 2, 4, 6, 8, 10 and 12) as described in Methods. Lanes 1, 3, 5, 7, 9 and 11 show the bands observed from the cellular lysates before analysis with GST-SRF. Shown below are diagrams of the Nkx3.1 proteins expressed in the CV-1 cells and their ability to associate with full-length or minimal binding segment of SRF as determined by probing 3–5 separate cellular lysates. (B) The association of SRF with segment of Nkx3.1 was evaluated by mammalian two-hybrid assay using the CheckMate™ system. Sequences encoding full length SRF was cloned with the pBind™ expression vector (GAL4-SRF) and sequences for full length or specific region of Nkx3.1 were cloned into the pACT™ vector (VP16-Nkx3.1). These expression vectors were co-transfected along with 0.5 μg/well of a luciferase reporter vector under the control of GAL4 DNA binding sites (×5), pG5-Luc, into CV-1 cells as detailed in Methods. All experimental transfections were performed in triplicate (n=15) and the average fold activation compared to pGL.3–basic plotted ± SEM (* = $P < 0.5$; ** = $P < 0.05$).

proteins, Nkx3.1 and SRF, is required for the synergistic activation of the SMGA transcription.

Multiple Nke Sites Are Present Within the Human SGMA Promoter. Within the avian SMGA promoter, the NK/CARG element at –120 bp is critical for SMGA transcription (16). Although there is strong overall homology between the SMGA gene proximal promoter among species (16, 20, 23), there are sequence differences.

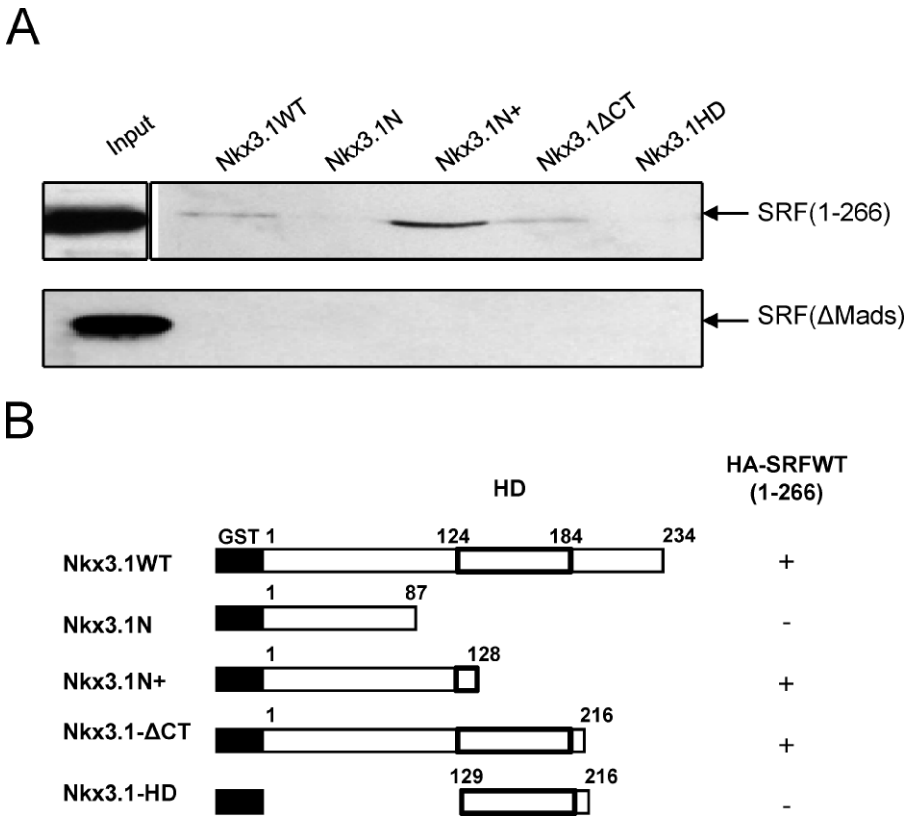

Figure 3. Fine mapping SRF association domains of Nkx3.1 using GST-Nkx3.1 fusion peptides. Full length and specific regions of Nkx3.1 were expressed as GST-fusion proteins in bacteria and used to probe lysates derived from cells that had been transfected with vectors expressing SRF domains fused to an HA-epitope which were identified by western blotting assay using an HA-specific antibody as described in Methods. (A) Shown are representative western blots of proteins associated SRF N-terminal domain (SRF 1–266) and an N-terminal segment in which the MADS box domain had been removed (SRF Δ MADS) following incubation with GST-Nkx3.1 fusion peptides. The input control shows the signal obtained from the original cellular lysate probed for HA-SRF peptides. (B) A diagram of the GST-Nkx3.1 fusion proteins used to examine SRF binding is shown. A summary of the SRF N-terminal domain binding observed from probing 3 independent HA-SRF cellular lysates is shown with + indicating SRF binding and – indicating no SRF binding was observed.

