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Structural and Functional Analysis of Domains Mediating Interaction Between NKX-3.1 and PDEF

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Abstract *NKX-3.1* is a suspected prostate tumor suppressor gene that encodes a homeodomain transcription factor. NKX-3.1 has been demonstrated to interact with prostate derived Ets factor (PDEF) and to suppress the ability of PDEF to transactivate the prostate specific antigen promoter. To dissect the molecular basis of the interaction between these transcription factors, deletion analyses were preformed using the yeast two-hybrid system. The interaction of NKX-3.1 with full-length PDEF requires part of the homeodomain and a tyrosine-rich 21 amino acid sequence that lies C-terminal to the homeodomain. The interaction of PDEF with full-length NKX-3.1 requires the Ets domain and a linker region that lies between the Ets and pointed domains. Deletion of the C-terminal 21 amino acids of NKX-3.1 completely disrupts the ability to suppress the transactivation function of PDEF in prostate tumor cells, demonstrating concordance between interaction in yeast and function in mammalian cells. These studies have identified novel protein–protein interaction domains within NKX-3.1 and PDEF that operate in concert with their respective DNA binding domains to mediate functional interactions between these growth regulatory transcription factors. J. Cell. Biochem. 94: 168–177, 2005.
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Key words: NKX-3.1; PDEF; interacting domains; prostate cancer; prostate specific antigen

Prostate cancer is the second leading cause of cancer deaths in men in the United States, and an estimated 28,900 were estimated to die from the disease in 2003 [Jemal et al., 2003]. Despite the prevalence of prostate adenocarcinoma and the extent of morbidity associated with it, the molecular basis of prostate cancer remains poorly defined. Multiple lines of evidence suggest that the homeodomain transcription factor NKX-3.1 plays a significant role in the etiology of most prostate tumors [Abate-Shen and Shen, 2000]. First described in mice as an androgen dependent, prostate-restricted gene [Bieberich et al., 1996], it is now apparent that in both humans and mice, NKX-3.1 has a growth suppressive function in adult prostate epithelial cells. The human gene maps to chromosomal region 8p21, an area that has long been suspected of carrying at least one tumor suppressor gene based on loss of heterozygosity analyses [He et al., 1997]. Knockout studies in mice have demonstrated that loss of *Nkx-3.1* function leads to prostate epithelial cell hyperplasia that resembles prostatic intraepithelial neoplasia, a suspected precursor to adenocarcinoma in humans [Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000; Abdulkadir et al., 2002; Magee et al., 2003]. In addition, loss of immunohistochemical staining for NKX-3.1 has been shown to correlate with human prostate tumor progression [Bowen et al., 2000].

Although it is a leading candidate for a prostate-specific tumor suppressor, the biochemistry of NKX-3.1 has not been extensively characterized. The 234-amino acid human NKX-3.1 protein has a structure typical of many homeodomain transcription factors, where the 60 amino acid DNA-binding homeodomain is located near the C-terminus and is followed by a short "tail." A study to identify protein partners of NKX-3.1, undertaken with a view towards defining the nature of the regulatory complexes in which NKX-3.1 participates, identified an epithelial specific Ets transcription factor as an interacting partner [Chen et al., 2002]. Prostate

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derived Ets factor (PDEF) was shown to interact with NKX-3.1 in yeast two-hybrid assays and by co-immunoprecipitation in cultured prostate tumor cells. PDEF has recently been implicated in controlling growth and invasiveness of mammary epithelial cells [Feldman et al., 2003], and it is possible that it plays similar roles in prostate epithelial cells [Nozawa et al., 2000; Tsujimoto et al., 2002].

Given that both NKX-3.1 and PDEF have been implicated in processes that are central to cancer progression, we sought to characterize in detail the regions of both proteins that are required to mediate their interaction. Using a yeast two-hybrid deletion analysis, we demonstrate here that the interaction is mediated by short amino acid stretches both within and outside of their respective DNA-binding domains. We further demonstrate that interaction between NKX-3.1 and PDEF is necessary for NKX-3.1 to modulate the function of PDEF in prostate epithelial cells. The identification of these interacting domains is an important step in elucidating the molecular basis whereby these transcription factors perform their functions in regulating prostate cell growth and motility.

MATERIALS AND METHODS

Yeast Two Hybrid Analysis

The yeast two-hybrid screen for interaction between full-length NKX-3.1 and PDEF and deletion derivatives of each was performed as described [Chen et al., 2002]. Truncated versions of NKX-3.1 were created by PCR and cloned into pLexA (Clontech, Palo Alto, CA). The following primers were used: for full-length NKX-3.1, 5′-GGAATTCATGCTCAGGGTTCCGGAGCCGCG-3′, 5′-CCGCTCGAGTTACCAAAAAGCTGGGCTCCAGC-3′; for construct N1 (1–123), 5′-GGAATTCATGCTCAGGGTTCCGGAGCCGCG-3′, 5′-CCGCTCGAGTTACGGCTGCTTAGGGGTTTGGGGAAG-3′; for construct N2 (1–183), 5′-GGAATTCATGCTCAGGGTTCCGGAGCCGCG-3′, 5′-CCGCTCGAGTTACTGCTTTCGCTTAGTCTTATAGC-3′; for construct N3 (1–213), 5′-GGAATTCATGCTCAGGGTTCCGGAGCCGCG-3′, 5′-CCGCTCGAGTTACACGGAGACCAGGGAGGCCC-3′; for construct N4 (184–234): 5′-GGAATTCCTCTCCTCGGAGCTGGGAGAC-3′, 5′-CCGCTCGAGTTACCAAAAAGCTGGGCTCCAGC-3′; for construct N5 (124–234), 5′-GGAATTCCAGAAGCGCTCCCGAGCTGCCTTC-3′, 5′-CCGCTCGAGTTACCAAAAAGCTGGGCTCCAGC-3′; for construct N6 (154–234), 5′-GGAATTCCGGGCCCACCTGGCCAAGAAC-3′, 5′-CCGCTCGAGTTACCAAAAAGCTGGGCTCCAGC-3′; for construct N7 (124–183), 5′-GGAATTCCAGAAGCGCTCCCGAGCTGCCTTC-3′, 5′-CCGCTCGAGTTACTGCTTTCGCTTAGTCTTATAGC-3′; for construct N8 (124–198), 5′-CCGCTCGAGTTACGGCAAAGAGGAGTGCTTCTC-3′; 5′-CCGCTCGAGTTACTGCTTTCGCTTAGTCTTATAGC-3′; for construct N9 (124–213), 5′-GGAATTCCAGAAGCGCTCCCGAGCTGCCTTC-3′; 5′-CCGCTCGAGTTACACGGAGACCAGGGAGGCCC-3′.

Constructs carrying truncated versions of PDEF were also generated by PCR and cloned into pB42AD (Clontech). The following primers were used: for full-length PDEF, 5′-CCGGAATTCATGGGCAGCGCCAGCCCGGG-3′, 5′-CCGCTCGAGTCAGATGGGGTGCACGAACTG-3′; for construct P1 (1–247), 5′-CCGGAATTCATGGGCAGCGCCAGCCCGGG-3′, 5′-ACGCGTCGACTCACTGCCCGGAGCATGATGAGT-3′; for construct P2 (248–335), 5′-GGAATTCCCCATCCACCTGTGGCAGTTCC-3′, 5′-CCGCTCGAGTCAGATGGGGTGCACGAACTG-3′; for construct P3 (142–335), 5′-GGAATTCAACATCACCGCAGATCCCATGG-3′, 5′-CCGCTCGAGTCAGATGGGGTGCACGAACTG-3′; for construct P4 (142–247), 5′-GGAATTCAACATCACCGCAGATCCCATGG-3′, 5′-CCGCTCGAGTCACTGCCCGGAGCATGATGAGTC-3′; for construct P5 (142–289), 5′-GGAATTCAACATCACCGCAGATCCCATGG-3′, 5′-CCGCTCGAGTCACCGGGCCACCTGGGCTGAGTC-3′; for construct P6 (211–335), 5′-GGAATTCTCAGCGGCCTGGATGAAAGAG-3′, 5′-CCGCTCGAGTCAGATGGGGTGCACGAACTG-3′.

Western Blot Analysis

To analyze the expression of NKX-3.1 or its deletion derivatives, yeast transformants were inoculated into dextrose containing –His–Trp–Ura liquid synthetic media and grown at 30°C overnight. Cultures were centrifuged and the cell pellets were stored at −80°C. To analyze the expression of PDEF and its deletion derivatives, the yeast transformants were inoculated into galactose-and raffinose-containing –His–Trp–Ura liquid synthetic media and grown at 30°C overnight. Single yeast colonies were then inoculated into YPD media and grown for 4 h. Cells were collected by centrifugation and stored at

−80°C. For Western blot analysis, the cell pellets were denatured with 2× SDS loading dye at 100°C for 5 min. The denatured pellets were briefly vortexed and centrifuged. The supernatants were separated on 12% SDS–polyacrylamide gels and gels were electroblotted onto PVDF membranes using a Bio-Rad mini Trans-Blot System (Bio-Rad Laboratories, Hercules, CA). An anti-mouse Pdef polyclonal antibody that cross reacts with human PDEF [Chen et al., 2002] was used to detect expression of PDEF and an anti-LexA polyclonal antibody (Invitrogen, Carlsbad, CA) was used to detect expression of NKX-3.1 deletion derivatives fused in frame with Lex A. Western blot signal was detected using ECL reagents (Amersham Biosciences, Piscataway, NJ).

Cell Culture, Transfection, and Luciferase Reporter Gene Assay

LNCaP cells were maintained in RPMI-1640 media (Biofluids, Rockville, MD) containing 10% fetal bovine serum (Biofluids) at 37°C in a humidified chamber with 5% CO_2. The cells were seeded into culture dishes 2 days before transfection. The Myc-tagged full-length human PDEF expression vector and the HA-tagged full-length NKX-3.1 expression vector have been described [Chen et al., 2002]. The HA-tagged NKX-3.1 deletion construct NKX (1–213) was also derived by PCR using primers 5′-CCGGAATTCATGCTCAGGGTTCCGGAGCCG-3′ and 5′-CCGCTCGAGTTAAGAGGCATAATCTGGCACATCATAAGGGTACACGGAGACCAGGGAGGCCC-3′ and was cloned into pcDNA3 (Invitrogen). All constructs were transfected into LNCaP cells using LipofectAMINE Plus reagents (Invitrogen) following the manufacturer's instructions with minor modifications. Transfected cells were harvested after 48 h. The luciferase reporter gene assay was performed as described [Chen et al., 2002].