One difference is within the NKE segment of the NK/CARG, being *CATCACTTAG* in chicken and *CGTCAGCTGG* in human (16, 20), which would change the consensus AAGTG NKE sequence in chick to AGCTG in human. We reasoned that this change might change Nkx3.1 binding with the human NK/CARG element. Moreover, when we examined the human SMGA proximal promoter for potential NKE binding sites we observed three additional consensus sites within the initial 300 bp, not found in the avian promoter (Fig. 5A). When we tested the human SMGA –205 promoter (approximately equivalent to the –176 avian promoter needed for Nkx3.1/SRF co-activation, (16, 19) and a promoter containing the initial 285 base pairs in front of (5′) to the human gene, both showed Nkx3.1/SRF synergistic transcriptional activation (Fig. 5B). To evaluate if Nkx3.1 bound the sequences within the –285 base pairs proximal to the human SMGA gene we performed EMSA analysis using this fragment as a probe. As shown in Figure 5C, the Nkx3.1 homeodomain demonstrated multiple binding species with the human promoter –285 bp fragment. To assess if the homeodomain is capable of binding with the multiple NKE segments within this proximal promoter, we fashioned oligonucleotide probes comprising each potential NKE and surrounding 10–15 bases for EMSA experiments (Fig. 5B). A probe that we termed NKE-A containing the sequence GCAAGTG bound the Nkx3.1 homeodomain (Lane 2), and this binding was abolished when 100× molar concentration of unlabeled fragment (NKE–self, Lane 3) or PSA NKE (Lane 4) was included in the reaction. Similar results were obtained with an oligonucleotide probe containing a consensus NKE on the opposite strand as that of NK–A; 5′–CACTGAG–3′ (NKE-B, Fig. 5C). Within the human SMGA proximal promoter there is a sequence adjacent to the initial CArG/SRE that is similar but not exactly the same as the NK/CArG we have demonstrated previously as the functional unit in the avian SMGA promoter (16). The oligonucleotide probe containing this segment of the human promoter was capable of specifically binding the Nkx3.1 homeodomain (Lanes 9–12); however, the binding was not as efficient as that found for the equivalent region of the avian promoter (Lanes 17 and 18). This suggests that the Nkx3.1 bound the SMGA NK/CArG within the human promoter, but