Immunofluoresent Staining and Confocal Microscopy

Transiently transfected LNCaP cells were detached from cell culture dishes using trypsin, resuspended in PBS, and added to coverslips coated with Cell-TAK Cell and Tissue Adhesive (BD Biosciences, Bedford, MA). The coverslips were incubated at 37°C for 30 min then rinsed with PBS to remove floating cells. Cells were then fixed with 4% paraformaldehyde for 10 min and washed extensively with PBS. Coverslips were incubated with 0.2% Triton X-100 for 3 min to permeabilize cells and were subsequently incubated with 1% BSA in PBS for 20 min to block non-specific antibody binding. A rat anti-HA monoclonal antibody (Roche, Indianapolis, IN) was diluted in 1% BSA in PBS and incubated for 1 h at room temperature. After extensive washing with PBS, coverslips were incubated with Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. The nuclei were counterstained with TOTO-3 (Molecular Probes, Eugene, OR). Images of fluorescent staining were captured with a confocal laser-scanning microscope (Leica, Exton, PA).

Purification of Recombinant NKX-3.1

NKX-3.1 was cloned in frame into the Bam H I and Pst I sites of vector pQE80L (Qiagen, Inc., Valencia, CA), transformed into *E. coli* strain BL21, and induced with IPTG at 30°C. Soluble his-tagged recombinant protein was enriched by standard nickel column chromatography. Fractions containing NKX-3.1 were loaded onto a Q sepharose anion exchange column, and NKX-3.1 was present predominantly in the flow through. The flow through was adjusted to 100 mM NaCl, 50 mM Tris, and the sample was loaded to a 5 ml heparin sepharose column. After washing with three column volumes of 100 mM NaCl, 50 mM Tris, NKX-3.1 was eluted with 300 mM NaCl and 50 mM phytic acid. Column chromatography was performed using a Bio-Rad Biologic HR liquid chromatography system with flow rates of 1 ml/min.

PDEF Electrophoretic Mobility Shift Assay

PDEF was generated by in vitro translation using The TNT® T7 coupled reticulocyte lysate system (Promega, Madison, WI). Oligonucleotides 5′-TCGAGAAGCAGGATGTGATAG-3′ and 3′-CTTCGTCCTACACTATCAGCT-5′ were annealed at room temperature to generate the PSA E binding site for PDEF [Oettgen et al., 2000] and end labeled with ^{32}P. Electrophoretic mobility shift assays were conducted as described [Udvadia et al., 1992].

RESULTS

Defining the Domains of NKX-3.1 Required for Interaction With PDEF

A previous study demonstrated interaction between NKX-3.1 and PDEF in yeast two-

hybrid assays as well as by immunoprecipitation in prostate tumor cells [Chen et al., 2002]. To determine the regions of NKX-3.1 that mediate interaction with PDEF, a deletion approach using the yeast two-hybrid system was employed. Human NKX-3.1 is a 234 amino acid protein in which the homeodomain occupies positions 124–183. Deletion of the homeodomain and the 51 amino acids C-terminal to the homeodomain (Fig. 1A, construct N1) completely abrogated the interaction with full-length PDEF (Fig. 1A) indicating that regions within the C-terminal 111 amino acids of NKX-3.1 are required for the interaction. Western blot analyses demonstrated that construct N1 and all other NKX-3.1 deletion derivatives described here were stably expressed in EGY48 yeast cells (Fig. 1B). Adding back the homeodomain (Fig. 1A, construct N2) failed to restore the ability to interact with PDEF, nor did the addition of the homeodomain and the N-terminal 30 amino acids of the C-terminal tail (Fig. 1A, construct N3). These data demonstrated the C-terminal-most 21 amino acids of NKX-3.1 are necessary for interaction with PDEF. However, the C-terminal tail alone which contains the necessary 21 amino acid domain (Fig. 1A, construct N4) was not capable of interacting with PDEF in the two-hybrid system, demonstrating that this region was not sufficient. In contrast, when the full homeodomain or the second half of the homeodomain was added back to the C-terminal tail (Fig. 1A, construct N5, N6), interaction was restored. Although the second half of the homeodomain was required for interaction, the homeodomain by itself (construct N7) was not sufficient to mediate interaction with PDEF. The importance of the C-terminal-most 21 amino acids was further demonstrated by deletion of this region in the context of the homeodomain (construct N8, N9), which also eliminated the ability to interact with PDEF. In summary, these data demonstrated that the 30 amino acids that constitute the second half of the homeodomain and the 21 C-terminal–most amino acids of NKX-3.1 are required to mediate the interaction with PDEF.

Defining the Regions of PDEF Required for Interaction With NKX-3.1

A yeast two-hybrid deletion analysis was also used to identify regions of PDEF required to mediate interactions with full-length NKX-3.1. PDEF contains 335 amino acids and has two conserved domains, the 69-amino acid pointed domain (positions 142–210) and the 84-amino acid Ets domain (positions 248–331). Deletion of the Ets domain, which lies at the C-terminus of PDEF (Fig. 2A, construct P1), eliminated the ability to interact with NKX-3.1 in yeast cells. Western blot analyses demonstrated that the Ets domain deletion mutant and the other PDEF deletion derivatives described here were stably expressed in EGY48 yeast cells (Fig. 2B). However, in a manner analogous to the NKX-3.1 homeodomain described above, the Ets domain alone (Fig. 2A, construct P2,) was not sufficient to mediate interaction. When 106 N-terminal amino acids were added back to the Ets domain (Fig. 2A, construct P3), the ability to interact was restored. This region included the 69-amino acid pointed domain as well as a 37-amino acid linker region. These data suggested that motifs present within the pointed domain or the linker region between the pointed domain and the Ets domain or both were required together with the Ets domain to mediate interaction. However, the 106-amino acid region alone (Fig. 2A, construct P4) was not able to interact with NKX-3.1. To further narrow the domains required for interaction, the second half of the Ets domain was deleted from construct P3, which abrogated the ability to interact with NKX-3.1 (Fig. 2A, construct P5). Surprisingly, the pointed domain was dispensable for interaction in the context of a complete Ets domain and linker region (Fig. 2A, construct P6). These data demonstrate that the 46 C-terminal amino acids of PDEF which include the second half of the Ets domain and four additional amino acids together with the 37-amino acid linker region were required to mediate the interaction with NKX-3.1. In addition to interacting with full-length NKX-3.1, this region is also capable of interacting with the minimal interacting region of NKX-3.1 (amino acids 154–234) encoded by construct N6 (data not shown).

Correlation Between Interaction and Function in Prostate Epithelial Cells

It has previously been demonstrated that NKX-3.1 can repress the ability of PDEF to transactivate the prostate-specific antigen (PSA) promoter in LNCaP prostate tumor cells [Chen et al., 2002]. To determine whether the ability of NKX-3.1 to abrogate the transactivation