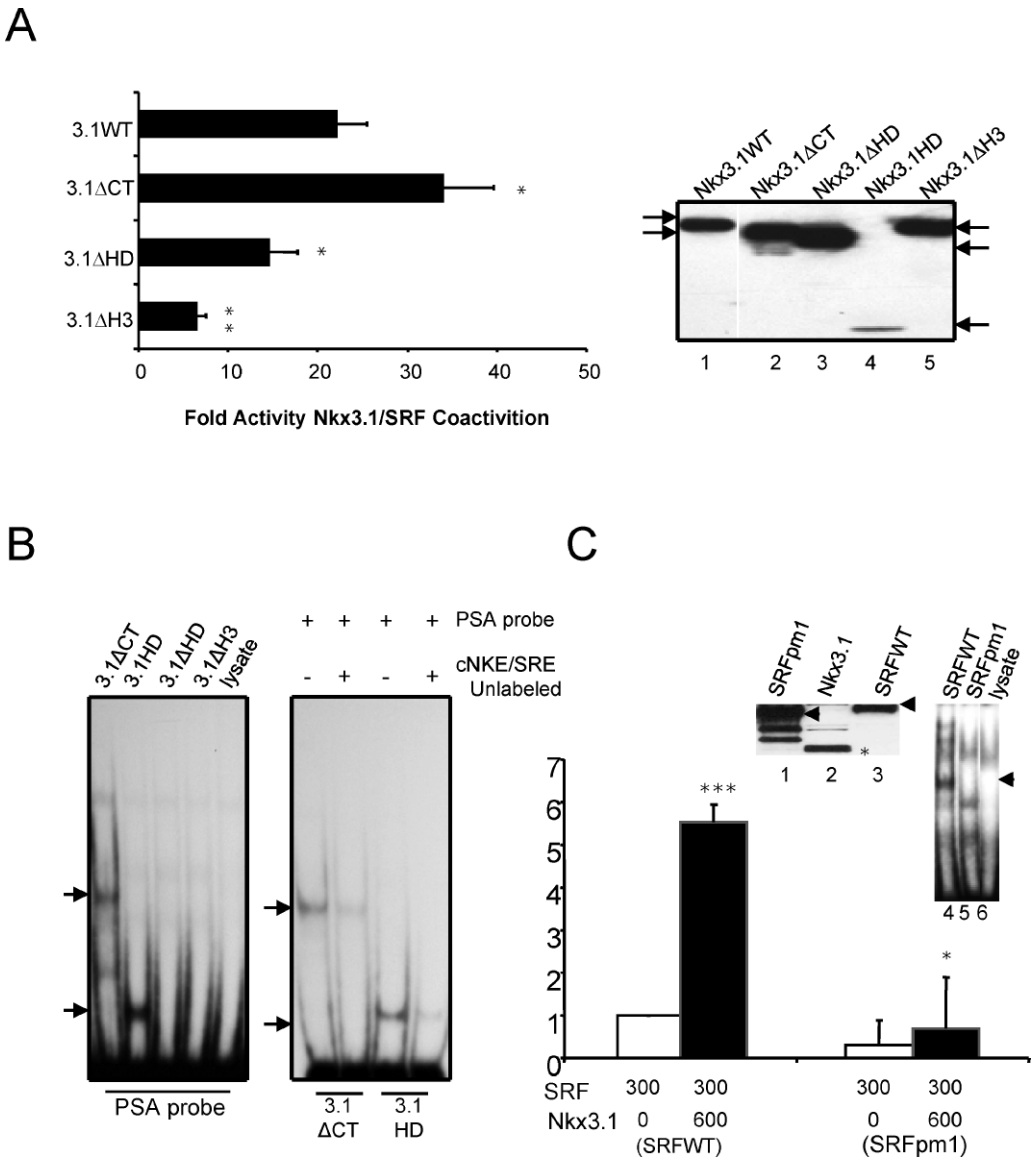

Figure 4. Nkx3.1 and SRF DNA binding is required for synergistic activation SMGA transcription. (A) Transcription of the human SMGA promoter activated by Nkx3.1 and SRF was determined by co-transfection analysis using the HSMGA-luciferase vector in CV-1 cells as detailed in Methods. Luciferase activity was measured with each experimental assay performed in triplicate (n=18) and the activity was evaluated relative to HSMGA3 co-transfected with 1 μg of empty pCGN vector and the mean fold activation plotted ± SEM (* = $P < 0.5$; ** = $P < 0.05$). The inset shows a western blot analysis of lysates obtained from transfected cells to demonstrate expression of the Nkx3.1 proteins. (B) DNA binding analysis of Nkx3.1 mutant proteins was evaluated by gel shift (EMSA) assay. The PSA Nkx3.1 oligonucleotide binding site was used to probe lyastes from cells transfected with vectors expressing Nkx3.1 mutant protein lacking the C-terminus (3.1 ΔCT), homeodomain (3.1 ΔHD) and the third helix of the homeodomain (3.1 ΔH3). Expressed Nkx3.1 homeodomain alone (3.1 HD) was used as a control for our experiments. Representative autoradiographs are shown with the PSA-Nkx3.1 binding complexes marked by the arrows. To the right is an autoradiogram demonstrating the specificity of the Nkx3.1 ΔCT and Nkx3.1 HD binding shown by competition of the bound complex using 100× molar concentration of unlabeled NKE/CArG from the avian SMGA gene. (C) The synergistic activation of the HSMGA3 promoter was evaluated by co-transfection assay in CV-1 cells. Fold activation was evaluated with SRF alone and SRF plus Nkx3.1. The same experiment was performed using the dominant/negative PM1-SRF expression vector (SRF PM1), which demonstrated no transactivation of the SMGA reporter (* = $P < 0.5$; ** = $P < 0.05$; *** = $P < 0.005$). The insert shows control experiments demonstrating the expression of the SRF (WT and PMI, arrowheads) and Nkx3.1 (asterisk) in lysates using western blotting (Lanes 1–3) and an EMSA experiment (Lanes 4–6) demonstrating that the SRF PM1 is incapable of binding an oligonucleotide probe fashioned from the NKE/CArG of the avian SMGA gene promoter (arrowhead).

the observed sequence differences between the species appear to influence the strength of this binding. The specificity of binding was demonstrated by the ability of known Nkx3.1 binding sites to compete for binding and by the experiment; using a control oligonucleotide probe of identical length and containing similar sequence structures did not exhibit binding (control, Lanes 13–16). Thus, these data demonstrate that there are multiple binding sites for Nkx3.1 within the promoter of the human SMGA gene, one that is conserved in location but not sequence to the SMGA promoter of other species and at least two that are functional but not found in the avian SMGA promoter.

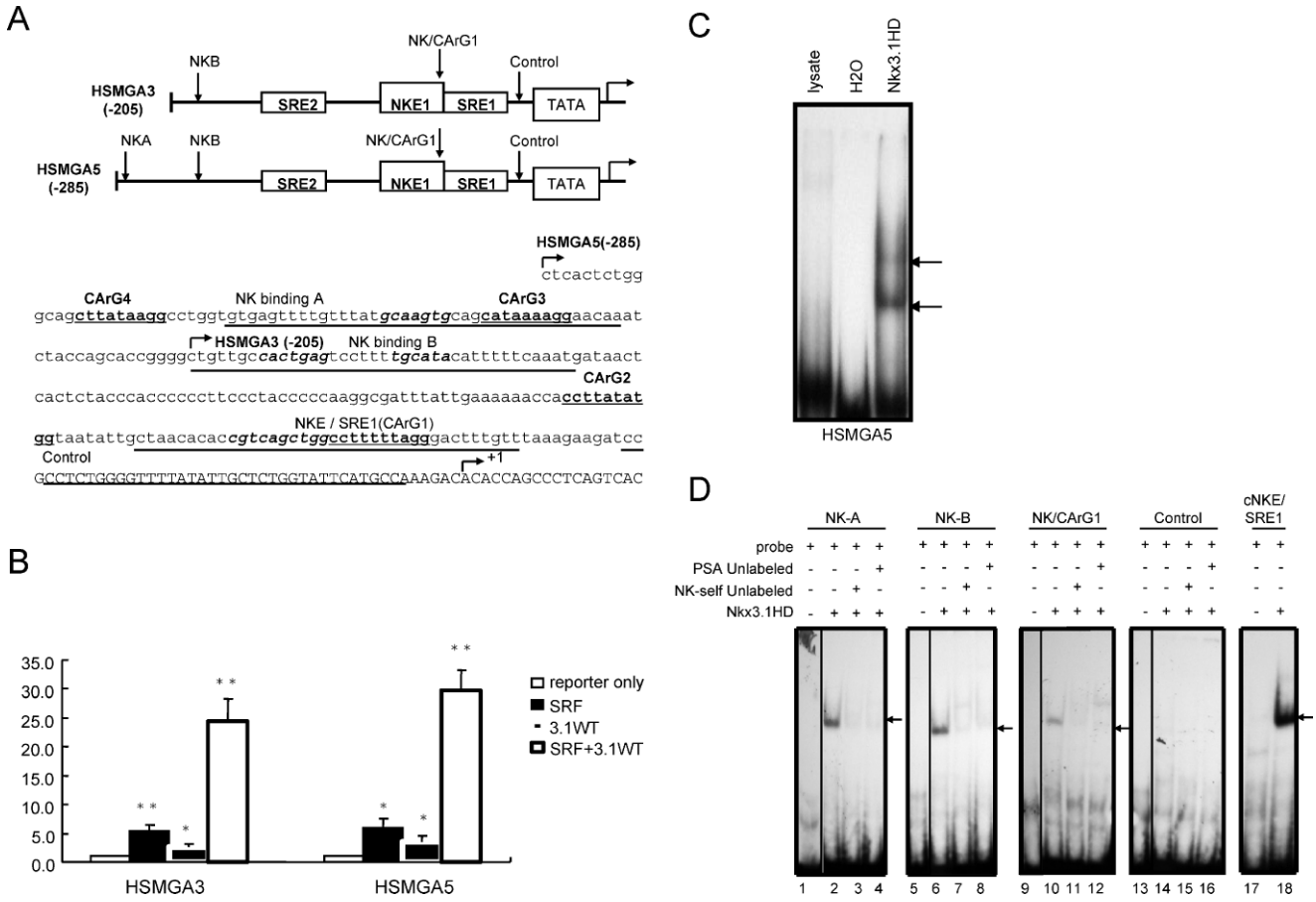

Figure 5. Human SMGA promoter contains multiple Nkx3.1 binding sequences. (A) A schematic view of the human SMGA gene promoter is shown illustrating the relative position of Serum Response Elements (SRE) and potential NK binding elements (NKA, NKB, NK/CArG). The top diagrams sequences found in HSMGA3 and HSMGA5 vectors, and the DNA sequence from the promoter is shown below the diagrams. (B) Reporters were co-transfected with the various activators into CV-1 cells and luciferase activity was measured in lysates derived from the cells as detailed in Methods. The activity generated from the activators was evaluated relative to the activity observed from cells that received the luciferase promoter vectors and empty expression plasmid, pCGN, and is plotted ± SEM ($n = 12$; * = $P < 0.5$; ** = $P < 0.05$). (C) EMSA analysis was performed using the sequences found in the HSMGA5 promoter. The HSMGA5 sequences were used to probe lysates derived from cells expressing the Nkx3.1 homeodomain by transfection with pCGN-Nkx3.1 HD as detailed in Methods. The arrows mark the multiple migrating complexes observed only in transfected cells (Nkx3.1 HD) and not found in samples from cells transfected with empty pCGN vector (lysates). (D) EMSA analysis was performed using oligonucleotides representing each of the potential NKE sequences in the HSMGA5 promoter segment. Oligonucleotides representing the individual NKE sites shown in A were labeled and used to probe lysates from cells expressing the NKX3.1 HD segment. The arrow denotes the migration of the NKX3.1HD/NKE complex. In each experiment the first lane (1, 5, 9, 13, 17) shows the result of probing lysate from untransfected cells which were run in separate lanes of the same gels, the second lane (2, 6. 10, 14, 18) from cells expressing the Nkx3.1 HD, the third lane (3, 7, 11, 15) a control experiment which included 100× molar excess of unlabeled oligonucleotide, and the last lane (4, 8. 12, 16) a control in which 100× molar excess of an oligonucleotide containing the NKX3.1 binding element from the PSA gene was added to the reaction. The experiment labeled control shows the results from a oligonucleotide probe from the HSMGA3 promoter that contains a sequence that does not bind Nkx3.1, and a positive control experiment was performed using a probe that represented the avian SMGA NK/CArG (cNKE/SRE1) previously shown to bind Nkx3.1 (Lanes 17 and 18).