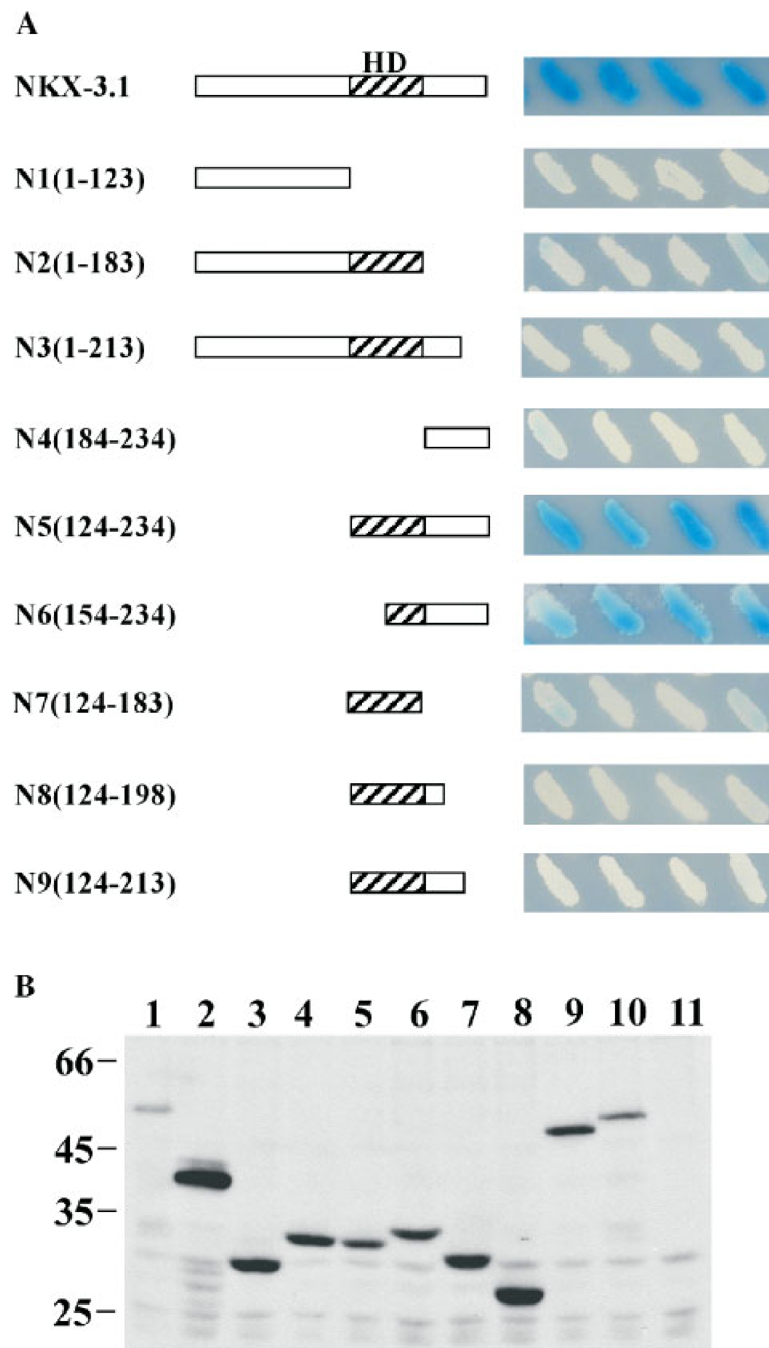

Fig. 1. Yeast two-hybrid analysis of the interaction between NKX-3.1 deletion mutants and full-length prostate derived Ets factor (PDEF). **A**: A series of NKX-3.1 deletion constructs were constructed and transformed into EGY48/[p8op-lacZ] together with full-length PDEF. Colonies were patched onto dextrose containing plates (−His−Trp−Ura) and then replica plated onto galactose- and raffinose-containing plates (−His−Trp−Ura + X-gal) to assay reporter gene (lacZ) expression. Blue color indicates an interaction. Numbers in parentheses indicate the beginning and ending amino acids. Hatched box indicates the homeodomain. **B**: Western blot analysis to assay expression of NKX-3.1 deletion constructs in the yeast two-hybrid system. NKX-3.1 or its deletion constructs were fused in frame with LexA protein. Anti-LexA antibody was used to detect the fusion proteins. **Lane 1**, Full-length NKX-3.1; **Lane 2**, N1 (1–123); **Lane 3**, N7 (124–183); **Lane 4**, N8 (124–198); **Lane 5**, N9 (124–213); **Lane 6**, N5 (124–234); **Lane 7**, N6 (154–234); **Lane 8**, N4 (184–234); **Lane 9**, N2 (1–183); **Lane 10**, N3 (1–213). **Lane 11**, EGY48 yeast extract with no transformed constructs. Faint low molecular weight bands present in all lanes represent yeast proteins that cross-react with the anti-LexA antibody. HD, homeodomain.

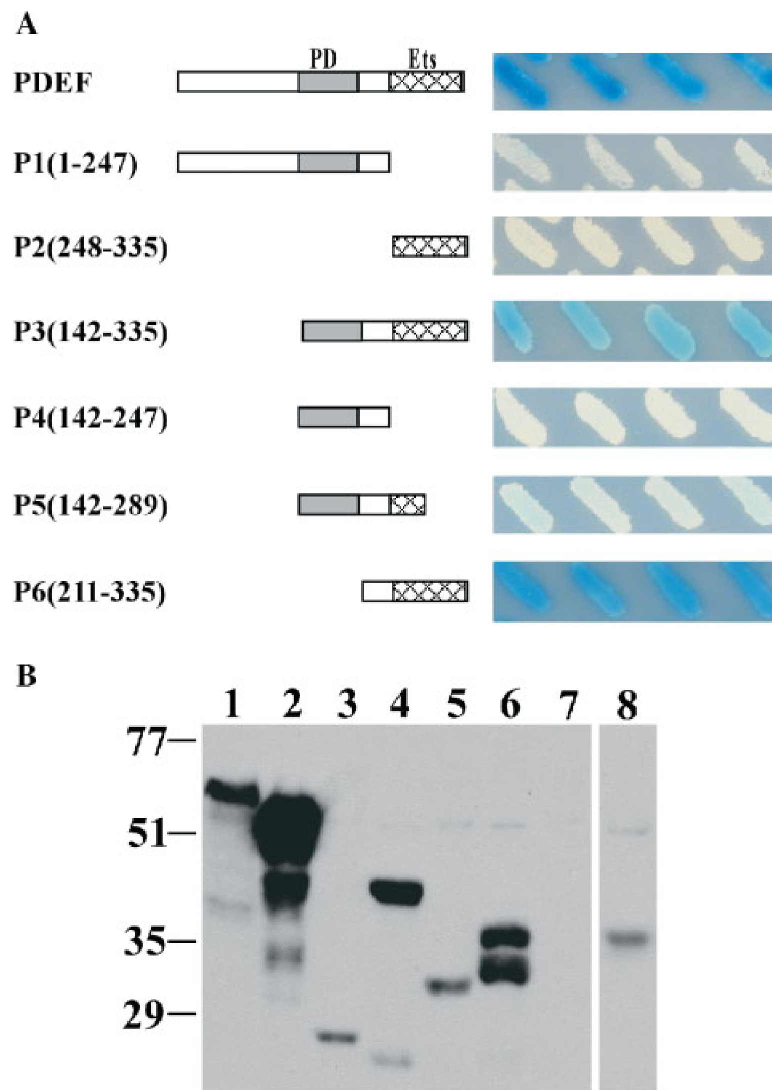

Fig. 2. Yeast two-hybrid analysis of the interaction between PDEF deletion mutants and full-length NKX-3.1. **A**: A series of PDEF deletion constructs were transformed into EGY48/[p8op-lacZ] with full-length NKX-3.1. Colonies were patched onto dextrose containing plates (–His–Trp–Ura) and replica plated onto galactose- and raffinose-containing plates (–His–Trp–Ura + X-gal) to assay lacZ expression. Blue color indicates an interaction. Numbers in parentheses indicate the beginning and ending amino acids encoded by the deletion constructs. The gray box indicates the pointed domain and the cross-hatched box indicates the Ets domain. **B**: Western blot analysis to assay expression of PDEF deletion constructs. PDEF or its deletion constructs were fused in frame with B42AD. A rabbit anti-mouse Pdef polyclonal antibody was used to probe Western blots. **Lane 1**, full-length PDEF; **Lane 2**, P1 (1–247); **Lane 3**, P2 (248–335); **Lane 4**, P3 (142–335); **Lane 5**, P4 (142–247); **Lane 6**, P6 (211–335). **Lane 7**, EGY48 yeast extract with no transformed constructs; **Lane 8**, P5 (142–289). PD, pointed domain; Ets, Ets domain.