Discussion

We (16) and others (28) have previously shown that Nkx3.1 can functionally collaborate to regulate transcription from the avian SMGA gene promoter. A function for Nkx3.1 transcriptional regulatory properties is not well elucidated, although it likely plays a role in early structures derived from somitic mesoderm where expression is initially observed (6, 7). This is supported by our recent demonstration that somite cells that had expressed Nkx3.1 can be found to populate bones and other mesoderm-derived structures using an Nkx3.1/CRE knock in allele and the ROSA 26R CRE reporter mice (8). In addition to embryonic expression in somatic mesoderm, Nkx3.1 has been shown to be critical for appropriate differentiation and maintenance of prostate epithelia (3, 10, 12, 17, 18, 29). Furthermore, the demonstration that the SMGA gene is regulated in an androgen responsive fashion in prostate epithelia (19) underscores the importance of the functional collaboration of Nkx3.1 and SRF upon gene regulatory paradigms in prostate cells. Here we demonstrate that this functional collaboration requires both protein:protein interactions as well as protein:DNA interactions between these two transcription factors. Nkx3.1 interaction site on SRF was

narrowed to approximately a 30 amino acid stretch at the beginning of the α1–helix of the MADS box DNA binding domain (AAs 142–171; Fig. 6A). Interestingly, multiple sites upon the Nkx3.1 molecule interact with SRF; including segments before (AAs 87–128) and after (AAs 185–234) the homeodomain (Fig. 6B). Although the homeodomain was found to participate minimally in the interactions with SRF, the DNA binding component of Nkx3.1 was absolutely necessary for cooperative transcriptional activation of the SMGA promoter (Figs. 2, 3 and 4). Therefore, synergistic Nkx3.1/SRF transcriptional activation includes complex interactions at the protein and DNA levels.

Serum response factor is a multifunctional transcriptional activator protein that cooperates with other factors to achieve cell specific gene regulation. The MADS box forms the DNA binding and dimerization domain for the protein. Structural studies have shown this to form a stratified structure above the DNA with an α-helix (α1 helix, AAs 153–179) forming the base of the structure and a second helix referred to as the α2 helix (AAs 207–220) laying atop the stratified structure (30). Residues of the SRF α-1 helix have been shown to make actual DNA contacts and bend the DNA (30, 31). A segment that overlaps the SRF α-1 helix domain, residues 45–171, has been determined to be a transcriptional inhibitory segment of the SRF molecule (32, 33). Therefore, our mapping the site of the functional positive transcriptional activation site upon SRF to this region may have a consequence in both the specificity of DNA binding/bending activity as well as masking of an auto-inhibitory portion of the molecule. The composite effects of this interaction might introduce enhanced DNA interactions and the release of a negative input, leading to an enhancement or augmentation of transcriptional activity. This prediction is completely consistent with our data in which Nkx3.1 synergistically activates the SMGA promoter in combination with SRF due to specific protein: protein associations. The Nkx3.1/SRF interaction is different from that mapped for the SRF–ternary complex factor (TCF) interaction sites upon the beta sheet segment of the SRF MADS box, C-terminal to the α-1 helix (31, 34, 35) which stabilizes SRF DNA binding (36). We show here that DNA binding by Nkx3.1 is a critical component of its synergistic activity with SRF on SMGA gene transcription. In addition, our previous work demonstrated that Nkx3.1 altered DNA confirmation upon binding the NKE/CArG within the avian SMGA promoter which facilitates SRF binding (16). Since DNA-binding mutants of Nkx3.1 can associate with SRF (Fig. 2) but not activate transcription, our data support a model in which DNA binding is required for Nkx3.1 transcriptional activity, which can be modified through select protein:protein interactions. In the case of SMGA, a product of the differentiated prostate epithelial cell, Nkx3.1 interactions with SRF promote or enhance the differentiated status of the cell by selectively altering transcription of cell-specific gene sequences.

Contrary to the single interaction site on SRF, we demonstrate that multiple sites of Nkx3.1are involved in contacting SRF. Similar to our studies, multiple sites were mapped upon the Nk2 homeobox protein, Nkx2.5, and its interaction with SRF that is an important step in the activation of heart specific genes such as α–cardiac actin (22). The interaction sites upon Nkx2.5 are within the homeodomain which contacts a region of the SRF molecule (AAs 142–171) that is similar to what we have shown for Nkx3.1, and in the case of α–cardiac gene activation both factors (Nkx2.5 and SRF) bind to overlapping segments of the CArG box (22). Although Nkx3.1 does weakly bind to the CArG sequence (16), we demonstrate here that the majority of the Nkx3.1 DNA-binding capability occurs via NKE sequences adjacent to the SRF binding site. Interestingly, while Nkx3.1/SRF synergistically activates both the avian (1, 16) and human SMGA promoters (Figs. 4 and 5) there appears to be a difference in the way the Nkx3.1 dependent activation is manifested. In the avian proximal promoter of approximately 400 bp there is a single NKE sequence adjacent to the first CArG element, forming the NK/CArG DNA element previously reported by our laboratory (16) whereas there are other multiple NKE sequences within the proximal promoter of the human SMGA gene. As illustrated in Figure 5, several of the NKE sequences identified within the human proximal promoter are capable of Nkx3.1 binding. Although Nkx3.1 DNA-binding is absolutely required for the functional synergism (Fig. 4), having multiple binding sites did not significantly alter the maximal activation (Fig. 5). Thus, it is likely that although there are multiple NKE binding sites within the human SMGA proximal promoter, the NK/CArG associated NKE at −70 bp of the promoter is the principal DNA segment for the Nkx3.1/SRF synergism of SMGA transcription.