function of PDEF in mammalian cells correlates with the ability to interact in yeast cells, co-transfection assays in LNCaP cells were performed. Lysates of transfected cells were divided and analyzed for luciferase reporter gene activity and by Western blot analyses to ensure that the transfected genes were stably expressed or co-expressed (Fig. 3B). As expected, PDEF readily transactivated the PSA-luciferase reporter, and, consistent with our previous study, this ability was almost complete blocked by the addition of full-length NKX-3.1 [Chen et al., 2002]. In contrast, co-transfection of an NKX-3.1 deletion mutant missing the C-terminal-most 21 amino acids shown to be critical for interaction in yeast failed to block the transactivation function of PDEF on the PSA promoter (Fig. 3A). Western blot analysis revealed that the mutant protein was expressed at a relatively high level in comparison with the

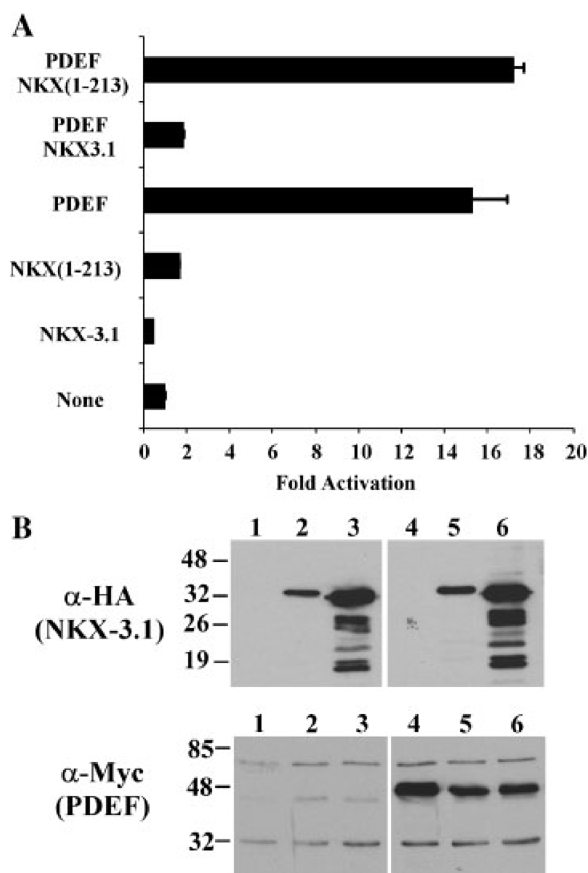

Fig. 3. NKX (1–213) fails to represses the PDEF-mediated transactivation of the PSA promoter. **A**: LNCaP cells were co-transfected with the indicated PDEF, NKX-3.1 and NKX (1–213) expression vector constructs and luciferase reporter constructs PSA/Luc and pRL-CMV. The pRL-CMV construct was used to normalize the transfection efficiency. Empty vectors were used to balance the total amount of DNA. Luciferase activity in the lysates was determined 48 h after transfection. **B**: Western blot analyses to measure expression of transfected genes in the cell lysates assayed in (A). **Upper panel**: Anti-HA monoclonal antibody was used to detect expression of NKX-3.1 and NKX (1–213). **Lower panel**: Anti-Myc antibody was used to detect expression of PDEF. For both **upper** and **lower panels**: **Lane 1**, vectors only; **Lane 2**: NKX3.1; **Lane 3**, NKX (1–213); **Lane 4**, PDEF only; **Lane 5**, NKX3.1 and PDEF; **Lane 6**: NKX (1–213) and PDEF.

full-length NKX-3.1 protein, ruling out the possibility that the failure to block PDEF function was due to a failure of the mutant protein to accumulate (Fig. 3B). These data support the conclusion that for NKX-3.1 to block the activity of PDEF, a physical interaction between the two proteins is required. However, it remained possible that the failure of the NKX-3.1 C-terminal deletion mutant to function was due to a failure of the mutant protein to accumulate in the nucleus. To rule out this possibility, LNCaP cells were transfected with either HA-tagged full-length NKX-3.1 or the HA-tagged C-terminal deletion mutant NKX (1–213). Expression of the full-length and mutant proteins was detected using a FITC-conjugated anti-rat secondary antibody, and nuclei were simultaneously labeled with TOTO-3. Confocal microscopic analysis of cells transfected with either full-length or the C-terminal NKX-3.1 deletion mutant demonstrated that in both cases, the transfected NKX-3.1 proteins localized predominantly in the nucleus (Fig. 4).

Recombinant NKX-3.1 Does Not Interfere With DNA Binding by PDEF

To begin to dissect the mechanism whereby NKX-3.1 blocks the transactivation function of PDEF on the PSA promoter, electrophoretic mobility shift assays were performed in the presence and absence of recombinant NKX-3.1. In vitro translated PDEF alone was able to bind to the PSA E site in agreement with a previous study (Fig. 5). In the presence of recombinant NKX-3.1 purified from bacteria, PDEF binding to the PSA E site was not diminished (Fig. 5) [Oettgen et al., 2000].

DISCUSSION

Tissue-specific gene regulation is often mediated by conserved families of transcription factors that bear highly homologous DNA binding domains [Graves and Petersen, 1998; Wilson and Desplan, 1999]. These domains often exhibit similar binding site preferences, which presents a paradox for achieving target specificity of gene regulation when multiple members of a family are co-expressed in the same cell. Current paradigms emphasize the role of protein partners that can modulate activity by altering sub-cellular localization, DNA binding activity, or function [Chariot et al., 1999; Wilson and Desplan, 1999; Li et al., 2000]. The identification and functional analysis of peptide domains that mediate these interactions is a requirement for understanding the molecular basis of regulated assembly of transcriptional complexes. In a previous study, we demonstrated that NKX-3.1 and PDEF physically interact in prostate epithelial cells, and that NKX-3.1 can abrogate the transactivation function of PDEF [Chen et al., 2002]. In the experiments reported here, we have identified the domains that are critical for mediating the interaction between NKX-3.1 and PDEF, two transcription

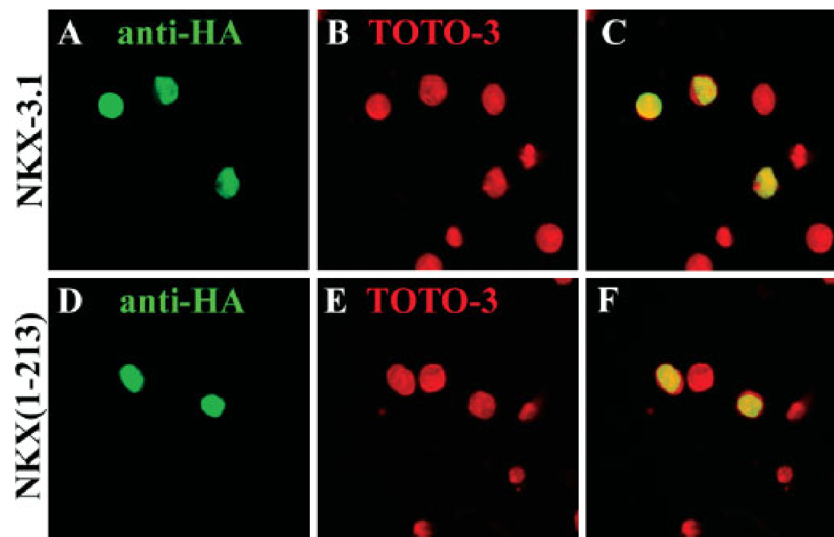

Fig. 4. Localization of NKX-3.1 and NKX (1–213) using confocal microscopy. LNCaP cells were transfected with HA-tagged NKX-3.1 or NKX (1–213). HA signal is shown in green and nuclear staining with TOTO-3 is shown in red (false color). **A**: High magnification view of LNCaP cells transfected with HA-tagged NKX-3.1. **B**: Same field view as **panel A** showing nuclear staining with TOTO-3. **C**: Overlay of **panels A** and **B**. **D**: High magnification view of LNCaP cells transfected with HA-tagged NKX (1–213). **E**: Same field view as **panel D** showing nuclear staining with TOTO-3. **F**: Overlay of **panels D** and **E**.

factors bearing distinct, highly conserved DNA binding domains that have been implicated in controlling the growth of prostate epithelial cells.

The domains of NKX-3.1 required for interaction with PDEF are the second half of the homeodomain, which includes the DNA recognition helix, and a tyrosine rich 21-amino acid region of the C-terminal tail. The fact that the NKX-3.1 homeodomain is involved is consistent with structural analyses that have demonstrated a similar protein–protein interaction function in other homeodomains [Chan et al., 1994]. Homeodomain-mediated interaction with cofactors plays a prominent role in regulating the activity and functional specificity of this class of transcription factors [Chariot et al., 1999; Bondos and Tan, 2001]. Our deletion analyses demonstrate that the homeodomain is necessary, but not sufficient to mediate interaction with PDEF. To date, the only other homeodomain known to interact with an Ets domain is that of the pituitary specific protein Pit-1/GHF-1, which, in contrast, is sufficient to mediate interaction with Ets-1 [Bradford et al., 2000]. Taken together with our demonstration that the homeodomain of NKX-3.1 is required for the interaction with PDEF, these data suggest that interactions between Ets domains and homedomains may be a general phenomenon. Although, we cannot rule out the possibility that the NKX-3.1 or PDEF deletion mutants that did not interact were misfolded in yeast cells, this concern is mitigated by the fact that all of the mutants were stably expressed.