From studies of Nkx3.1 knockout mouse models, it is clear that this Nk2 homeodomain is critical for directing differentiation of the prostate epithelia. As such, our previous studies have shown that it functions to regulate SMGA transcription in the prostate, thus indicating that SMGA expression is a product of the differentiated prostate epithelial cell (19). Our studies presented here demonstrate that there are multiple sites or domains of Nkx3.1 which mediate interactions with SRF. Our results are similar to the recent study from Ju et al. (1) that used solution NMR spectroscopy to examine changes in Nkx3.1 structure in the presence of DNA or the MADS domain of SRF. Both studies found that the homeodomain segment in Nkx3.1 did not significantly mediate SRF interactions. Thus, Nkx3.1 associates with SRF differently than that observed for Nkx2.5, which is dependent upon the homeodomain (22), indicating that NK2 proteins associate with other transcriptional proteins in a protein–specific manner. We mapped a strong SRF association site within the N-terminal sequences adjacent to the Nkx3.1 homeodomain (AAs 87–128). Using NMR it was observed that two segments of Nkx3.1 demonstrated enhanced structural changes with

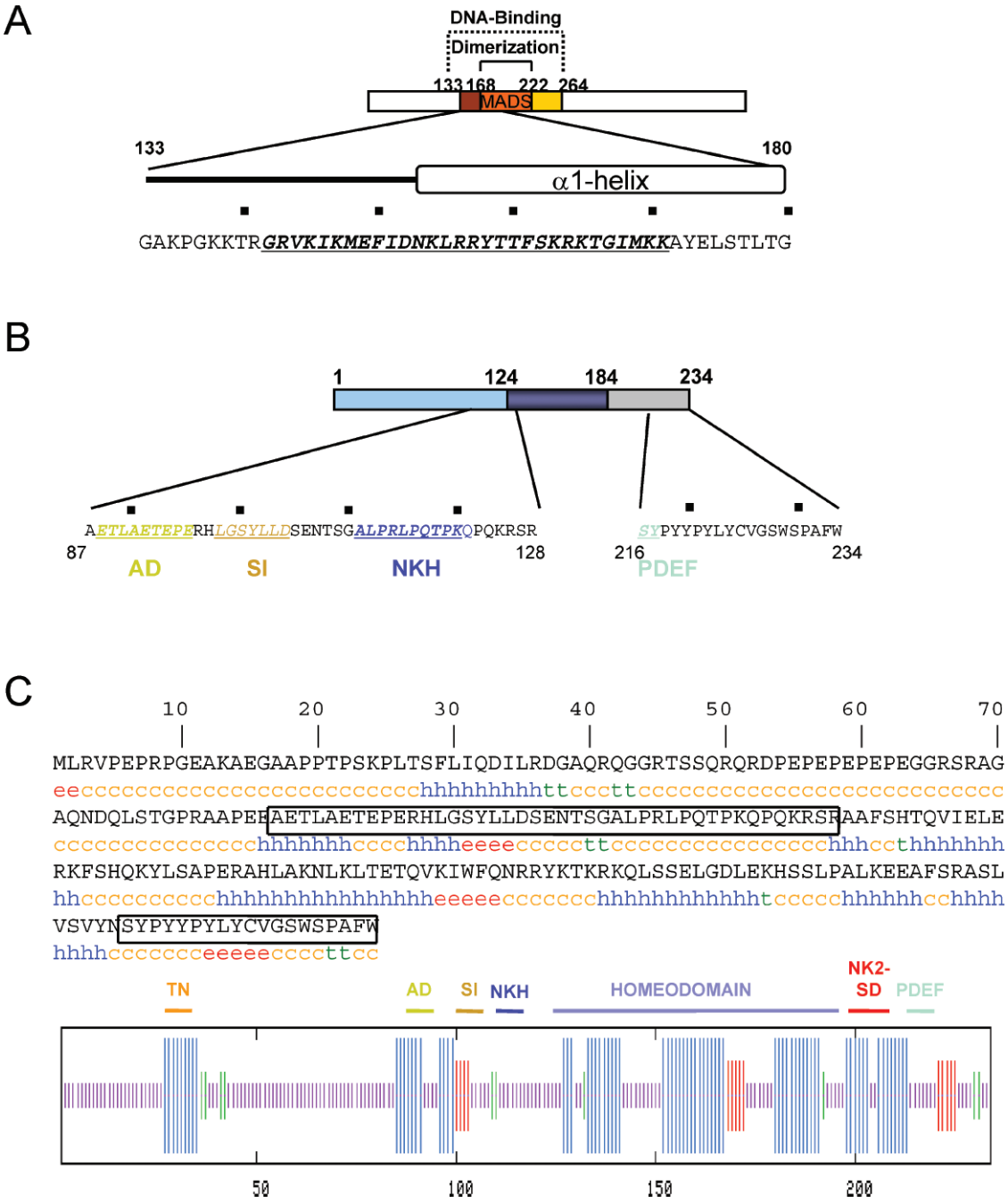

Figure 6. Summary of SRF and Nkx3.1 interacting domains. (A) The region of SRF required for Nkx3.1 inaction is shown. The top segment diagrams the SRF molecule showing the location of the MADS box DNA-binding /Dimerization region of the molecule. The N-terminal segment of this domain and juxtaposed sequences are shown below with the primary amino acid structure from AA 133 to AA 180 delineated. The segment within this sequence that is required for co-association with Nkx3.1 is shown in bold, italics and is underlined. (B) The regions of Nkx3.1 that are important for SRF are illustrated. The top bar is a diagram of the Nkx3.1 molecule with the N-terminal domain (AAs 1–124), homeodomain (AAs 124–185) and the C-terminal domain (AAs 185–234) shown. The sequence within the N-terminal domain that is required for SRF interaction is shown with the acidic domain (AD), SRF interacting (SI) and the NK2 homology (NKH) domains illustrated. Sequences within the C-terminal domain that are important for SRF association are shown, and the region identified as interacting with the prostate derived ets domain protein (PDEF) is illustrated. (C) Nkx3.1 secondary structure predictions based upon the self-optimized predictor method (SOMPA) is shown. The Nkx3.1 sequence was evaluated and is show with the predicted structural component listed below the amino acid sequence. The symbols are: e = extended strand; h = alpha-helix; c = random coil; and, t = beta-turn. The two regions of the molecule that are important for SRF association are shown by the rectangle. Below is a graphical representation of the predicted structural analysis. The identified domains of the Nkx3.1 protein are shown above the structural prediction which are: Tinman homology (TN); Acidic Domain (AD); SRF Interaction (SI); NK2 Homology (NKH); Homeodomain (homeodomain); NK2 homology (NK2-SD) and prostate derived ets Factor interaction domain (PDEF).

added SRF MADS domain (1). Structural prediction (Fig. 6) indicates that one region called the acidic domain or AD, is likely α–helical in character while the second, called SRF interacting or SI, exhibits β-strand qualities. Of these two regions, the SI β-strand is most similar to segments of other proteins that co-associate with SRF (37). In addition, structural analysis of the yeast MADS box factor, MCM1, bound with a Co-activating factor, the mating homeodomain factor MAT α2, documented that the MAT α2 contained a β-strand, hairpin structure within a segment N-terminal to the homeodomain that mediated contacts (38). Thus, our data is consistent with a prediction that the SI β–strand structure is the segment of Nkx3.1 within the N-terminal region of the protein that mediates SRF protein interactions. Our studies presented here differ from the NMR study (1) in two cases. First, we show that there is no significant interaction of the extreme N-terminal segment of Nkx3.1 (AAs 1–87, Fig. 3) with full-length SFR or its MADS domain. This data would be consistent with functional analysis from our lab (16) and that observed by Gelman and collaborators (1) which showed that deletion of the TN domain did not significantly alter the co-activation of SMGA transcription with SRF using a heterologous co-transfection assay system. However, the finding that mutations in this region of Nkx3.1, both *in vitro* (1) and naturally occurring (39), do have effects on SMGA transcription and prostate function implicating this region's importance for this process. Based upon our work this would occur without this Nkx3.1 segment contacting SRF.

A second difference between our studies and the observation of Gelman's group NMR studies is that we mapped an SRF interaction domain within the C-terminal sequences of Nkx3.1. The Nkx3.1 segment used for the NMR studies did not contain these C-terminal sequences and was thus not evaluated. Nkx3.1 C-terminal sequence has been shown to mediate protein–protein contacts with prostate-derived ETS factor (PDEF), and this interaction represses PDEF activation of PSA transcription (13). Structural predictions indicate that this segment of Nkx3.1 would be β-strand and largely hydrophobic in character (Fig. 6). This is identical to the structural components of the SI domain within the Nkx3.1 N-terminus, which we would predict mediates contacts through stacking with the SRF β-strand located above the α1 helix (1, 30, 38). Since the C-terminal segment of Nkx3.1 has been shown to house functional repressor capabilities (13, 16), it is possible that interactions of this segment with SRF would mask this effect, allowing for robust activation of SMGA transcription, and other potential target genes identified in Nkx3.1 knockout mouse models (17, 18). The elucidation of the exact roles of the structural components mediating Nkx3.1 protein co-associations will enhance our knowledge of Nkx function in prostate differentiation and disease as well as provide a rational basis for drug design as a potential prostate cancer therapeutic.