In addition to the second half of the homeodomain, 21 amino acids of the C-terminal tail of NKX-3.1 are necessary though not sufficient

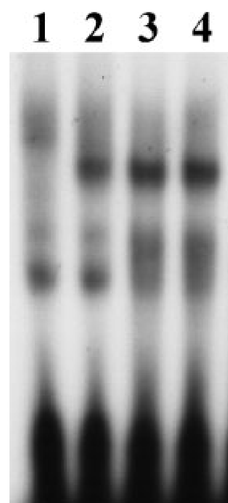

Fig. 5. Recombinant NKX-3.1 has no effect on DNA binding of in vitro translated PDEF. The PSA E PDEF binding site was used as a probe for the gel mobility shift assay. **Lane 1**: Negative control showing DNA binding by the in vitro translation system with an empty vector. **Lane 2**: DNA binding by in vitro translated PDEF. **Lanes 3** and **4**: DNA binding by in vitro translated PDEF with 1.6 μg of recombinant NKX-3.1.

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Mouse     1 YTSYPYYPYLYCLGSWHPSFW 21
Rat       1 YASYPYYPYLYCLGSWHPTFW 21
Human     1 YNSYPYYPYLYCVGSWSPAFW 21
Xenopus   1 YQNYHCYPYMYYLAGWPAPLW 21
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Fig. 6. Alignment of the essential C-terminal 21 amino acid interaction domain of mouse and human NKX-3.1 and their putative orthologs in rat and *Xenopus*. The accession numbers are: human, NP_006158; mouse, NP_035051; rat, XP_224360; *Xenopus*, AAH47968. ClustalW was used to align the sequences.

for interaction. This region is well conserved among the mouse, human, rat, and *Xenopus* NKX-3.1 orthologs (Fig. 6). Nine of the 21 residues are identical in all species, and five of these are tyrosines. The high density and conservation of tyrosine residues raises the possibility that this domain, and perhaps the interaction between NKX-3.1 and PDEF, may be regulated by phosphorylation. It is also interesting to note that the C-terminal tail of Ultrabithorax, a *Drosophila* homeotic protein, mediates interactions with its homeodomain-containing partner Extradenticle [Chan et al., 1994].

The co-transfection analyses presented here strongly suggest that the ability of NKX-3.1 to repress the function of PDEF requires a physical interaction between the two proteins. It remains possible that, despite the fact that the NKX-3.1 C-terminal deletion mutant co-localized in the nucleus with PDEF, it may not have been targeted to the correct subnuclear compartment. However, we consider this unlikely since the confocal microscopic analysis did not reveal a recognizable difference in the distribution of full-length NKX-3.1 compared to the C-terminal deletion mutant. The mechanism whereby NKX-3.1 represses the activity of PDEF remains unclear. In general, transcriptional repressors can work by interfering with DNA binding, by blocking the general transcription machinery, or by inhibiting an activation function. Recombinant full-length NKX-3.1 does not appear to block the ability of in vitro translated PDEF to bind to a binding site derived from the PSA promoter, arguing against the first mechanism although it is possible that NKX-3.1 requires post-translational modification to enable it to interfere with the DNA binding function of PDEF. The second mechanism also appears unlikely, since full-length NKX-3.1 did not repress the control renilla luciferase reporter gene driven by the CMV promoter. Hence, we favor the third mechanism, wherein NKX-3.1 may itself repress the activation function of PDEF or may act by recruiting another repressor protein. The AP-1 like protein MafB/Kreisler appears to repress Ets-1 by a similar mechanism [Sieweke et al., 1996]. Further experiments, for example, purification of native transcriptional complexes containing NKX-3.1 and PDEF will be required to clarify the molecular basis of the repression.

The regions of PDEF shown here to be required for interaction with NKX-3.1 are the C-terminal half of the 84 amino acid Ets domain, which includes the DNA recognition helix $\alpha3$, and the 37-amino acid linker region located between the Ets and pointed domains. Crystallographic studies of several other Ets/partner complexes have demonstrated the importance of the DNA recognition helix in the co-regulation of DNA binding and protein partner selection [Verger and Duterque-Coquillaud, 2002]. However, in those examples, adjacent DNA binding sites for the Ets protein and its partner are thought to play a pivotal role in the assembly of a functional complex. Although the PSA promoter does contain potential binding sites for both PDEF and NKX-3.1, they are not juxtaposed [Oettgen et al., 2000]. Chromatin immunoprecipitation experiments may help to determine whether the interaction between NKX-3.1 and PDEF occurs when PDEF is bound to sites in the PSA promoter.

The fact that the pointed domain is completely dispensable for the interaction is somewhat surprising given that pointed domain in other Ets factors is important for regulating interactions with partners [Sharrocks, 2001; Verger and Duterque-Coquillaud, 2002]. On the other hand, our observation that the 37 amino acid region between the Ets and pointed domains is required for interaction with NKX-3.1 is consistent with the location of interaction domains in other Ets factors [Yang et al., 1998; Guidez et al., 2000].

The results reported here have identified the minimal domains required to mediate interactions between NKX-3.1 and PDEF and have set the stage for further high-resolution structural analyses. This system may serve as a paradigm to study the regulation of Ets domain/homeodomain interactions.

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