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1. Ju JH, Maeng JS, Zemedkun M, Ahronovitz N, Mack JW, Ferretti JA, Gelmann E, Gruschus JM. Physical and functional interactions between the prostate suppressor homeoprotein NKX3.1 and serum response factor. J Mol Biol 360:989–999, 2006.
2. Tanaka M, Komuro I, Inagaki H, Jenkins NA, Copeland NG, Izumo S. Nkx3.1, a murine homolog of drosophila bagpipe, regulates epithelial ductal branching and proliferation of the prostate and palatine glands. Dev Dyn 219:248–260, 2000.
3. Schneider A, Brand T, Zweigerdt R, Arnold H. Targeted disruption of the Nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. Mech Dev 95:163–174, 2000.
4. Sciavolino PJ, Abrams EW, Yang L, Austenberg LP, Shen MM, Abate-Shen C. Tissue-specific expression of murine Nkx3.1 in the male urogenital system. Dev Dyn 209:127–138, 1997.
5. Shen MM, Abate-Shen C. Roles of the Nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis. Dev Dyn 228:767–778, 2003.
6. Tanaka M, Lyons GE, Izumo S. Expression of the Nkx3.1 homobox gene during pre and postnatal development. Mech Dev 85:179–182, 1999.
7. Kos L, Chiang C, Mahon KA. Mediolateral patterning of somites: multiple axial signals, including Sonic hedgehog, regulate Nkx-3.1 expression. Mech Dev 70:25–34, 1998.
8. Stanfel MN, Moses KA, Carson JA, Zimmer DB, DeMayo F, Schwartz RJ, Zimmer WE. Expression of an Nkx3.1-CRE gene using ROSA26 reporter mice. Genesis 44:550–555, 2006.
9. Bieberich CJ, Fujita K, He WW, Jay G. Prostate-specific and androgen-dependent expression of a novel homeobox gene. J Biol Chem 271: 31779–31782, 1996.
10. Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C, Shen MM. Roles for Nkx3.1 in prostate development and cancer. Genes Dev 13:966–977, 1999.
11. He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, Carter KC. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43:69–77, 1997.
12. Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, Abate-Shen C. Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res 62:2999–3004, 2002.
13. Chen H, Nandi AK, Li X, Bieberich CJ. NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the PSA promoter. Cancer Res 62:338–340, 2002.
14. Simmons SO, Horowitz JM. Nkx3.1 binds and negatively regulates the transcriptional activity of Sp-family members in prostate-derived cells. Biochem J 393:397–409, 2006.
15. Oettgen P, Finger E, Sun Z, Akbarali Y, Thamrongsak U, Boltax J, Grall F, Dube A, Weiss A, Brown L, Quinn G, Kas K, Endress G, Kunsch C, Libermann TA. PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. J Biol Chem 275: 1216–1225, 2000.
16. Carson JA, Fillmore RA, Schwartz RJ, Zimmer WE. The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3–1, and serum response factor. J Biol Chem 275:39061–39072, 2000.
17. Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK,

Humphrey PA, Milbrandt J. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol 22:1495–1503, 2002.

18. Magee JA, Abdulkadir SA, Milbrandt J. Haploinsufficiency at the Nkx3.1 locus. A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. Cancer Cell 3:273–283, 2003.
19. Filmore RA, Dean DA, Zimmer WE. The smooth muscle gamma-actin gene is androgen responsive in prostate epithelia. Gene Expr 10:201–211, 2002.
20. Kovacs AM, Zimmer WE. Cell-specific transcription of the smooth muscle gamma-actin gene requires both positive- and negative-acting cis elements. Gene Expr 7:115–129, 1998.
21. Browning CL, Culberson DE, Aragon IV, Fillmore RA, Croissant JD, Schwartz RJ, Zimmer WE. The developmentally regulated expression of serum response factor plays a key role in the control of smooth muscle-specific genes. Dev Biol 194:18–37, 1998.
22. Chen CY, Schwartz RJ. Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Mol Cell Biol 16:6372–6384, 1996.
23. Carson JA, Culberson DE, Thompson RW, Fillmore RA, Zimmer W. Smooth muscle gamma-actin promoter regulation by RhoA and serum response factor signaling. Biochim Biophys Acta 1628:133–139, 2003.
24. Geourjon C, Deleage G. SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput Appl Biosci 11:681–684, 1995.
25. Shore P, Sharrocks AD. The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. Mol Cell Biol 14:3283–3291, 1994.
26. Shore P, Sharrocks AD. The MADS-box family of transcription factors. Eur J Biochem 229:1–13, 1995.
27. Sadowski I, Ma J, Triezenberg S, Ptashne M. GAL4-VP16 is an unusually potent transcriptional activator. Nature 335:563–564, 1988.
28. Asatiani E, Huang WX, Wang A, Rodriguez Ortner E, Cavalli LR, Haddad BR, Gelmann EP. Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res 65:1164–1173, 2005.
29. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 14:2410–2434, 2000.
30. Pellegrini L, Tan S, Richmond TJ. Structure of serum response factor core bound to DNA. Nature 376:490–498, 1995.
31. Hassler M, Richmond TJ. The B-box dominates SAP-1-SRF interactions in the structure of the ternary complex. EMBO J 20: 3018–3028, 2001.
32. Johansen FE, Prywes R. Identification of transcriptional activation and inhibitory domains in serum response factor (SRF) by using GAL4-SRF constructs. Mol Cell Biol 13:4640–4647, 1993.
33. Johansen FE, Prywes R. Serum response factor: transcriptional regulation of genes induced by growth factors and differentiation. Biochim Biophys Acta 1242:1–10, 1995.
34. Marais R, Wynne J, Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73:381–393, 1993.
35. Messenguy F, Dubois E. Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene 316:1–21, 2003.
36. Zaromytidou AI, Miralles F, Treisman R. MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. Mol Cell Biol 26:4134–4148, 2006.
37. Herring BP, Kriegel AM, Hoggatt AM. Identification of Barx2b, a serum response factor-associated homeodomain protein. J Biol Chem 276:14482–14489, 2001.
38. Tan S, Hunziker Y, Pellegrini L, Richmond TJ. Crystallization of the yeast MATalpha2/MCM1/DNA ternary complex: general methods and principles for protein/DNA cocrystallization. J Mol Biol 297:947–959, 2000.
39. Rodriguez Ortner E, Hayes RB, Weissfeld J, Gelmann EP. Effect of homeodomain protein NKX3.1 R52C polymorphism on prostate gland size. Urology 67:311–315, 2006.